International Journal of

Dairy Science

ISSN 1811-9743

Academic
Journals Inc.

www.academicjournals.com

International Journal of Dairy Science 5 (2): 60-70, 2010
ISSN 1811-9743
© 2010 Academic Journals Inc.

Virulence Factors of Genotyped Bovine Mastitis *Staphylococcus aureus* Isolates in The Netherlands

[1]R. Ikawaty, [1]E.C. Brouwer, [2]E. Van Duijkeren,
[2,3]D. Mevius, [1]J. Verhoef and [1]A.C. Fluit
[1]Department of Medical Microbiology, University Medical Centre Utrecht,
Room G04.614, P.O. Box 85500, 3508 GA Utrecht, The Netherlands
[2]Faculty of Veterinary Medicine Utrecht University, Yalelaan 1,
Utrecht, The Netherlands
[3]Central Veterinary Institute, Lelystad, The Netherlands

Abstract: The pathogenesis of bovine mastitis caused by *Staphylococcus aureus* and the presence of *S. aureus* virulence genes in bovine mastitis are poorly understood. In this study, the virulence factor profiles from different genotyped clinical bovine mastitis *S. aureus* isolates from The Netherlands were investigated. A total of 76 *S. aureus* isolates from 2 institutions that represented regional and national strains were examined by PCR for 19 toxin genes, 12 adhesin genes, 3 immune evasion genes and 6 other genes. The presence of *hlb*, *ebps*, *efb*, *sspA* and *sspB* was observed in all isolates, *coa* in all isolates but one, while the presence of *sec3, seg, seh, sei, sel, sem, sen, tst, lukE, fnbA, fnbB*, *icaB*, *icaC*, *icaD*, *clfA, sdrE, cna, cap8*, *cap5* and *map* was variable. *Sak* and the genes for the human specific immune evasion proteins, SCIN and CHIPS were detected in only one isolate. In addition, none of isolates were *lukPV*-positive. Five major virulence factor profiles were observed which, with the exception of SaPIbov, were in general agreement with the clustering obtained from PFGE, MLST, *spa*-typing and MLVA. In conclusion, *hlb*, *ebps*, *efb*, *sspA* and *sspB* were present in all isolates and other virulence genes were variably present. The virulence factor profiles generally matched genotyping results for Dutch bovine mastitis isolates.

Key words: *Staphylococcus aureus*, bovine mastitis, virulence factors, PFGE, *spa*-typing

INTRODUCTION

Virulence of a microorganism can be defined as the capacity of the microorganism to damage the host which may lead to signs and symptoms of disease. A large array of virulence factors are produced by *Staphylococcus aureus*. *S. aureus* may produce a number of exotoxins including the classical Staphylococcal Enterotoxins (SEs) A to E, the recently characterized SE types (SEG-SEU) (Dinges *et al*., 2000; Smyth *et al*., 2005), toxic shock syndrome toxin 1 (TSST-1), β-hemolysin (*hlb*), the bi-component Panton-Valentine Leukocidin (Luk-PVL), proteases with the ability to induce T-cell proliferation and exfoliative toxin A and B (ETA and ETB). A number of Microbial Surface Components Recognizing

Corresponding Author: A.C. Fluit, Department of Medical Microbiology,
University Medical Centre Utrecht, Room G04.614, P.O. Box 85500,
3584 CX Utrecht, The Netherlands

Adhesive Matrix Molecules (MSCRAMMs) which promote adhesion to host extra-cellular matrix components (Foster, 2005). Clumping factor A and B (ClfA and ClfB) are two dominant fibrinogen-binding adhesins (Foster, 2005), while fibronectin-binding protein A and B (FnBPA and FnBPB) mediate binding to fibronectin and collagen adhesin (CNA) binding to collagen. Serine-aspartate repeat proteins (Sdr) are surface proteins related to ClfA and ClfB (Josefsson *et al*., 1998) which have been hypothesized to mediate interactions of *S. aureus* with the extra-cellular matrix (George *et al*., 2006). Coagulase induces polymerization of fibrinogen to fibrin. Distinct from coagulase, fibrinogen-binding protein (Efb) is an intracellular protein which specifically promotes binding to fibrinogen (Bodén and Flock, 1989). Elastin-binding protein of *S. aureus* (EbpS) mediates binding to soluble tropoelastin (Downer *et al*., 2002). Adherence of *S. aureus* is also enhanced by a MHC class II analog protein (MAP), a protein with broad binding activity to fibrinogen, collagen, fibronectin, vitronectin and elastin which may enable colonization of different tissues. Plasmin-sensitive protein (Pls) has an adhesive function by mediating interaction between bacterial cells, bacterial binding to immobilized fibronectin and immunoglobulin G and invasion of epithelial cells, as well as anti-adhesive functions at a certain stages during infection which allow the bacteria to spread. *S. aureus* can produce biofilm which is mediated by gene products of the *icaADBC* locus (Rohde *et al*., 2007). *S. aureus* exoenzymes including the extra-cellular serine protease (SspA) and cysteine protease (SspB) are involved in elastin degradation known as elastinolytic activity.

