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Genotyping of *Staphylococcus aureus* isolated from humans, bovine subclinical mastitis and food samples in Argentina

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Summary

The aim of the present study was to characterize genotypically 45 *Staphylococcus aureus* strains isolated from humans, bovine subclinical mastitis and food samples in Argentina by rep-PCR and PCR amplification of virulence genes. Resistances to various antibiotics could be observed for the human *S. aureus*, less pronounced for the bovine strains, but not for the eight *S. aureus* isolated from food samples. The strains could be classified genotypically by rep-PCR and by amplification of the genes encoding protein A, coagulase, clumping factor, the collagen adhesin domains A and B, capsular polysaccharide 5 and 8, the accessory gene regulator *agr* classes I to III, and the *S. aureus* gene regulator *sae*. rep-PCR analyses and the different gene patterns revealed that the strains could be divided into seven groups mostly matching with the origin of the isolates. The present study describes genotypic variations of *S. aureus* strains isolated from different origins in Argentina. The study provides a valuable insight into molecular specificities of this important pathogen.

Introduction

Staphylococcus aureus is recognized worldwide as an important pathogen. These bacteria have a great versatility in their ability to colonize different hosts and cause infections at different anatomic sites. The molecular processes responsible

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for disease and host specificity are poorly understood but are presumed to be caused in part by differences in gene content and by allelic variations between strains.

Genotypical differences between *S. aureus* isolated from humans and bovines had been observed by numerous authors (Kapur et al., 1995; Zadoks et al., 2000; Reinoso et al., 2004). In addition, genome sequencing of human and bovine strains identified notable differences in both genomes and provides a framework for the identification of specific factors associated with host specificity in this major human and animal pathogen (Herron et al., 2002). However, at present little is known about *S. aureus* populations isolated from different hosts.

The aim of the present study was to characterize genotypically *S. aureus* strains isolated from humans, bovines and food samples in Argentina by rep-PCR and PCR amplification of virulence genes.

Materials and methods

Bacterial strains and identification

This study included 45 unrelated *S. aureus* isolates collected from humans, bovines and food samples in Argentina between 1999 and 2000. Among these, 15 isolates were obtained from infections of humans (bloodstream $n = 3$, catheter tips $n = 6$, bone $n = 1$, respiratory tract $n = 1$, surgical wound $n = 1$, urine infection $n = 1$ and skin infections $n = 2$), seven isolates were obtained from anterior nares of apparently healthy persons, 15 isolates from bovine subclinical mastitis and eight from food samples (ice cream $n = 7$; cake $n = 1$). All 15 isolates from human infections were collected in different private clinics. Bovine isolates were collected from cases of subclinical mastitis in different herds located in Buenos Aires according to the recommendations of the National Mastitis Council methods (National Mastitis Council, 1987). All isolates were previously identified as *S. aureus* by a standard microbiological procedure.

Antibiotic susceptibility testing

Antibiotic sensitivity of the bacteria was tested by the standardized agar diffusion test (Bauer et al., 1966) on Müller-Hinton agar (Laboratorios Britania, Buenos Aires, Argentina) using the following antibiotic disks: cephalothin (30 μg), erythromycin (15 μg), kanamycin (30 μg), novobiocin (30 μg), streptomycin (10 μg), penicillin (10 IU) and tetracycline (30 μg) (Laboratorios Britania). The antibiotic susceptibility test was performed as recommended by the National Committee for Laboratory Standards (NCCLS) (1999) with *S. aureus* ATCC 25923 as reference strain.

rep-PCR

Genomic DNA was isolated as previously described (Reinoso et al., 2004). A rep-PCR assay was carried out as described by van Belkum et al. (1995). For the rep-PCR assay, approximately 25 ng chromosomal DNA were used per reaction. Amplifications were conducted in 25 μl buffer solution containing 3 μM oligonucleotide primers, 200 μM of each deoxynucleoside triphosphate (Promega, Madison, WI, USA), 3.5 mM $MgCl_2$ and 2.5 U DNA *Taq* polymerase (Promega). Table 1 shows the sequences of the oligonucleotide primers and the temperature programme. The amplified products were separated by electrophoresis in 1.5% agarose gels (Promega) in 40mM Tris, 1 mM EDTA, 1.14 ml/l glacial acetic acid (pH 7.6) at constant voltage of 4 V/cm, and stained with 0.5 μg/ml ethidium bromide. Pattern analysis was performed as described (Reinoso et al., 2004).

