FULL PAPER *Bacteriology*

Characterization of Genes Encoding Virulence Determinants and Toxins in *Staphylococcus aureus* from Bovine Milk in Central Italy

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(Received 15 April 2010/Accepted 28 June 2010/Published online in J-STAGE 9 July 2010)

ABSTRACT. The aim of this study was to evaluate the genotypic characteristics of *Staphylococcus aureus* isolates (n=170) from bovine milk collected from seven dairy farms in Italy. On the basis of cultural and biochemical properties and by amplification of the 23S rRNA specific to *S. aureus*, all isolates were identified as *S. aureus*. To genotypically characterize *S. aureus* isolates, genes encoding virulence determinants (*nuc, clfA, spa-IgG-binding, spa*-X-region, *fnbA* and *fnbB*, *cap5* and *cap8*) and staphylococcal enterotoxins (*sea, seb, sec, sed, see, seg, seh, sei, sej*) were investigated using a PCR technique. The results showed that the isolates of *S. aureus* in each farm had the same genotypic characteristics, while the isolates genotipically differed between the different farms. The present study might help to understand the distribution of prevalent *S. aureus* strains in dairy farms.

KEY WORDS: bovine, PCR, staphylococcal enterotoxins, *Staphylococcus aureus*, virulence determinants.

J. Vet. Med. Sci. 72(11): 1443–1448, 2010

Staphylococcus aureus is recognized worldwide as a major pathogen causing subclinical intramammary infections (IMI) and considerable economic loss to dairy farmers. Some authors have shown that in different countries, only a few *S. aureus* clones are causing most of the cases of bovine mastitis and that these clones have a broad geographic distribution [2]. The main reservoir of *S. aureus* seems to be the infected quarter, and transmission between cows usually occurs during milking.

S. aureus produces a spectrum of extracellular protein toxins (exotoxins) and other virulence factors, but not all factors are produced by each strain [3]. *S. aureus*, like other Gram-positive bacteria, has numerous surface proteins, called "*microbial surface components recognizing adhesive matrix molecules*" (MSCRAMMs), that mediate adherence to host tissues. MSCRAMMs bind molecules such as collagen, fibronectin and fibrinogen, and different MSCRAMMs may adhere to the same host-tissue component. Different *S. aureus* strains may have different constellations of MSCRAMMs and so may be predisposed to causing certain kinds of infections [27].

Some strains of *S. aureus* have the ability to produce a variety of particular exoproteins, called Staphylococcal Enterotoxins (SEs) [3]. The SEs are resistant to inactivation by gastrointestinal proteases such as pepsin. Additionally, they display strong thermoresistance, a very important property of SEs for food safety considerations and a potential problem for public health. In fact, enterotoxigenic *S. aureus* is one of the major pathogens causing food poisoning cases worldwide. The SEs have been divided into five serological types (SEA to SEE) on the basis of their antigenicities [12]. Recently, the occurrence of new types of SEs (SEG to SE1V) has been reported [4, 5, 17, 22, 35]. The objective of this study was to characterize *S. aureus* isolates from milk samples for virulence genes in order to identify strains in different farm settings.

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MATERIALS AND METHODS

Phenotypic characterization: The research work was carried out between 2008 and 2009 on seven dairy farms (Fig. 1). A total of 170 *S. aureus* isolates were cultured from 1257 quarter milk samples collected from 320 cows using standard procedures of the National Mastitis Council (NMC, 1999). The dairy cows were affected by subclinical mastitis, as assessed by the increase of the somatic cell count (SCC).

One hundred μl of milk sample was plated on 5% defibrinated sheep blood agar (Blood Agar Base, BAB, Oxoid, Milan, Italy), on Mannitol Salt Agar (MSA, Oxoid, Milan, Italy) with 5% Egg Yolk Emulsion (Oxoid, Milan, Italy) and on Baird-Parker Agar Base (BPA, Oxoid, Milan, Italy) with RPF Supplement (RPF Supplement, Oxoid, Milan, Italy) and incubated aerobically at 37°C for 24 hr. The isolates were identified as *S. aureus* on the basis of morphology of colonies, hemolytic properties, Gram staining, catalase reaction, tellurite reduction, lecithinase activity, ability to coagulate rabbit plasma (tube coagulase test) and ability to produce DNase and TNase (DNase agar and T-test, Oxoid, Milan, Italy, respectively). The identification was confirmed by biochemical tests (API 20 Staph, BioMérieux Ita-

Fig. 1. Geographical distribution of the farms (Copyright Google Maps).

lia S.p.A., Florence, Italy).