Capsular Polysaccharide (CP) produced by *S. aureus* renders the bacteria resistant to phagocytosis. Eleven CP serotypes have been described and CP type 5 and 8 were predominantly found in clinical isolates (O'Riordan and Lee, 2004; Poutrel *et al*., 1988; Sordelli *et al*., 2000). Furthermore, *S. aureus* phages encoding immune evasion molecules such as staphylokinase (SAK), chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS) and staphylococcal complement inhibitor (SCIN) may provide *S. aureus* the ability to escape the human immune system. In addition, an Arginine Catabolic Mobile Element (ACME) which inhibits polymorphonuclear cell production, is considered to enhance the virulence and colonization by *S. aureus* (Diep *et al*., 2006a, b).

S. aureus is generally recognized as the most common pathogen of contagious bovine mastitis and it causes significant economic losses in the diary industry. Chronic and deep infections in the mammary glands of bovines are often associated with poor success of treatment. Different suites of virulence factors may result in differences in severity and course of mastitis. However, little is known about the presence of *S. aureus* virulence genes in bovine mastitis and so far, dominant virulence factors among bovine mastitis *S. aureus* have not been identified. In this study, we investigated the virulence factor profiles from different genotyped clinical bovine mastitis *S. aureus* isolates from The Netherlands.

MATERIALS AND METHODS

Bacterial Isolates

The study was conducted between September 2007 and September 2009 at the Department of Medical Microbiology of the University Medical Center Utrecht, Utrecht, The Netherlands. Seventy six *Staphylococcus aureus* isolates from clinical cases of bovine mastitis were included. Thirty isolates were obtained by the Faculty of Veterinary Medicine Utrecht University, Utrecht, The Netherlands, from 26 farms near Utrecht. Isolates from farms sampled twice were taken at least one year apart. Another 46 isolates were collected by the Animal Health Service in Deventer, The Netherlands for the Central Veterinary Institute

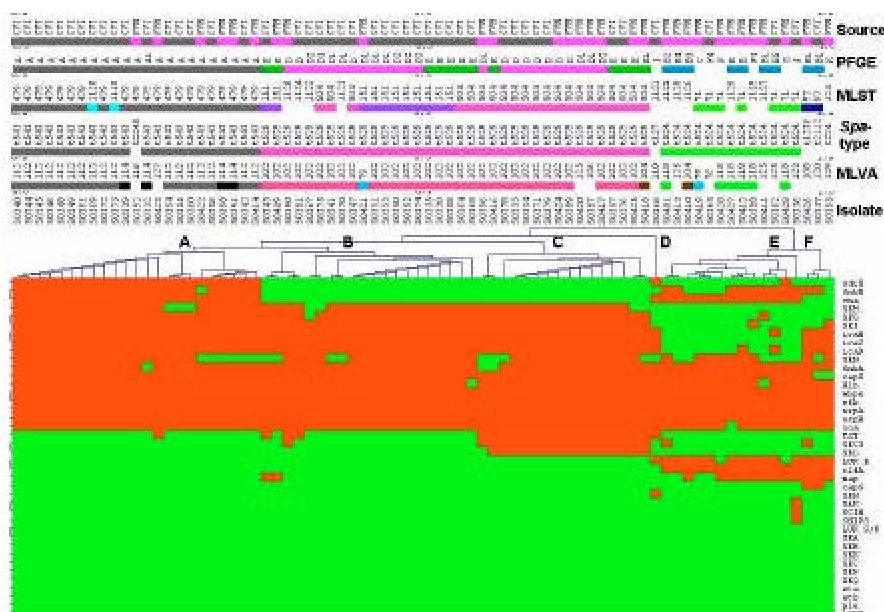

Fig. 1: Virulence factor profiles observed within various typing methods. Place where the isolates were taken. FVM: Veterinary Faculty Utrecht University; CVI: Central Veterinary Institute, Lelystad, The Netherlands. The presence and absence of genes is indicated by red and green spots. Virulence factor profiles were defined based on the presence and absence of the genes, and resulted 5 main profiles, A, B, C, D and E

(CVI), Lelystad and The Netherlands from farms throughout The Netherlands. The sources were individual teat milk samples from dairy cattle from all over the country from clinical or subclinical cases of mastitis. Each isolate from CVI represents one farm, the location of the farms is unknown but they are distributed all over The Netherlands. All isolates were methicillin-susceptible *S. aureus* (MSSA) and collected between 1988 and 2005.

These isolates were genotyped by Multilocus Sequence Typing (MLST), staphylococcus protein A (*spa*)-typing, Multiple Locus Variable Number Tandem of Repeat Analysis (MLVA) and Pulsed-Field Gel Electrophoresis (PFGE) (Fig. 1) (Ikawaty *et al*., 2008). Most of the 76 bovine *S. aureus* isolates used in this study belonged to 4 dominant MLST types. We observed 3 dominant *spa*-types and 2 dominant MLVA types. DNA of 76 isolates was digested with *Sma*I and showed 21 PFGE profiles, 4 dominant profiles, 10 profiles related to the dominant profiles and 7 unique PFGE profiles (Fig. 1). No particular difference was observed between regionally and nationally obtained *S. aureus* bovine mastitis strains in terms of PFGE profile, MLST and *spa*-types, except for MT118 that was only present among regionally obtained isolates. Despite the fact that isolates could belong to the same cluster, they were not considered to belong to local outbreaks as at least 90% of the isolates were obtained on different farms whereas the remaining isolates were obtained at least one year apart when sampled on the same farm. MLST generally showed a distinct cluster of bovine *S. aureus* compared to isolates from the human population, although a little sharing of strains between the bovine and human population was observed. In addition, the ST or Clonal

Complexes (CC) of bovine mastitis isolates from The Netherlands differed from those described elsewhere with the exception of 4 isolates (S0337, S0426, S0431 and S0436) that belonged to CC97 which has previously been reported to be obtained from humans.