Genotypic characterization

DNA amplification of the genetic determinants for virulence or regulatory traits was investigated using oligonucleotide primers derived from published sequences. This analysis included the genes encoding the immunoglobulin G binding region and X region of staphylococcal protein A (*spa*), coagulase (*coa*), clumping factor (*clfA*), the collagen adhesin domains A (*cna*A) and B (*cna*B), the capsular polysaccharide 5 (*cap*5) and 8 (*cap*8), the acessory gene regulator (*agr*) classes I to III and the *S. aureus* exoprotein expression gene (*sae*). The sequences of the oligonucleotide primers, thermocycler programmes, and the corresponding references are summarized in Table 1.

The presence of PCR products was determined by electrophoresis of 10 μl of the reaction products in 2% agarose gels in 89 mM Tris-borate, 2 mM EDTA, pH 8.3, at 120 V and visualized under UV light using the Gel Doc 1000 system (BioRad, München, Germany).

Statistics

A χ^2 test was used to determine the significance of occurrence of genes in a host specific group by

Table 1. Oligonucleotide primers and PCR programmes

Target gene	Sequence (5′–3′)	PCR programme[a]	Reference
rep	TCG CTCA AAA CAA CGA CAC C	1	van Belkum et al. (1995)
spa (X-region)	CAA GCA CCA AAA GAG GAA CAC CAG GTT TAA CGA CAT	2	Frénay et al. (1996)
spa (IgG-binding region)	CAC CTG CTG CAA ATG CTG CG GGC TTG TTG TTG TCT TCC TC	3	Seki et al. (1998)
coa	ATA GAG ATG CTG GTA CAG G GCT TCC GAT TGT TCG ATG C	3	Hookey et al. (1998)
*clf*A	GGC TTC AGT GCT TGT AGG TTT TCA GGG TCA ATA TAA GC	4	Stephan et al. (2000)
cna (A domain)	ATA TGA ATT CGA GTA TAA GGA AGG GGT T TTT GGA TCC CTT TTT CAG TAT TAG TAA CCA	5	Switalski et al. (1993)
cna (B domain)	AGT GGT TAC TAA TAC TG CAG GAT AGT TGG TTT A	5	Switalski et al. 1993
cap 5	ATG ACG ATG AGG ATA GCG CTC GGA TAA CAC CTG TTG C	6	Moore and Lindsay (2001)
cap 8	ATG ACG ATG AGG ATA GCG CAC CTA ACA TAA GGC AAG	7	Moore and Lindsay (2001)
agr I	CAC TTA TCA TCA AAG AGC C CCA CTA ATT ATA GCT GG	8	Moore and Lindsay (2001)
agr II	GTA GAG CCG TAT TGA TTC C GTA TTT CAT CTC TTT AAG G	9	Moore and Lindsay (2001)
agr III	CTG CAT TTA TTA GTG GAA TAC G GTT TCA TTT CTT TAA GAG	10	Moore and Lindsay (2001)
sae	TGT GGG GTT CAG GAA TTG TT ATT GAT GAG AAG GAT GCC CA	11	Giraudo et al. (1999)

