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Occurrence of enterotoxin genes and macrorestriction analysis of *Staphylococcus aureus* isolated from bovine mastitis and bulk-tank milk samples in Italy. An epidemiological study

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ABSTRACT

The goal of the study was to genotypically compare *S. aureus* isolates from mastitis milk and raw milk to identify the relation between strains and to assess the enterotoxigenicity of the isolates. Eighty-three *Staphylococcus aureus* isolates recovered from cows and bulk tank milk of five farms in northern Italy were compared genotypically. The genes for the enterotoxins A, D, G and I, but not for B, C, E and H and the toxic shock syndrome toxin 1 (TSST-1), were detected by PCR amplification. Macrorestriction analysis with the restrictions enzyme *Sma*I revealed 14 pulsed-field gel electrophoresis patterns. These were in part different from each other only in a few fragments and thus displayed a close clonal relation. The results of the present investigation showed that identical or closely related clones seemed to be responsible for the cases of bovine mastitis in the farms investigated and partly responsible for contamination of bulk tank milk.

Key Words: Staphylococcus aureus, Milk, Enterotoxins, Polymerase Chain Reaction, Pulsed-Field Gel Electrophoresis

RIASSUNTO

PRESENZA DI GENI CODIFICANTI PER LE ENTEROTOSSINE E ANALISI DI RESTRIZIONE DI CEPPI DI S. AUREUS ISOLATI DA MASTITI BOVINE E CAMPIONI DI LATTE CRUDO IN ITALIA: UNO STUDIO EPIDEMIOLOGICO

Lo scopo del lavoro è di comparare dal punto di vista genotipico ceppi di S. aureus *isolati da mastite bovina e da latte crudo al fine di evidenziarne una eventuale correlazione e di valutare la presenza di geni codificanti per la produzione di enterotossine negli isolati. Sono state analizzate le correlazioni genetiche di 83 ceppi di* Staphylococcus *aureus isolati dal latte di vacche con mastite subclinica e delle cisterne di raccolta in 5 aziende zootecniche. Nel genoma dei ceppi sono*

state amplificate le sequenze responsabili della sintesi delle SE A, D, G ed I mentre non sono state riscontrate quelle della B, C, E, H e TSST-1. L'elettroforesi in campo pulsante ha classificato gli isolati in 14 profili. L'analisi genotipica dei ceppi suggerisce che la maggior parte dei casi di mastite da S. aureus *in una azienda siano causati da cloni batterici strettamente correlati tra loro, e che gli stessi rappresentino la principale causa di contaminazione del latte in aziende con elevato numero di animali infetti.*

Parole chiave: Staphylococcus aureus*, Latte, Enterotossine, Reazione a catena della polimerasi (PCR), Elettroforesi in campo pulsante (PFGE).*

Introduction

Staphylococcus aureus is known worldwide as a frequent cause of mastitis in dairy cattle (Cardoso et al., 1999; Akineden et al., 2001; Stephan et al., 2001) and also as a principal contaminant of raw milk (Asperger, 1994). The contamination of milk by *S. aureus* can be of endogenous origin, following excretion from the udder of an infected animal (Bryan, 1983). Contamination may also be of exogenous origin, through direct contact with infected persons or through the environment (Brisabois et al., 1997). Some *S. aureus* cultures have the ability to form one or more toxins, including enterotoxins (SE), which play an important role in food poisoning and staphylococcal toxic shock. Staphylococcal food poisoning is one of the leading causes of foodborne illness caused by microbial intoxication (Balaban and Rasooly, 2000; Dinges et al., 2000). However, growth of *S. aureus* to a population of at least 10^5 cells per gram of food is considered essential for production of a sufficient amount of SE to induce symptoms of food poisoning (Asperger, 1994). The knowledge of the contamination source can help in applying control programs for food safety and reduce the risk of foodborne illnesses for the consumer, as contamination can be caused by strains of both animal and human origin. The present study was designed to investigate *S. aureus* isolated from raw milk by PCR determination for various toxin genes and by genotyping by macrorestriction analysis of their chromosomal DNA by pulsed-field gel electrophoresis (PFGE). It should provide information both on the spreading of clones within farms and between farms, as well as about the relation of *S. aureus* isolated from mastitis milk and from bulk tank milk.