Genotypic characterization: The genomic DNA of each *S. aureus* isolate was extracted using a Charge SwitchgDNA Mini Bacteria Kit (Invitrogen, Carlsbad, CA, U.S.A.), as described by the manufacturer. The PCR reaction mix (30 μl) included 2.5 μl DNA, 10X PCR buffer, 1.5 mM $MgCl_2$, 200 μM of dNTPs, 0.2 μM of each primer (Invitrogen, Carlsbad, CA, U.S.A.) and 1.5 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.). Amplification of the genes was carried out with a GeneAmp PCR System 2400 thermal cycler (Applied Biosystems, Foster City, CA, U.S.A.). Amplified products were separated by agarose gel electrophoresis (1.5%–2% agarose gel containing 4 μl of GelRed^{TM} Nucleic Acid Gel Stain, 10,000X in water per 100 m*l*) (Biotium, Hayward, CA, U.S.A.) at 5 V/cm for 2 hr and photographed in an ultraviolet transilluminator system (Euroclone S.p.A., Milan, Italy) using a DigiDoc-It system (UVP, Celbio S.p.A., Milan, Italy).

Molecular identification was conducted for the detection of the *S. aureus* 23S rRNA gene by using species-specific primers described by Salasia *et al.* [28].

The genetic determinants for the following virulence traits were investigated using oligonucleotide primers derived from the published sequences: this included the genes encoding clumping factor (*clfA*) [34], X-region [16] and IgG binding-region of protein A (*spa*) [29], SEs: *sea* [37], *seb, sec, sed, see* [21], *seg, seh, sei* [19] and *sej* [25], thermonuclease (*nuc*) [8], fibronectin binding protein A (*fnbA*) and fibronectin binding protein B (*fnbB*) [6] and capsular polysaccharide 5 (*cap5*) and 8 (*cap8*) [26]. The amplified products were sequenced. The sequences of the oligonucleotide primers, sizes of amplified products and temperature programs are summarized in Table 1.

RESULTS

According to the results of tests for cultural and biochemical (coagulase, DNase and TNase tests) properties as well as amplification of the 23S rRNA specific to *S. aureus*, all 170 isolates analyzed in the present investigation were identified as *S. aureus*. The genotypic characteristics of the 170 *S. aureus* isolates are summarized in Table 2.

Overall, 17 genes were analyzed, and all the isolates were negative for seven of them, particularly for the *cap8*, *seb, sec, see, seg, seh* and *sei* genes (Fig. 2). All the other investigated genes were observed in the isolates from farm A and farm B that only differed each other in the amplicon of *spa-IgG-binding*. In farm A, the amplicon was a product of 970 bp, whereas in farm B the amplicon was a product of 810 bp, showing size polymorphisms that have already been described [1, 23, 28]. Farm C showed the same profile as farm B. Farm D showed the same profile as farm A, with the exception of the *fnbA* and *cap5* genes. The isolates from farm E showed the poorest profile, being positive only for the *nuc*, *spa X-region* and *cap5* genes. The isolates from farm F showed the same profile as farm A, with the exception of *sed* and *sej*. The isolates from farm G were positive only for *nuc, spa-IgG-binding* (810 bp), *spa X-region*, *fnbA* and *sea*. It seems clear that the isolates of *S. aureus* from each farm have the same genotypic profile, while they are genotypically distinct between the different farms, with the only constant features that all the isolates were negative for *cap8*, *seb, sec, see, seg, seh* and *sei* and positive for the *nuc* and *spa X-region* genes.

Size polymorphism has been reported [1, 23, 28] for *spa-IgG-binding* and *clfa* genes, but in the present study, it was detected only for *spa-IgG-binding*, while amplification of the *clfa* gene resulted in a single amplicon (approximately 1,000 bp). It should be noted that *seh* gene was produced as an amplicon of approximately 600 bp rather than 375 bp in the isolates from farms A, B and F. These results require further studies because the 600 bp amplicon has not been reported previously. The PCR product sequences were compared with NCBI database sequences and showed 99% homology with *nuc, clfA, spa IgG-binding, spa X-region, fnbA, fnbB, cap5, sea, sed* and *sej*.