[a]1: 36 times 93 °C–1 min, 54 °C–1:30 min, 72 °C–1.30 min; 2: 30 times 94 °C–1 min, 60 °C–1 min, 72 °C–1 min; 3: 30 times 94 °C–1 min, 58 °C–1 min, 72 °C–1 min; 4: 35 times 94 °C–1 min, 57 °C–1 min, 72 °C–1 min; 5: 30 times 94 °C–30 s, 50 °C–30 s, 72 °C–1 min; 6: 20 times 94 °C–15 s, 57 °C–15 s, 72 °C–30 s; 7: 20 times 94 °C–15 s, 52 °C–15 s, 72 °C–30 s; 8: 20 times 94 °C–30 s, 57 °C–30 s, 72 °C–1 min; 9: 20 times 94 °C–15 s, 60 °C15 s, 72 °C–15 s; 10: 20 times 94 °C–30 s, 55 °C–45 s, 72 °C–1 min; 11: 29 times 93 °C–1 min, 62 °C–30 s, 72 °C–1 min.

Table 2. Antibiotic resistances among 45 *S. aureus* strains isolated from different origins

Isolates from	Resistance to													
	Cp		Em		Km		Nb		Pn		Sm		Tc	
Human infections	1	7%	7	47%	3	20%	2	13%	13	87%	7	47%	6	40%
Anterior nares	—	—	1	14%	1	14%	—	—	6	86%	2	28%	1	14%
Bovine mastitis	—	—	—	—	—	—	—	—	4	27%	4	27%	—	—
Food samples	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Cp = cephalothin, Em = erythromycin, Km = kanamycin, Nb = novobiocin, Pn = penicillin, Sm = streptomycin, Tc = tetracycline.

use of Statgraphics version 5.1. The nominal *P* value for statistical significance was 0.05.

Results

In this study, 45 *S. aureus* strains collected from different origins were characterized genotypically. The results from the antibiotic resistance studies of the investigated *S. aureus* strains are shown in Table 2. Among the human strains, 19 (86%) were resistant to penicillin, nine (41%) to streptomycin, eight (36%) to erythromycin, seven (32%) to tetracycline, four (18%) to kanamycin, two (9%) to novobiocin and one to cephalotin (4%). Among the bovine mastitis strains, four (27%) strains were resistant to penicillin and four (27%) to streptomycin. The remaining *S. aureus* strains isolated from food samples were susceptible to all antibiotics tested.

S. aureus strains were assayed genotypically by rep-PCR (Fig. 1). The subsequent cluster analysis suggested the existence of 35 rep profiles which

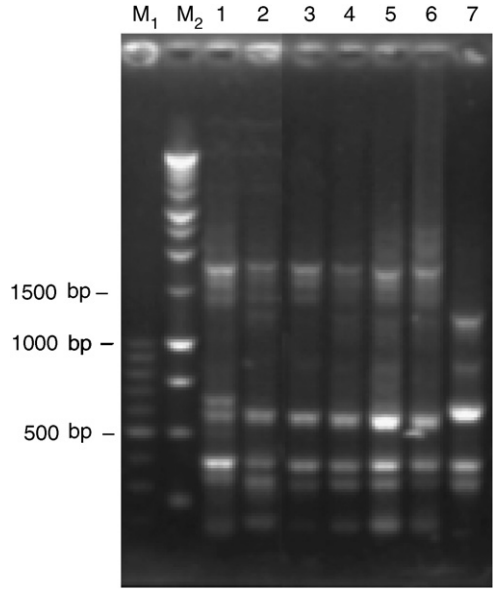

Figure 1. Typical rep-PCR fingerprints of seven *S. aureus* strains representing rep-group II (1–6) and III (7). M_1: 100 bp molecular weight marker (Promega). M_2: 1 kb molecular weight (Promega).

could be divided by dendrogram analyses into seven different groups at 60% of relative genetic similarity designated I–VII. The groups generally matched with the origin of the isolates. Group II consists of 13 (87%) human infection strains, groups III and IV of 14 (93%) bovine mastitis strains and groups V and VII of five (71%) strains isolated from anterior nares of healthy people. Six (75%) food sample strains were grouped together with the human strains in group II. The rep-PCR group of the remaining *S. aureus* strains did not match with the origin of the strains (Fig. 2).