Genomic DNA Preparation

Preparation of bacterial genomic DNA was performed using the NucleoSpin kit (Macherey-Nagel) following the protocols from the manufacturer with the exception that the bacterial pellet is resuspended in 20 mM Tris/HCl, 2 mM EDTA and 1% Triton X-100 supplemented with 0.2 mg mL^{-1} of lysostaphin, achromopeptidase and RNase.

PCR Amplifications

PCRs were performed to detect the genes of 40 potential virulence factors including 19 staphylococcal toxin genes, 12 adhesion genes; 3 genes encoding extra-cellular proteases, 3 immune evasion genes, 2 genes for capsular polysaccharide and SCC_{ACME} (Table 1). Table 1 lists the oligonucleotide primers, positive and negative control strains used in this study.

Table 1: Oligonucleotide primers used

Items	Primer sequence (5'-3')	Pos. Ctrl.[1]	Neg. Ctrl.[2]	Ref[3]
Toxin genes				
sea	ttaaccgaaggttctgtaga agatcattcgtggtataacg	MSSA476	COL	(Van Wamel *et al.*, 2006)
seb	tacacccaacgttttagcagaga aaggcgagttgttaaattcatagagt	COL	-	(Mahmood and Khan, 1990)
sec3	aaaaattatgacaaagtgaaaacagagt gcaggcatcatatcataccaaaaagt	Mu50	COL	(Regassa *et al.*, 1991)
seg	aactatgggaaatgtaatgaatctt gccagtgtcttgctttgtaatc	MRSA252	MSSA476	UMCU
seh	cacatcatatgcgaaagc cgaatgagtaatctctagg	MSSA476	-	UMCU
sei	atctaataattgggacgaa aaaaacttacaggcactc	MRSA252	COL	UMCU
sek	tactcctatagctaatcaact ggtaacccatcatctcc	USA 300	-	UMCU
sel	taacggcgatgtaggtccag attttgaagaagtgccgtatt	N315	COL	UMCU
sem	ggataattcgacagtaac tttcagtttcgacagttt	N315	MSSA476	UMCU
sen	tatatccgtacttaaaacttcta aaaaactctgctcctactg	N315	MSSA476	UMCU
seo	agaccctattgctttaca tctttatgctccgaatga	N315	MRSA252	UMCU
sep	aatcataaccaaccgaatca tcataatggaagtgctataa	N315	MSSA476	(Van Wamel *et al.*, 2006)
seq	tctagcatatgctgatgtagg caatctcttgagcagttacctc	MRSA252	MSSA476	(Diep *et al.*, 2006a)
hlb	gttggtgcacttactgacaa tgtgtaccgataacgtgaac	COL	-	(Van Wamel *et al.*, 2006)
tst-1	attttacccctgttcccttatcatc taggtggtttttcagtattgtattca	Mu50	MSSA476	UMCU
luk-E	gcaactttgtcagtaggactg gtctacttcactgacataactc	COL	MSSA476	UMCU
luk-S/F	cacagtggtttcaatccttc cacctgataagccgttagag	USA 300	-	UMCU
eta	gttggtgcacttactgacaa tccacggattttattttatttattac	COL	-	UMCU
etb	atattatttttacacccgctcaa ttccccaaagtgtctccaaaagta	S0202	-	UMCU
efb	cgaaggatacggtccaagag ctgccttttgtgcttttctg	COL	-	UMCU