DISCUSSION

Identification of the 170 *S. aureus* isolates from dairy milk was performed by conventional methods and by using PCR technology. The latter uses primers targeted at species-specific parts of the gene encoding the 23S rRNA and the genes encoding clumping factor (*clfa*), segments encoding the immunoglobulin G-binding region of protein A (*spa-IgG-binding*) and X-region of protein A (*spa*-X-region) and stable thermonuclease activity (*nuc*). Comparable PCR-

Table 1. Primers and PCR conditions for amplification of staphylococcal genes

Gene	Sequence (5'–3')	PCR program*	Size of amplified products (bp)
23S rRNA	Forward:ACG GAG TTA CAA AGG ACG AC Reverse AGC TCA GCC TTA ACG AGT AC	9	1250
clfA	Forward:GGCTTCAGTGCTTGTAGG Reverse:TTTTCAGGGTCAATA TAAGC	1	1042
spa (IgG-binding)	Forward: CACCTGCTGCAAATGCTGCG Reverse: GGCTTGTTGTTGTCTTCCTC	1	810, 970
spa (X-region)	Forward: CAAGCACCAAAAGAGGAA Reverse: CACCAGGTTTAACGACAT	2	253
nuc	Forward:TGCTATGATTGTGGTAGCCATC Reverse:TCTCTAGCAAGTCCCTTTTCCA	3	420
sea	Forward: AAAGTCCCGATCAATTTATGGCTA Reverse: GTAATTAACCGAAGGTTCTGTAGA	3	216
seb	Forward:TCGCATCAAACTGACAAACG Reverse:GCAGGTACTCTATAAGTGCC	4	478
sec	Forward:GACATAAAAGCTAGGAATTT Reverse:AAATCGGATTAACATTATCC	4	257
sed	Forward:CTAGTTTGGTAATATCTCCT Reverse:TAATGCTATATCTTATAGGG	4	317
see	Forward:TAGATAAGGTTAAAACAAGC Reverse:TAACTTACCGTGGACCCTTC	4	170
seg	Forward:AATTATGTGAATGCTCAACCCGATC Reverse:AAACTTATATGGAACAAAAGGTACTAGTTC	4	642
seh	Forward:CAATCACATCATATGCGAAAGCAG Reverse:CATCTACCCAAACATTAGCACC	4	375
sei	Forward:CTCAAGGTGATATTGGTGTAGG Reverse:AAAAAACTTACAGGCAGTCCATCTC	4	576
sej	Forward:CATCAGAACTGTTGTTCCGCTAG Reverse:CTGAATTTTACCATCAAAGGTAC	5	142
fnbA	Forward:GCGGAGATCAAAGACAA Reverse:CCATCTATAGCTGTGTGG	6	1279
fnbB	Forward:GGAGAAGGAATTAAGGCG Reverse:GCCGTCGCCTTGAGCGT	6	812
cap5	Forward:ATGACGATGAGGATAGCG Reverse:CTCGGATAACACCTGTTGC	7	880
cap8	Forward:ATGACGATGAGGATAGCG Reverse:CACCTAACATAAGGCAAG	8	1147

*1: 35 cycles 94°C × 60 sec, 57°C × 60 sec, 72°C × 60 sec.
2: 30 cycles 94°C × 60 sec, 60°C × 60 sec, 72°C × 60 sec.
3: 30 cycles 94°C × 3 min, 58°C × 30 sec, 72°C × 5 sec.
4: 30 cycles 94°C × 2 min, 55°C × 2 min, 72°C × 1 min.
5: 40 cycles 94°C × 1 min, 60°C × 1 min, 72°C × 1 min.
6: 30 cycles 94°C × 30 sec, 50°C × 30 sec, 72°C × 1 min.
7: 30 cycles 94°C × 2 min, 56°C × 2 min, 72°C × 1 min.
8: 40 cycles 94°C × 2 min, 52°C × 2 min, 72°C × 1 min.
9: 37 cycles 94°C × 40 sec, 64°C × 1 min, 72°C × 75 sec.
Initial denaturation: 94°C × 5 min; final extension: 72°C × 10 min.

based systems for identification of *S. aureus* isolates from various origins have been used by numerous authors [2, 8, 34]. All these target genes allowed for rapid identification of this species with high sensitivity and specificity.