The 45 *S. aureus* strains were additionally investigated for various virulence genes. PCR amplification of the gene segment encoding the IgG binding region of protein A revealed a size of approximately 900 bp (five repeats according to Seki et al., 1998) for 42 isolates and a size of approximately 700 bp (four repeats) for three of the investigated isolates (Fig. 3a). The amplification of the gene segments encoding the X region of protein A revealed typical size polymorphisms ranging from 100 to 315 bp (2–10 repeats according to Frénay et al., 1996) (Fig. 3b). Strains isolated from humans had different size amplicons and consequently different numbers of repeats compared to the strains isolated from bovines and food samples. Twelve (80%) bovine mastitis strains showed 300 and 280 bp indicating that strains of this origin possessed 10 and 9 repeats. Six (75%) strains isolated from food samples had 300 and 315 bp amplified fragment indicating that food samples strains possessed 10 and 11 repeats.

PCR amplification of the gene *coa* yielded seven different *coa* types with amplicon sizes ranging from 400 to 1000 bp (according to Hookey et al., 1998) (Fig. 3c). Seven (42%) human infection strains had *coa* polymorphic regions with an amplicon of 700 bp, indicating the presence of five repeats. Six (40%) of the bovine strains showed an amplicon of 900 bp, indicating the presence of seven repeats and four (50%) strains from food samples showed four repeats. PCR amplification of the clumping factor gene *clf*A yielded an amplicon size of 1000 bp for 40 of the investigated *S. aureus*. Four strains from bovine origin and one strain from food sample had an amplicon size of 900 bp. The amplification of the genes *cna* (A and B domain) resulted in five strains of human origin with amplicons with sizes of approximately 1200 and 1700 bp, respectively. The gene *cap5* could be observed for 21 (47%) *S. aureus* strains (11 human, nine bovine and one food sample strain), whereas the gene *cap8* for seven (15%) *S. aureus* strains (four human and three food sample strains). Seventeen cultures (38%) were non-*cap5* or -*cap8*. All bovine strains were negative with capsular polysaccharide type 8 oligonucleotide primers. With the oligonucleotide primers for the genes *agr*-classes I–III, eight (36%) human strains were *agr* class III, the remaining 14 strains belonged to *agr* classes I and II (14% and 27%, respectively). Seven (47%) bovine mastitis strains were assigned to *agr* class II and the remaining eight strains belonged to *agr* classes I and II (27% and 13%, respectively). Ten (22%) *S. aureus* strains were negative for the *agr*-classes I to III oligonucleotide primers. An amplification of the *sae* gene could be observed in 23 (51%) strains (12 human, eight bovine and three from food sample strains). Genes *spa X*-region, *cna* and *sae* were significantly associated with a specific host ($P<0.007$, $P<0.01$ and $P<0.05$, respectively). All other genes were not significant at the 0.05 significance level. The genotypic characteristics of the *S. aureus* strains are summarized in Table 3.

Discussion

Antibiotic resistances appeared to be more pronounced among the *S. aureus* isolated from humans compared to the *S. aureus* isolated from bovine subclinical mastitis or from food samples. This enhanced antibiotic resistance of the human *S. aureus* strains might be caused by horizontal gene transfer under the pressure of an enhanced anti-