Material and methods

Bulk-tank milk from 75 farms showing high somatic cells count (SCC >400.000) was aseptically sampled. A 50 ml sample was collected from each farm utilizing an automatic milk sampler, cooled at 4°C and investigated within 24 hours for coagulase-positive staphylococci. Farms that showed negative results for staphylococci at the first sampling were sampled again within a month. The isolation of coagulase-positive staphylococci from bulk tank milk was performed according to the ISO 6888 – 1:99 standard, using Baird Parker Agar (Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with Egg Yolk Tellurite emulsion (Oxoid); if present, 5 Egg Yolk (EY) positive colonies and 5 EY negative colonies from each sample were chosen for identification. Suspected colonies were identified phenotypically by OF test, determination of urease, acetoin and arginine dihydrolase production and by determination of fermentative and oxidative cleavage of the carbohydrates maltose, D-mannitol, D-trehalose, D-xylose, D-turanose and lactose on purple agar (Difco Laboratoires, Detroit, MI, USA).

Within two weeks after a positive result in bulk-tank milk, a sampling of cow milk was performed: milk samples of all the lactating cows with an individual SCC higher than 400,000 and with milk macroscopic alterations were aseptically collected from each quarter, plated on sheep blood agar (Difco) and incubated at 37°C for 24 h. One suspect colony from each plate was phenotypically identified as described above.

Five farms (designated with numbers 1 to 5) which tested positive for *S. aureus* at cow and bulk tank milk sampling, and chosen based on the increasing distances from each other, were selected for further characterization of isolates. The shortest distance between farm number 1 and number 2 was 2 km and the greatest distance between farm number 1 and number 5 was 200 km. The number of milking cows present in each farm at the time of sampling, the number of cows sampled and the number of lactating cows positive for *S. aureus* are summarized in Table 1.

Molecular identification and further characterization

The 83 isolates were additionally identified by PCR amplification of the gene encoding a *S. aureus* specific part of the 23 S rRNA (Straub et al., 1999). A PCR amplification of the genes encoding staphylococcal enterotoxins was performed for SEA to SEE, SEG to SEI and for TSST-1 (Akineden et al. 2001). Control strains possessing the various genes were included.

Total genomic DNA of the *S. aureus* cultures was obtained with the DNeasy tissue kit as described by the manufacturer (DNeasy Tissue kit 250, Qiagen S.p.A. Milano, Italy). The sequence of the oligonucleotide primers and the temperature programs used were described by Johnson et al. (1991), Tsen and Chen (1992), Jarraud et al. (1999) and Straub et al. (1999); a detail of the primer used for PCR amplification is reported in Table 2. The presence of PCR products was determined by electrophoresis of 8 µl of the reaction product in a 1.5% agarose gel, with tris-acetate electrophoresis buffer TAE (40 mM Tris-HCl, 1 mM EDTA, 1.14 ml/l glacial acetic acid pH 7.6) and a 100 bp DNA ladder (GIBCO BRL Life technologies, Eggenstein, Germany) as a molecular marker.

Pulsed-field gel electrophoresis

A macrorestriction analysis of the chromosomal DNA of the isolates was performed using the restriction enzyme SmaI (New England BioLabs., Beverly, MA, U.S.A.) and subsequent PFGE. DNA restriction fragments were separated in 1% agarose in 0.5% TBE using a CHEF DR II (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). Gels were stained with ethidium bromide, visualized using a UV transilluminator and photographed. The isolation of the chromosomal DNA and the PFGE program was performed as described (Toshkova et al., 1997). For a final fragment analysis, their relative positions were evaluated visually on paper prints of the gels and compared with those generated with Low range PFG marker and Lambda ladder PFG marker (New England BioLabs). To determine the clonal relationship among the isolates, the criteria of Tenover et al. (1995) were used. PFGE patterns that differed in more than three fragments were recorded as types and were identified with a capital letter. Patterns that differed in one to three fragments were recorded as different subtypes of the pattern and identified with a capital letter (type) followed by an Arabic numeral.

Results and discussion

A total of 83 *S. aureus* isolates were identified phenotipically and used for genotypical characterization. Of these bacteria, 61 and 22 strains were isolated from subclinical mastitis and from farm bulk tank milk, respectively. According to the phenotypic results and to amplification of the *S.*

Table 1. Further information of the number of cows in the five farms sampled.