The capability of *S. aureus* to adhere to extracellular matrix proteins is thought to be essential for colonization

Table 2. Genotypic characteristics of *S. aureus* isolates from various farms in Central Italy

Farm	No. of cows tested [No. of] isolates	Gene (bp)																		
				Spa region								*SE*								
		nuc (420)	*clfA* (1042)	*IgG binding* (970)	*IgG binding* (810)	*X* (253)	*fnbA* (1279)	*fnbB* (812)	*fnb/A/B*	*cap5* (880)	*cap8* (1147)	*a* (216)	*b* (478)	*c* (257)	*d* (317)	*e* (170)	*g* (642)	*h* (375)	*i* (576)	*j* (142)
A	73 [40]	40	40	40		40	40	40	40	40	–	40	–	–	40	–	–	–	–	40
B	56 [30]	30	30		30	30	30	30	30	30	–	30	–	–	30	–	–	–	–	30
C	58 [30]	30	30		30	30	30	30	30	30	–	30	–	–	30	–	–	–	–	30
D	35[20]	20	20	20		20	–	20	–	–	–	20	–	–	20	–	–	–	–	20
E	38 [20]	20	–	–	–	20	–	–	–	20	–	–	–	–	–	–	–	–	–	–
F	41 [20]	20	20	20		20	20	20	20	20	–	20	–	–	–	–	–	–	–	–
G	19 [10]	10	–		10	10	10	–	–	–	–	10	–	–	–	–	–	–	–	–
TOTAL (%)	320 [170]	170 (100%)	140 (80%)	80 (47%)	70 (41%)	170 (100%)	130 (76%)	140 (82%)	120 (71%)	140 (82%)	0	150 (88%)	0	0	120 (71%)	0	0	0 (0%)	0	120 (71%)

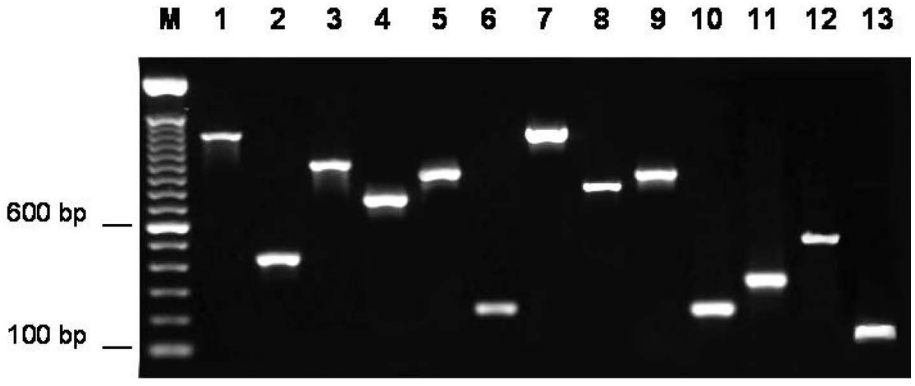

Fig. 2. Amplicons of the genes encoding staphylococcal virulence determinants and toxins genes. Lane M: DNA molecular weight, 100 bp. Lane 1: 23S rRNA. Lane 2: *nuc*. Lane 3: *clfA*. Lane 4: *spa* Ig-G binding 810 bp. Lane 5: *spa* Ig-G binding 970 bp. Lane 6: *spa* X-region. Lane 7: *fnbA*. Lane 8: *fnbB*. Lane 9: *cap5*. Lane 10: *sea*. Lane 11: *sed*. Lane 12: *seh*. Lane 13: *sej*.

and the establishment of infections. *S. aureus* possesses various adhesion genes such as *clfA, fnbA, fnbB, ebpS, cna, fib, fbpA* and *ma* [30]. The gene *clfA* was detected in 82.4% of the isolates in agreement with the rates observed by other authors [23, 28, 34]. It should be noted that this gene has an important role in the pathogenicity in bovine mastitis [33].

The fibronectin-binding proteins of *S. aureus* are important virulence factors and contribute to bacterial adhesion and invasion of the bovine mammary gland. For *S. aureus*, two fibronectin-binding proteins (*fnbA, fnbB*) have been described, and their corresponding genes have a high degree of sequence similarity [20]. Salasia *et al.* [28] detected *fnbA* in all isolates but only found *fnbB* in 3% of *S. aureus* isolated in Germany. Booth *et al.* [6] observed that 89.7% of the strains they investigated possessed *fnbA*, whereas only 20.1% had *fnbB*. In our study, the *fnbA* and *fnbB* genes were observed in high percentages, 76.5% and 82.4%, of the isolates, respectively; moreover 120 isolates were positive for both genes. Comparison of our results with those reported in the literature shows that the distribution of *fnbA* and *fnbB* varies widely.