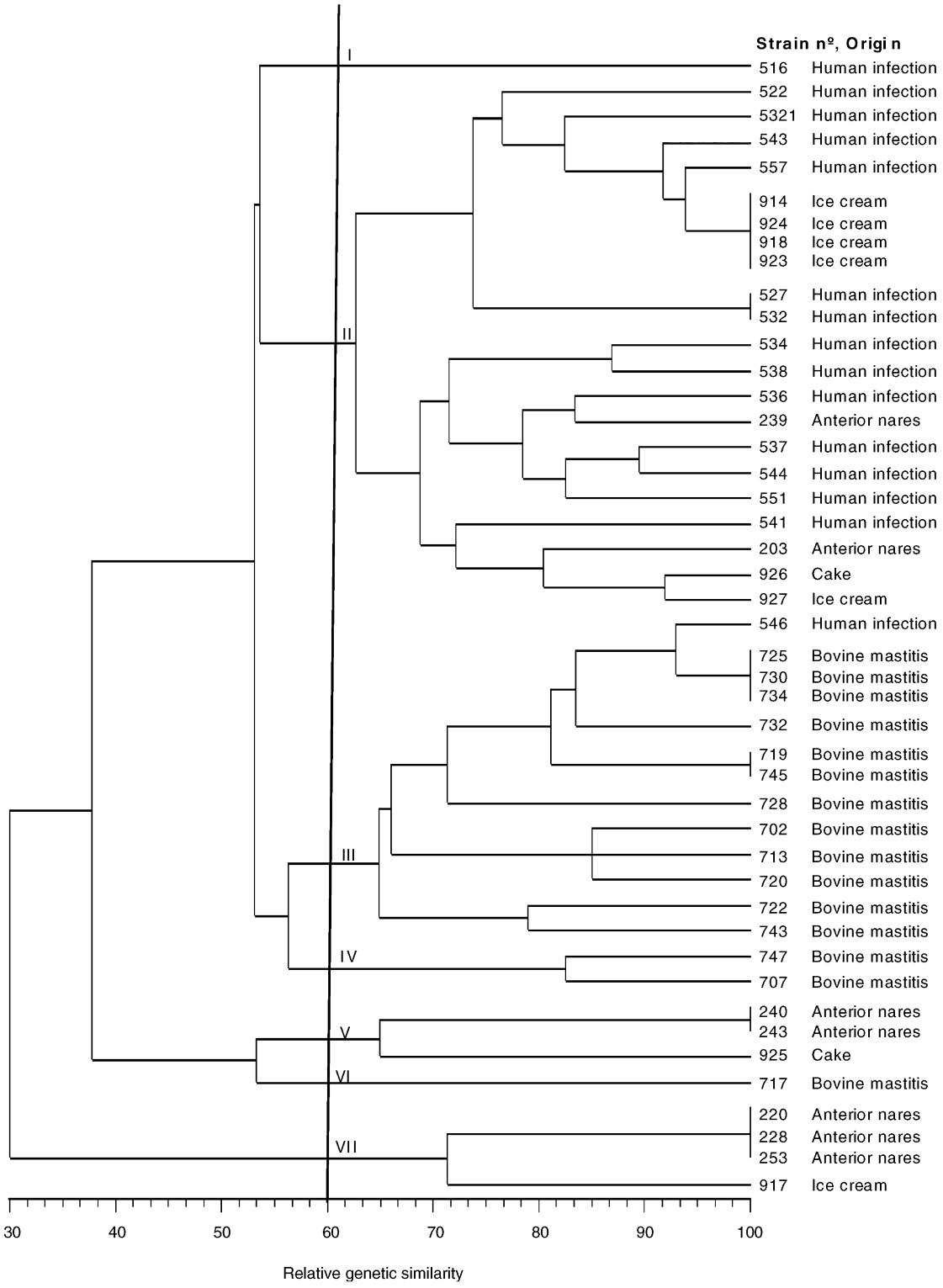

Figure 2. Genetic relationship between the *S. aureus* strains determined by rep-PCR clustering analysis.

biotic treatment of the host. These results also indicate that *S. aureus* of bovine origin or from food samples, at least the strains investigated in the present study, seemed to be of minor importance for the resistance situation of *S. aureus*. The food sample strains differ in antibiotic resistances compared to the strains isolated from humans or bovines.