Farm	Number of lactating cows	Number of lactating cows sampled	Number of lactating cows positive for *S. aureus*
1	48	12	6
2	18	6	2
3	75	54	9
4	18	12	1
5	50	34	17

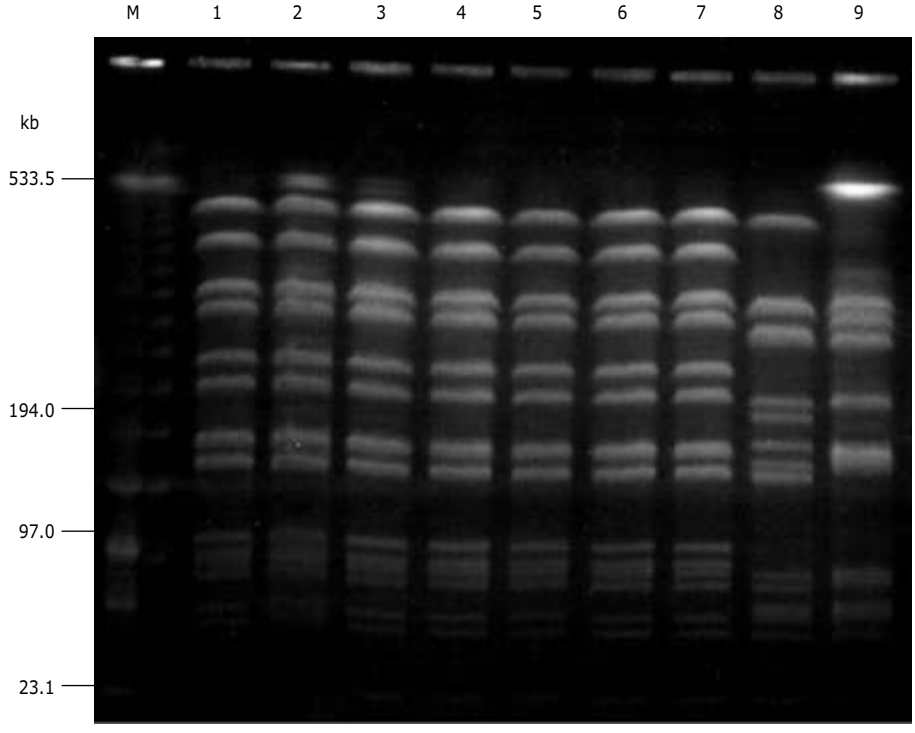
Table 2. Primers used for PCR amplification.

Target gene	PRIMERS	Sequence	Reference
sea	SEA-1	AAAGTCCCGATCAATTTATGGCTA	Tsen e Chen (1992)
	SEA-2	GTAATTAACCGAAGGTTCTGTAGA	
seb	SEB-1	TCGCATCAAACTGACAAACG	Johnson et al. (1991)
	SEB-2	GCAGGTACTCTATAAGTGCC	
sec	SEC-1	GACATAAAAGCTAGGAATTT	Johnson et al. (1991)
	SEC-2	AAATCGGATTAACATTATCC	
sed	SED-1	CTAGTTTGGTAATATCTCCT	Johnson et al. (1991)
	SED-2	TAATGCTATATCTTATAGGG	
see	SEE-1	TAGATAAAGTTAAAACAAGC	Johnson et al. (1991)
	SEE-2	TAACTTACCGTGGACCCTTC	
seg	SEG-1	AATTATGTGAATGCTCAACCCGATC	Jarraud et al. (1999)
	SEG-2	AAACTTATATGGAACAAAAGGTACTAGTTC	
seh	SEH-1	CAATCACATCATATGCGAAAGCAG	Jarraud et al. (1999)
	SEH-2	CATCTACCCAAACATTAGCACC	
sei	SEI-1	CTCAAGGTGATATTGGTGTAGG	Jarraud et al. (1999)
	SEI-2	AAAAAACTTACAGGCAGTCCATCTC	

aureus specific 23S rRNA gene, all 83 isolates used in the present investigation could be identified as *S. aureus*. The amplicons of the 23S rRNA gene had a uniform size of approximately 1250 bp (data not shown). This species-specific gene part had already been used to identify this species (Straub et al., 1999; Akineden et al., 2001). By PCR amplification the genes for SEA, D, G and I, but not for SEB, C, E and H and TSST-1, could be detected. The *S. aureus* isolates of farm 5 were negative throughout. The combined detection of the genes for the enterotoxins SEA and SED could be observed for isolates of farm 1 and 3 and for SEG and SEI could be demonstrated for the isolates of farm 2 and 4. The results are summarized in Table 3. Digestion of the chromosomal DNA of the 83 isolates collected from cow and bulk tank milk with SmaI and subsequent separation of the fragments by PFGE yielded 15 to 20 fragments. A further analysis of the fragments revealed four major PFGE patterns which were designated as types W, X, Y and Z. The PFGE patterns W1, W2, W3, W4, W5, W6, W7 and W8 and the PFGE patterns Y1, Y2, Y3 and Y4, respectively, were different from each other in two or three fragments and thus displayed close clonal relations (Table 3). A typical picture of pattern W2, Y1 and Z is shown in Figure 1. Data of distribution of PFGE patterns within the five farms are detailed in Table 3.