Table 1: Continued

Items	Primer sequence (5'-3')	Pos. Ctrl.[1]	Neg. Ctrl.[2]	Ref[3]
Adhesion genes				
clfA	caactttgaccatgcccgcttat ccaggctcatcaggttgttcagg	COL	MSSA476	UMCU
cna	taaaacgggagatatgctaccag gatttcccgttcacttcttccta	MSSA476	USA300	(Blevins *et al*., 1999)
coa	atattattagccgttacaggtg aatgcgcgtttatcttga	COL	-	(McDevitt *et al*., 1992)
ebpS	atgtatttgcttggttag aaatcgtcaatcgataga	N 315	COL	(Downer *et al*., 2002)
fnbA	attgggagcagcatcagtattcttagga gatcttgtcacacgtggcttactttctg	COL	-	UMCU
fnbB	taaagcaagcgaaacacaaacaactac atctcccgccttaattccttctc	COL	MRSA252	UMCU
icaB	ttgatcatattgcctgtaagc aatcgtgggtatgtgtttca	MSSA476	-	(Peacock *et al*., 2002)
icaC	atggtatggctattttatcgttgtta gtgatatcgctagaaagaccattgta	MSSA476	-	UMCU
icaD	atcgctatatcttgtgtcttt tctctgccatttttgaa	MSSA476	-	UMCU
cap5a	aagtggtgggagaaaacgcctgat ttcttttgctgcatgacttactgttacg	N315	MRSA252	UMCU
cap8	atgacgatgaggatagcg cacctaacataaggcaag	MW 2	COL	UMCU
map	tagaggtatcggggaacg aatctttttaatatctttggcacta	COL	MRSA252	UMCU
pls	caagttggtcaaaatgccctaaata ttcaccacgttcaactacatctcct	COL	MRSA252	UMCU
sspA	ttattgcttccggtgtagttgtag atctgggttattaggttggtcatc	COL	-	UMCU
sspB	agaagatggcaaagttagattagt tgtgggtcattagggttttg	COL	-	UMCU
sdrE	atcaaagttggcgatggt ccatggaccattggatct	Newman	MRSA252	UMCU
Immune evasion and other proteins				
sak	aaggcgatgacgcgagttat gcgcttggatctaattcaac	MRSA252	-	(Van Wamel *et al*., 2006)
scin	agcacaagcttgccaacatcg ccggaattcttaatatttactttttagtgc	MRSA252	-	(Van Wamel *et al*., 2006)
chips	gaaaaagacattagcaacaacag cataagatgatttagactctcc	MRSA252	-	(Van Wamel *et al*., 2006)
SCC_{ACME}	gaatgaacgtggatttaatgtcc cgttatggaggtgctctg	USA300	MRSA252	UMCU

[1]Positive control strains; [2]Negative control strains; [3]References. UMCU: Primers were designed at Dept. of Medical Microbiology, University Medical Centre Utrecht, Utrecht, The Netherlands

RESULTS AND DISCUSSION

Among the 18 toxin genes tested, *hlb*, *sspA* and *sspB* were detected in all isolates. The genes for enterotoxins A (*sea*), B (*seb*), K (*sek*), O (*seo*), P (*sep*), Q (*seq*), leukocidin encoded by *LukS/F*, exfoliative toxin A (*eta*) and B (*etb*) could not be amplified while the genes encoding the other 8 toxins were present in 1-80% of the isolates. The recently described enterotoxins G and I were the most prevalent (78 and 80% of the isolates, respectively). We observed that 56 of 76 (76%) isolates encoded both SEI and SEG. Forty (52.6%) isolates encoded SEM and SEN, although these two genes were not present simultaneously in 27 (35.5%) isolates. The *seh* gene was present in only 1 isolate (1%). The SaPIbov pathogenicity island signature combination of *sec3*, *sel* and *tst* was found in 15 (19.7%) isolates. Two (2.6%) isolates encoded *sec3* and *tst* but lacked *sel,* while 6 (7.9%) other isolates encoded either *sec3*, *sel* or *tst* only. In addition, *lukE* was detected in 20% of isolates.

Ebps and *efb* could be amplified for all isolates, while *pls* was absent. PCR specific for *fnbA* and *fnbB* showed that 73 (96%) isolates possessed *fnbA* and 33 (43%) isolates had *fnbB*. Three gene targets involved in biofilm formation, *icaB*, *icaC* and *icaD*, were found in 64 (84%), 63 (83%) and 65 (86%) isolates, respectively. *ClfA* was detected in 16 (21%) isolates, 33% (n = 16) isolates were positive for *sdrE*, 49% (n = 37) were *cna* positive, 18% (n = 14) contained *map* and most of isolates (99%, n=75) obtained *coa*.

The three immune evasion genes, *sak, scin* and *chips*, could not be amplified in almost all isolates, except for one isolate (S0336). SCC_{ACME} was not amplified in any of the isolates. *Cap8* was detected in 73 isolates, while *cap5* was present in 3 isolates.

Based on the presence and absence of virulence factors five main clusters (A, B, C, E and F) were identified among the 76 bovine mastitis *S. aureus* isolates (Fig. 1). In addition, a unique gene pattern (called D) was observed for 1 isolate (S0366). There is general agreement between the 4 typing methods used (PFGE, MLST, *spa*-typing and MLVA) and the 6 clusters of isolates, but exceptions were obviously present. Cluster B and C could not be identified based on PFGE profiles, since some of the isolates with an identical PFGE profile were distributed across both virulence profile clusters. These isolates were also considered closely related based on the other typing methods (MLST, *spa*-typing and MLVA).

Cluster A isolates mostly possessed *sdrE*, *finbB* and *cna* genes which were totally absent in cluster B and C isolates and occasionally present in cluster D and E isolates (Table 2). Identical and closely related isolates identified by MLST, *spa*-typing and MLVA in cluster B and C had similar virulence gene patterns, except that SaPIbov (*tst, sec3* and *sel*) were predominantly detected in cluster C isolates. Virulence gene patterns detected in cluster E and F isolates mostly lack SaPIbov similar to isolates of cluster A. The gene for enterotoxin *sem* was absent, while *sen, seg* and *sei* were occasionally detected. Almost all isolates from both clusters contained *lukE, clfA* and *map*, while other clusters did not. *icaB-D* and *cap8* were detected in all clusters (except cluster E isolates). The only isolate in cluster D showed a unique gene pattern with the presence of *seh* and a unique combination of other virulence genes.