The *S. aureus* strains were further analyzed by rep-PCR fingerprinting. This allowed a division of

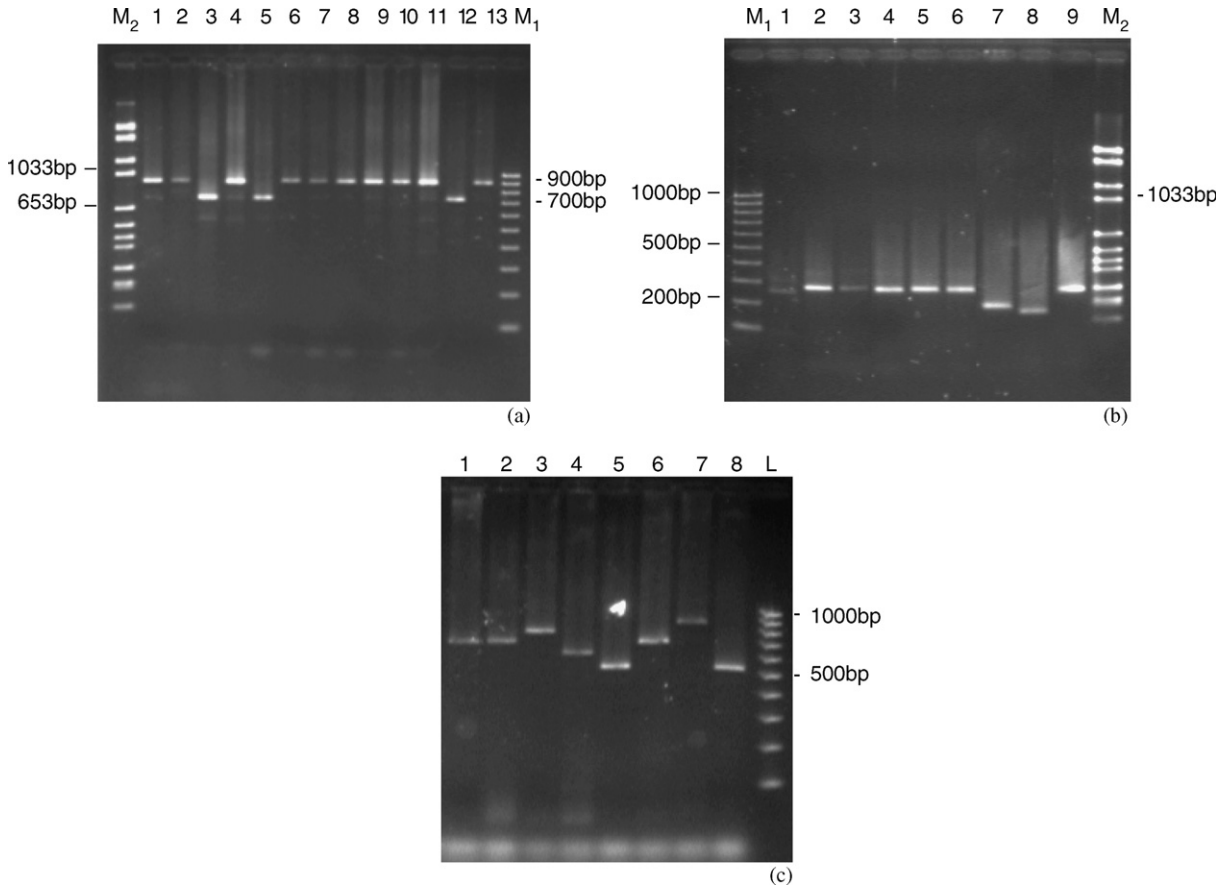

Figure 3. Typical amplicons of *spa* gene segments encoding the IgG binding region with sizes of 700 bp (3, 5 and 12) and 900 bp (1, 2, 4, 6–11, 13) (a) and the X-region of protein A with sizes of 150 bp (8), 180 bp (7) and 280 bp (1–6 and 9) (b); the genes encoding staphylococcal coagulase with sizes of 500 (5 and 8), 600 (4), 700 bp (1, 2 and 6), 800 (3). M_1 = 100 bp molecular weight marker (Gibco). M_2 = molecular weight marker VI (Roche).

the strains into seven groups. The rep-profiles of the *S. aureus* isolated from humans were clearly distinct from the profiles of the bovine strains. Comparable findings were reported earlier assaying *S. aureus* strains by RAPD-PCR (Reinoso et al., 2004). The results are also in agreement with Herron et al. (2002) who reported that the genome of bovine *S. aureus* is characterized by numerous genes which appear to be host specific.