The genes *spa*-X-region and *spa-IgG-binding* were detected in 100% and 88.2% of the isolates, respectively, in agreement with the rates described by several authors [16, 34]. Amplification of the gene segment encoding *spa-IgG-binding* produced amplicons of 810 and 970 bp, and amplification of the gene encoding *spa*-X-region produced an amplicon of 253 bp, unlike other authors who observed high polymorphism for both genes [1, 23, 28].

The capsular polysaccharide participates in the masking of the bacterial target for most of the naturally acquired opsonins present in normal bovine serum, resulting in an increased resistance to phagocytosis by polymorphonuclear leukocytes. Most *S. aureus* strains have been shown to have either *cap5* or *cap8*. However, the occurrence of *cap5* or *cap8* varies in isolates from different geographical regions [28, 32]. The gene *cap5* was observed in our study in 82.4% of the isolates of *S. aureus*, while the gene *cap8* was not detected. This result is not surprising because not all bovine *S. aureus* isolates harbour the *cap5(8)* locus; Cocchiaro *et al.* [10] observed that the *cap5(8)* locus was present in 38% of bovine isolates. Moreover, strains of *S. aureus* may lack a capsule or may produce a heterologous capsule type; other serotypes have been proposed, but specific detection methods are not available [36].

Staphylococcal food borne diseases resulting from inges-

tion of food contaminated with staphylococcal toxins are a major cause of food borne illnesses. The enterotoxins produced by *S. aureus* are 23- to 29-kDa single-chain proteins. These toxins also have immunomodulating properties [3] and are mostly carried on mobile genetic elements, which enable them to transfer horizontally among bacterial populations [14].

In the present study, 150 strains (88%) contained *sea,* which encodes the most common enterotoxin recovered from cases of food poisoning in humans [3]. In several studies, *sea* was detected [18, 33, 35], while other authors did not detect it [9, 34]; the reason for the observed discrepancy is unclear. This could be due to the fact that *sea* is carried by a family of temperate bacteriophages whose genomes incorporate and replicate with that of *S. aureus* [24]; moreover, the geographical distribution of these phages is irregular.

The high prevalence of *S. aureus* isolates harboring enterotoxin genes (*sea* and *sed*) could suggest a potential problem for public health. Whether the *se*-harboring isolates produce these toxins in amounts sufficient to cause food poisoning remains in questions.

A close genetic association has been observed between *sea, sed* and *sej* [15] and between *sed* and *sej*, according to the results of Vimercati *et al.* [38]. Both genes are located on plasmid pIB485 and are separated by an intergenic region of less than 1 Kb [39].

In regard to *se* the results obtained by several authors differ greatly [1, 31]. This could be explained by geographical variation in the occurrence of enterotoxigenic *S. aureus* isolated from bovine milk and by different methods used to detect the presence of SEs. At the moment, the role of virulence factors of *S. aureus* in mammary gland pathogenicity remains unclear [11]. Ferens *et al.* [13] suggested that SEs might facilitate immunosuppression that promotes the persistence of bacteria in cattle and contributes to chronic IMI. In our study, the most common SEs in the isolates from subclinical bovine mastitis were *sea*, *sed* and *sej*, which are closely associated genes, as described above. Our results are in accordance with those of Boynukara *et al.* [7], who observed that the most common *se* detected in *S. aureus* isolates from subclinical bovine mastitis was *sea*. The high frequencies of these factors in the majority of the isolates analyzed in this study suggest that the virulence factors may play a role in IMI pathogenesis. Our results showed that the isolates in each farm had the same virulence gene profile, while the gene profiles of the isolates were different between the farms. Consequently, each strain is limited to a restricted geographical area, and thus each strain can be better controlled in the case of outbreak of disease. These results might be of great interest for farmers, because the health of dairy cows is closely related to public health and hygiene.

ACKNOWLEDGMENT. The authors are thankful to Prof. Paolo Moroni for scientific support.

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