Thus in general, there is an agreement between the virulence profile and the typing methods and the clusters with exception of clusters B and C. Cluster C contains SaPIbov-positive isolates, whereas this is absent in cluster B isolates, but the overall virulence gene profiles are similar for both clusters.

By analyzing the presence of all virulence factors tested in all isolates *fnbA, hlb, ebps, sspA, sspB* and *coa* were always present in Dutch isolates, while *cap8, sem, seg, sei, icaB, icaC, icaD* and *sen* were variable present.

The presence of virulence factor encoding genes and variation in their presence in extensively genotyped *S. aureus* isolates collected from bovine mastitis was investigated. *Hlb*, *ebps*, *efb*, *sspA* and *sspB* were found in all isolates tested and *coa* in all isolates except one, while *sea, seb, sek, seo, sep, seq, PV- LukS/F, eta, etb, pls* and SCC_{ACME} were completely absent. Variation in presence was shown for 9 toxin genes: *sec3, seg, seh, sei, sel, sem, sen, tst* and *lukE* and 9 adhesion related genes: *fnbA*, *fnbB*, icaB, *icaC*, *icaD*, *clfA, sdrE*, *cna* and *map* and other genes such as *scin, chips*, *cap8* and *cap5*. The clustering of tst, sec3 and sel genes is specific for a bovine pathogenicity island known as SaPIbov (Smyth *et al*., 2005) was present in only part of the isolates. From these data a minimum set of genes which may be required for *S. aureus* bovine mastitis was distilled which includes *hlb, efb, ebps, sspA, sspB* and *fnbA*. However, *efb* has been reported to be present in only part of the isolates by others (Zecconi *et al*., 2006). Probably other virulence factors do contribute to the development and severity of disease. Details of the pathogenesis of bovine mastitis may differ for different gene profiles.

Table 2: Presence and absence of toxin and adhesion gene profiles in different bovine PFGE-types

Virulence gene profile	PFGE profiles (No. of isolate)	*hlb*	SaPIbov[a]	Enterotoxin gene cluster (*egc*)[b]	Other toxin genes	Capsular type	*clfA*	Adhesin genes present
A	A (13)	(+)	(-)	(+)	(-)	8	(-)	*fnbA, fnbB, sdrE, cna, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
A	A1 (1)	(+)	(-)	(+)	(-)	8	(-)	*fnbB, sdrE, cna, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
A	A (1)	(+)	*tst*	(+)	(-)	8	(-)	*fnbA, fnbB, sdrE, cna, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
A	A (3)	(+)	(-)	*seg, sei, sen*	(-)	8	(-)	*fnbA, fnbB, sdrE, cna, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
A	A (4)	(+)	(-)	*seg, sei, sem*	(-)	8	(-)	*fnbA, fnbB, sdrE, cna, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
A	A (1)	(+)	(-)	*seg, sei, sem*	(-)	8	(-)	*fnbA, sdrE, cna, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
B	E (1)	(+)	*tst*	*seg, sei, sem*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, map, coa*
B	E (1)	(+)	(-)	*seg, sei, sem*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, map, coa*
B	D2 (1)	(+)	(-)	*sei, sen*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
B	D3 (1)	(+)	(-)	*seg, sei, sen*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
B	D (1)	(+)	*sec3, tst*	(+)	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
B	D (1)	(+)	*tst*	(+)	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
B	D1 (2)	(+)	(-)	*seg, sei, sem*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
B	D (1), D1 (3), D2 (3), E (4)	(+)	(-)	(+)	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
B	E (1)	(-)	(-)	(+)	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
C	D1 (1)	(+)	*sec3, tst*	*seg, sei, sem*	(-)	8	(-)	*ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
C	E (1)	(+)	(+)	*seg, sei, sem*	(-)	8	(-)	*ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
C	D (1)	(+)	(+)	*seg, sei, sem*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
C	D (6), D1 (2), E (2)	(+)	(+)	(+)	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
C	E (1)	(+)	(+)	*seg, sei, sen*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
C	E1 (1)	(+)	(+)	*seg, sei*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
D	I (1)	(+)	*tst*	(-)	*seh, lukE*	8	(-)	*fnbA, sdrE, cna, ebps, efb ,icaB, icaC, icaD, sspA, sspB, map, coa*
E	B3 (1)	(+)	(-)	(-)	*lukE*	8	(+)	*fnbA, cna, ebps, efb, sspA, sspB,map, coa*
E	C (1), F4 (1)	(+)	(-)	*sen*	*lukE*	8	(+)	*fnbA, cna, ebps, efb, sspA, sspB, coa*
E	F (1)	(+)	(-)	*sen*	(-)	8	(+)	*fnbA, cna, ebps, efb, sspA, sspB,map, coa*
E	B2 (1)	(+)	*sec3*	*sen*	*lukE*	8	(+)	*fnbA, fnbB cna, ebps, efb, sspA, sspB, coa*
E	B4 (1)	(+)	(-)	(-)	*lukE*	8	(+)	*fnbA, fnbB cna, ebps, efb, sspA, sspB, coa*
E	B (1)	(+)	(-)	*sen*	*lukE*	8	(+)	*fnbA, fnbB cna, ebps, efb, sspA, sspB, map*
E	B (1)	(+)	(-)	*sen*	*lukE*	8	(+)	*fnbA, fnbB cna, ebps, efb, icaD, sspA, sspB, map, coa*
E	F3 (1)	(+)	(-)	*sei, sen*	*lukE*	8	(+)	*fnbA, fnbB cna, ebps, efb, sspA, sspB, map, coa*