A molecular identification and characterization of the *S. aureus* of the present investigation was additionally performed by PCR amplification of virulence genes. The determination of the size of the IgG binding region encoding segments of the *spa* gene revealed an identical amplicon size of about 900 bp for 42 *S. aureus* investigated, indicating that the *spa* gene of these strains encodes five IgG binding domains (Uhlen et al., 1984). However, the *spa* gene of three human strains yielded amplicon sizes of 700 bp; a lack of one domain may be assumed for these strains. Amplification of the *X* region encoding part of *spa* gene yielded different amplicon sizes for human, bovine and food sample strains, with a specific relation between the repeat number and the origin.

Amplification of the *coa* gene of the *S. aureus* strains yielded various gene polymorphisms. An amplicon of 700 bp was present in seven (47%) of the human infection strains, indicating the presence of five repeats. On the other hand, an amplicon of 900 bp was present in six (50%) *S. aureus* strains isolated from bovine. Shopsin et al. (2000) analyzed human MRSA strains which varied in *coa* gene size from three to seven repeats while *coa* genes with five repeats predominated. Schlegelova et al. (2003) analyzed *S. aureus* strains colonizing cows and humans by restriction fragment length polymorphism analysis of the *coa* gene and reported that *S. aureus* isolated from dairy cows and humans from one farm differed in coagulase genotype.

Table 3. Genotypic characteristics of the 45 *S. aureus* strains

Strain designed	Origin	Rep group	*spa* (IgG-binding region) size (bp)	Repeat number	*spa X*- region (bp)	Repeat number	Coa (bp)	Repeat number	*clfA* (bp)	*cna*[a] *(A and B)*	*cap*[b]	*agr* class[c]	*sae* (bp)[d]
516	HI	I	900	5	250	8	700	5	1000	–	–	III	–
522	HI	I	900	5	100	2	700	5	1000	–	–	–	–
527	HI	I	900	5	280	9	700	5	1000	+	5	II	+
532	HI	I	700	4	180	5	600	4	1000	+	8	I	+
532–1	HI	I	900	5	280	9	400	1	1000	–	5	–	+
534	HI	I	900	5	280	9	700	5	1000	–	5	–	+
536	HI	I	900	5	280	9	500	2	1000	+	–	III	–
537	HI	I	700	4	300	10	1000	8	1000	+	5	II	+
538	HI	I	900	5	280	9	700	5	1000	–	5	II	–
541	HI	I	900	5	280	9	700	5	1000	–	5	II	+
543	HI	I	900	5	280	9	1000	8	1000	–	5	–	+
544	HI	I	900	5	280	9	800	6	1000	–	5	III	+
546	HI	II	900	5	180	5	600	4	1000	–	5	II	+
551	HI	I	900	5	280	9	500	2	1000	–	8	III	+
557	HI	I	900	5	150	4	700	5	1000	+	8	III	+
220	AN	VI	900	5	100	2	500	2	1000	–	–	II	–
228	AN	VI	900	5	150	4	900	7	1000	–	–	III	–
253	AN	VI	900	5	300	10	500	2	1000	–	8	III	–
203	AN	I	900	5	300	10	900	7	1000	–	5	I	–
239	AN	I	700	4	150	4	900	7	1000	–	5	I	+
240	AN	IV	900	5	150	4	900	7	1000	–	–	III	–
243	AN	IV	900	5	250	8	500	2	1000	–	–	–	–
702	BM	II	900	5	300	10	500	2	1000	–	5	II	–
713	BM	II	900	5	280	9	500	2	1000	–	5	–	+
719	BM	II	900	5	300	10	1000	8	1000	–	5	II	+
720	BM	II	900	5	300	10	400	1	900	–	–	II	–
722	BM	II	900	5	280	9	600	4	900	–	–	II	–
725	BM	II	900	5	280	9	900	7	1000	–	5	III	+
728	BM	II	900	5	250	8	900	7	1000	–	5	I	+
730	BM	II	900	5	280	9	400	1	900	–	–	I	–
732	BM	II	900	5	280	9	900	7	1000	–	5	I	+
734	BM	II	900	5	300	10	900	7	1000	–	5	II	+
743	BM	II	900	5	280	9	500	2	900	–	–	I	–
745	BM	II	900	5	300	10	900	7	1000	–	5	III	+
747	BM	III	900	5	100	2	600	4	1000	–	5	II	+
707	BM	III	900	5	100	2	900	7	1000	–	–	–	–
717	BM	V	900	5	280	9	600	4	1000	–	–	II	–
914	FS	I	900	5	315	11	600	4	1000	–	8	II	+
917	FS	VI	900	5	300	10	400	1	900	–	–	–	–
918	FS	I	900	5	315	11	600	4	1000	–	8	II	–
923	FS	I	900	5	100	2	1000	8	1000	–	–	–	–
924	FS	I	900	5	315	11	600	4	1000	–	8	II	+
925	FS	IV	900	5	300	10	600	4	1000	–	–	–	–
926	FS	I	900	5	300	10	700	5	1000	–	5	II	+
927	FS	I	900	5	200	6	700	5	1000	–	–	II	–

HI = human infection, AN = anterior nares of healthy humans, BM = bovine mastitis, FS = food sample.
[a]Uniform sizes of 1700 bp (*cna*A domain), 1200 bp (*cna*B domain).
[b]880 bp (*cap5*), 1150 bp (*cap8*).
[c]350 bp, 460 bp and 550 (*agr* classes I–III, respectively).

According to the amplicons size displayed by amplification of the X region and the *coa* gene, the *S. aureus* food sample strains seemed to be related to bovine strains. This result may indicate a cross contamination of these strains, which should be confirmed with a larger number of strains.

In agreement with previous studies (Stephan et al., 2000), the amplification of clumping factor (*clfA*) gene revealed a single amplicon with a size of approximately 1000 bp for most of the strains investigated, indicating no size polymorphisms and no significant differences among *S. aureus* isolates from different origins.

The additionally investigated gene *cna* could only be detected for five human strains. According to Patti et al. (1992) *cna* is the only recognized gene that encodes an adhesin that specifically binds collagen. Corresponding to the present findings, Smeltzer et al. (1997) reported that *S. aureus* strains do not generally possess *cna*.

In the present study, 21 and seven *S. aureus* strains harboured the genes *cap5* and *cap8*, respectively. Seventeen strains (38%) were negative for both genes. These results are in agreement with the findings of Guidry et al. (1998) suggesting that capsular types 5 and 8 are the most prevalent capsular types in human and bovine *S. aureus*. Corresponding to Sordelli et al. (2000), who found a low prevalence of *cap5* and *cap8* among bovine *S. aureus* strains in Argentina, *cap8* could not be observed among the bovine *S. aureus* strains of the present study.

Investigation of the *S. aureus* strains for the genes *agr* I to III revealed that the occurrence of the *agr* class seemed not to be related to the origin of the strains. These results are in agreement to Gilot and van Leeuwen (2004), who reported that the repartition of human isolates in the *agr* interference groups (I–IV) was similar to what was found for bovine isolates. 21 *S. aureus* strains of the present investigation were negative for *sae*, possibly indicating a variant allele of this gene. Virulence gene expression in *S. aureus* is controlled by regulator genes like *agr* and *sae*. However, little is know about the distribution of *agr* types in strains of different origin and, to our knowledge, this is the first report that characterizes the *sae* gene of *S. aureus* strains isolated from different origins.

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