Table 2: Continued

Virulence gene profile	PFGE profiles (No. of isolate)	*hlb*	SaPIbov[a]	Enterotoxin gene cluster (*egc*)[b]	Other toxin genes	Capsular type	*clfA*	Adhesin genes present
E	B1 (1)	(-)	(-)	*seg*	*lukE*	8	(+)	*fnbA, fnbB cna, ebps, efb, sspA, sspB, map, coa*
E	B5 (1)	(+)	(-)	(-)	*lukE*	8	(+)	*fnbA, fnbB cna, ebps, efb,icaB, icaD, sspA, sspB, map, coa*
E	G (1)	(+)	(-)	(-)	(-)	8	(+)	*fnbA, fnbB cna, ebps, efb, sspA, sspB,sdrE, map, coa*
E	J (1)	(+)	(-)	(-)	*lukE, sak, scin, chips*	8	(+)	*fnbA, fnbB cna, ebps, efb, sspA, sspB, map, coa*
F	B1 (1)	(+)	*sec3*	*sen*	*lukE*	5	(+)	*fnbA, fnbB, ebps, efb, icaB, icaC, icaD, sspA, sspB, map, coa*
F	B1 (1)	(+)	(-)	(-)	*lukE*	5	(+)	*fnbA, fnbB, ebps, efb, icaB, icaC, icaD, sspA, sspB, map, coa*
F	K (1)	(+)	(-)	*sei, sen*	*lukE*	5	(+)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, map, coa*

[a]Bovine staphylococcal pathogenicity island characterized by the presence of *sec*, *sel*, and *tst*. The (+) means *sec*, *sel* and *tst* genes were amplified, while the (-) means none of the genes were detected. Written gene/s depicted their presence. [b]Enterotoxin gene cluster (*egc*) consists of 5 SEs, *seg, sei, sem, sen*, and *seo*. Since all of our isolates lack of *seo*, the (+) indicated the presence of *seg, sei, sem* and *sen*

The virulence factor gene profiles in general agreed with the clustering obtained from the 4 genotyping methods. The exception of cluster B and C was caused by the presence of SaPIbov in a part of the isolates belonging to PFGE types D, D1, D2 and E. This result indicates that SaPIbov was acquired independently by several isolates belonging to different genetic lineages. SaPIbov was present in 19.7% of the isolates and10.5% of all isolates carried either 1 or 2 genes representative for SaPIbov. The *tst* gene has also been reported on other pathogenicity islands (Lindsay *et al*., 1998). Whether the presence of *sec3* and *sel* in two isolates and *sec3* or *sel* in other isolates implicates that SaPIbov is incomplete or that the *sec3* and *sel* can be present on other locations is unknown. Although SaPIbov may contribute to mastitis it not required, because other lineages appear to be equally widespread as cluster C (Fig. 1).

The presence of *hlb, coa* in nearly all isolates is in agreement with other reports (Dinges *et al*., 2000; Zecconi *et al*., 2006). The genes that were not identified among Dutch bovine mastitis isolates were also generally absent from isolates from other countries with the exception of *sea*, *sek*, *seo* and *eta* and *etb* (Fournier *et al*., 2008; Haveri *et al*., 2007, 2008; Monecke *et al*., 2007; O'Riordan and Lee, 2004; Vautor *et al*., 2009; Zecconi *et al*., 2006). *Sea* and *seo* for example have been reported to be present in 77 and 52% of mastitis isolates in Switzerland (Fournier *et al*., 2008). However, Turkish isolates also lacked *sea*, but *eta*, *etb*, *sek* and *seo* were not tested (Karahan *et al*., 2009). The variability of the presence of other toxin genes reported here is in general agreement with other reports (Fournier *et al*., 2008; Haveri *et al*., 2007; Kenny *et al*., 1993; Monecke *et al*., 2007; Zecconi *et al*., 2006). The coexistence of the toxin genes *seg* and *sei* has been described before and further explained by the presence of both genes in a tandem orientation and belong to an operon of enterotoxin gene cluster (*egc*) (Jarraud *et al*., 2001; Karahan *et al*., 2009). In addition, we detected *sem* and *sen* within *seg*- and *sei*- harboring *S. aureus* isolates without the presence of *seo*. This finding is in contrast to those studies which demonstrated that *sem, sen, seg, sei* and *seo* predominate in *S. aureus* from animal hosts (Haveri *et al*., 2007; Smyth *et al*., 2005). The reason for these discrepancies is not known, although the number of different genetic clusters observed among the Dutch isolates was low. In addition, many of the virulence factors are encoded on mobile genetic elements which can easily be lost or exchanged.

None of our isolates were *lukPV*-positive in agreement with the study done by Fueyo *et al.* (2005), although, Zecconi *et al.* (2006) identified *pvl* in more than 50% of isolates from bovine mastitis (Zecconi *et al.*, 2006). However, also among human methicillin-susceptible *S. aureus* only a few percent of the isolates carry the *pvl* genes.

One of the common features of invasive bacterial pathogens is a capsule. In this study, the *cap8* was predominant compared to the *cap5*. Poutrel *et al.* (1988) described the difference in distribution of CP5 and CP8 between clinical *S. aureus* isolates collected from humans and bovines. He observed 51.4% of *S. aureus* from bovine origin were CP5 positive and 18% were CP8 positive, while the percentages of human *S. aureus* harboring CP5 and CP8 were 20 and 60%, respectively. Several studies described that CP5 and CP8 were commonly found in the clinical human *S. aureus* isolates (Sordelli *et al.*, 2000). Since CP 5 and 8 could be recovered from either human or animal derived *S. aureus* isolates, we conclude that CP5 and 8 are non-host specific.

Only one isolate carried the genes for CHIPS and SCIN, which interfere with activation of the human complement system but not the bovine complement system. Both genes are located on the same bacteriophage that is integrated in the *hlb* gene (Van Wamel *et al.*, 2006). The *hlb* product is poorly effective against human erythrocytes, but highly effective against bovine erythrocytes and bacteriophage insertion disrupts the *hlb* gene. Therefore, we consider this isolate as human derived. ST97 isolates have been obtained from both humans and bovines (Smith *et al.*, 2005). The gene profiles of these isolates resemble those of bovine mastitis isolates and lack the human complement specific interference factors (Table 2 and Fig. 1). Based on these data we believe that ST97 *S. aureus* isolates are from bovine origin.

CONCLUSION

In summary the virulence factor profiles with the exception of SaPIbov were generally in agreement with the typing results. This suggests independent mobilization of this pathogenicity island. Five major virulence factor profiles were observed. Of the tested virulence genes *hlb, ebps, sspA, sspB, fnbA* and *coa* were present in all isolates from bovine mastitis.

REFERENCES

Blevins J.S., A.F. Gillaspy, T.M. Rechtin, B.K. Hurlburt and M.S. Smeltzer, 1999. The Staphylococcal accessory regulator (*sar*) represses transcription of the *Staphylococcus aureus* collagen adhesin gene (*can*) in an agr-independent manner. Mol. Microbiol., 33: 317-326.

Bodén, M.K. and J.I. Flock, 1989. Fibrinogen-binding protein/clumping factor from *Staphylococcus aureus*. Infect. Immun., 57: 2358-2363.

Diep, B.A., H.A. Carleton, R.F. Chang, G.F. Sensabaugh and F. Perdreau-Remington, 2006a. Roles of 34 virulence genes in the evolution of hospital- and community-associated strains of methicillin-resistant *Staphylococcus aureus*. J. Infect. Dis., 193: 1495-1503.

Diep, B.A., S.R. Gill, R.F. Chang, T.H. Phan and J.H. Chen, 2006b. Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. Lancet, 367: 731-739.

Dinges, M.M., P.M. Orwin, P.M. Schlievert, 2000. Exotoxins of *Staphylococcus aureus*. Clin. Microbiol. Rev., 13: 16-34.

Downer, R., F. Roche, P.W. Park, R.P. Mecham and T.J. Foster, 2002. The elastin-binding protein of *Staphylococcus aureus* (EbpS) is expressed at the cell surface as an integral membrane protein and not as a cell wall-associated protein. J. Biol. Chem., 277: 243-250.

Foster, T.J., 2005. Immune evasion by staphylococci. Nat. Rev. Microbiol., 3: 948-958.

Fournier, C., P. Kuhnert, J. Frey, R. Miserez and M. Kirchhofer *et al*., 2008. Bovine *Staphylococcus aureus*: Association of virulence genes, genotypes and clinical outcome. Res. Vet. Sci., 85: 439-448.

Fueyo, J.M., M.C. Mendoza, M.R. Rodicio, J. Muñiz, M.A. Alvarez and M.C. Martín, 2005. Cytotoxin and pyrogenic toxin superantigen gene profiles of *Staphylococcus aureus* associated with subclinical mastitis in dairy cows and relationships with macrorestriction genomic profiles. J. Clin. Microbiol., 43: 1278-1284.

George, N.P.E., Q. Wei, P.K. Shin, K, Konstantopoulos and J.M. Ross, 2006. *Staphylococcus aureus* adhesion via Spa, ClfA and SdrCDE to immobilized platelets demonstrates shear-dependent behavior. Arterioscler. Thromb. Vasc. Biol., 26: 2394-2400.

Haveri, M., A. Roslöf, L. Rantala and S. Pyörälä, 2007. Virulence genes of bovine *Staphylococcus aureus* from persistent and nonpersistent intramammary infections with different clinical characteristics. J. Appl. Microbiol., 103: 993-1000.

Haveri, M., M. Hovinen, A. Roslöf and S. Pyörälä, 2008. Molecular types and genetic profiles of *Staphylococcus aureus* strains isolated from bovine intramammary infections and extramammary sites. J. Clin. Microbiol., 46: 3728-3735.

Ikawaty, R., E.C. Brouwer, M.D. Jansen, E. van Duijkeren, D. Mevius, J. Verhoef and A.C. Fluit, 2008. Characterization of Dutch *Staphylococcus aureus* from bovine mastitis using a multiple locus variable number tandem repeat analysis. Vet. Microbiol., 136: 277-284.

Jarraud, S., M.A. Peyrat, A. Lim, A. Tristan and M. Bes *et al*., 2001. *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. J. Immunol., 166: 669-677.

Josefsson, E., K.W. McCrea, D. Ní Eidhin, D. O'Connell, J. Cox, M. Höök and T.J. Foster, 1998. Three new members of the serine-aspartate repeat protein multigene family of *Staphylococcus aureus*. Microbiology, 144: 3387-3395.

Karahan, M., M.N. Açýk and B. Cetinkaya, 2009. Investigation of toxin genes by polymerase chain reaction in *Staphylococcus aureus* strains isolated from bovine mastitis in Turkey. Foodborne Pathog. Dis., 6: 1029-1035.

Kenny, K., R.F. Reiser, F.D. Bastida-Corcuera and N.L. Norcross, 1993. Production of enterotoxins and toxic shock syndrome toxin by bovine mammary isolates of *Staphylococcus aureus*. J. Clin. Microbiol., 31: 706-707.

Lindsay, J.A., A. Ruzin, H.F. Ross, N. Kurepina and R.P. Novick, 1998. The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. Mol. Microbiol., 29: 527-543.

Mahmood, R. and S.A. Khan, 1990. Role of upstream sequences in the expression of the staphylococcal enterotoxin B gene. J. Biol. Chem., 265: 4652-4656.

McDevitt, D., P. Vaudaux and T.J. Foster, 1992. Genetic evidence that bound coagulase of *Staphylococcus aureus* is not clumping factor. Infect. Immun., 60: 1514-1523.

Monecke, S., P. Kuhnert, H. Hotzel, P. Slickers and R. Ehricht, 2007. Microarray based study on virulence-associated genes and resistance determinants of *Staphylococcus aureus* isolates from cattle. Vet. Microbiol., 125: 128-140.

O'Riordan, K. and J.C. Lee, 2004. *Staphylococcus aureus* capsular polysaccharides. Clin. Microbiol. Rev., 17: 218-234.

Peacock, S.J., C.E. Moore, A. Justice, M. Kantzanou and L. Story *et al*., 2002. Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. Infect. Immun., 70: 4987-4996.

Poutrel, B., A. Boutonnier, L. Sutra and J.M. Fournier, 1988. Prevalence of capsular polysaccharide types 5 and 8 among *Staphylococcus aureus* isolates from cow, goat and ewe milk. J. Clin. Microbiol., 26: 38-40.

Regassa, L.B., J.L. Couch and M.J. Betley, 1991. Steady-state staphylococcal enterotoxin type c mRNA is affected by a product of the accessory gene regulator (*agr*) and by glucose. Infect. Immun., 59: 955-962.

Rohde, H., E.C. Burandt, N. Siemssen, L. Frommelt and C. Burdelski *et al*., 2007. Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. Biomaterials, 28: 1711-1720.

Smith, E.M., L.E. Green, G.F. Medley, H.E. Bird and L.K. Fox *et al*., 2005. Multilocus sequence typing of intercontinental bovine *Staphylococcus aureus* isolates. J. Clin. Microbiol., 43: 4737-4743.

Smyth, D.S., P.J. Hartigan, W.J. Meaney, J.R. Fitzgerald, C.F. Deobald, G.A, Bohach and C.J. Smyth, 2005. Superantigen genes encoded by the *egc* cluster and SaPIbov are predominant among *Staphylococcus aureus* isolates from cows, goats, sheep, rabbits and poultry. J. Med. Microbiol., 54: 401-411.

Sordelli, D.O., F.R. Buzzola, M.I. Gomez, L. Steele-Moore and D. Berg *et al*., 2000. Capsule expression by bovine isolates of *Staphylococcus aureus* from Argentina: Genetic and epidemiologic analyses. J. Clin. Microbiol., 38: 846-850.

Van Wamel, W.J.B., S.H.M. Rooijakkers, M. Ruyken, K.P. van Kessel and J.A. van Strijp, 2006. The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on β-hemolysin-converting bacteriophages. J. Bacteriol., 188: 1310-1315.

Vautor, E., V. Magnone, G. Rios, K. Le Brigand and D. Bergonier *et al*., 2009. Genetic differences among *Staphylococcus aureus* isolates from dairy ruminant species: A single-dye DNA microarray approach. Vet. Microbiol., 133: 105-114.

Zecconi, A., L. Cesaris, E. Liandris, V. Daprà and R. Piccinini, 2006. Role of several *Staphylococcus aureus* virulence factors on the inflammatory response in bovine mammary gland. Microb. Pathog., 40: 177-183.