For *S. aureus* the PCR technology allows the detection of SE genes in a relatively short period (Monday and Bohach, 1999; Akineden et al., 2001). In this study genes for SE, including the newly described enterotoxins G and I, could be detected for *S. aureus* isolated from milk in Italy. The involvement of enterotoxin G and I producing *S. aureus* had been previously demonstrated for *S. aureus* isolated from humans with staphylococcal toxic shock syndrome and staphylococcal scarlet fever (Jarraud et al.,1999) and seemed to be frequent also in bovine isolates of this species (Lämmler et al., 2000; Akineden et al., 2001). However, there was no close relationship between pulsotype and the presence of genes encoding for SE; in detail, out of 45 isolates belonging to pulsotype W, 15 harbored *sea*, 7 *sed*, 16 *sea* + *sed* and 7

Figure 1. Typical pulsed-field electrophoretic restriction patterns of chromosomal DNA of *S. aureus* isolates after digestion with the restriction enzyme SmaI (lane M: marker; lane 1 to 7 pattern W2; lane 8 pattern Y1 and lane 9 pattern Z).



were negative. On the contrary, all Y pulsotype harbored no SE genes and all X and Z pulsotype strains harbored *seg* + *sei*.

In order to demonstrate the epidemiological relation of strains isolated in cow milk and bulk tank milk the *S. aureus* strains of the present investigation were genotyped by PFGE. The distribution of the PFGE patterns in the present study revealed close relations between *S. aureus* isolated from cow and bulk tank milk in farms 1, 3 and 5, respectively. However, there was a clear difference in the genotypic properties of the isolates collected from cow and bulk tank milk in farms 2 and 4. These strains could be distinguished unequivocally on the basis of genotyping results as different clones. According to the results in farms 2 and 4, the milk of the cows investigated was not the primary source of staphylococcal contamination of bulk tank milk. This could be explained by the fact that in farms with a high number of infected cows, as could be seen in farms 1, 3 and 5, these cows could represent the main source of contamination. Contrarily, in farms in which there is a lower number of infected cows an environmental or human contamination may become predominant. The macrorestriction analysis revealed four major PFGE patterns. The results of the present investigation showed that identical or closely related clones with PFGE pattern W and Y seemed to be responsible for the cases of bovine mastitis of three and two farms, respectively, and also for bulk milk contamination of at least three farms. This corresponded to the findings of Annemüller et al. (1999), Akineden et al. (2001) and Stephan et al. (2001) in Germany and Switzerland, respectively, and to the information given by Matthews et al. (1994) and Fitzgerald et

Table 3. Relation between origin, presence of SE genes and PFGE pattern.

Farm	Origin	n.	Presence of SE genes					PFGE pattern
			A	D	A + D	G + I	negative	
1	c	16		6	10			W1
	b	5		1			4	W2
2	c	4	4					W2
	b	9				9		X
3	c	16	11		5			W3(9)*,W4(5), W5(1), W6(1)
	b	4			1		3	W7(1), W8(3)
4	c	1					1	Y1
	b	3				3		Z
5	c	24					24	Y2(20), Y3(3), Y4(1)
	b	1					1	Y2

n. = number of cultures
** = number of cultures with the respective pattern*
c = cow
b = bulk tank milk

al. (1997) indicating that in single farms only a few specialized clones seem to be responsible for the cases of bovine mastitis.

The comparison of results of enterotoxin gene PCR amplification and PFGE patterns (Table 3) shows that, in farms 1 and 2, pulsotype W1 includes 6 and 10 isolates harboring sed and sea + sed, respectively, and that pulsotype W2 includes 1 isolates harboring sed, 4 isolates harboring sea and 4 isolates harboring no gene encoding for enterotoxin production; on the other hand, in farms 3 and 5 isolates harboring the same enterotoxin encoding genes are identified as different clones by PFGE. These results confirm that the use of only one typing method doesn't make it possible to distinguish all the genetic differences between isolates. Nevertheless, PFGE is considered a highly discriminatory method for the typing of bacteria and has been used successfully for the typing of isolates of *S. aureus* (Vanderlinde et al., 1999).

S. aureus is one of the leading causes of foodborne disease; for example De Buyser et al. (2001) estimated that 85.5% of outbreaks associated with consumption of milk and dairy products, especially raw milk and raw milk products, were caused by *S. aureus*. Many sources of milk contamination exist, including infected cows, animal and human skin, food handlers, milking machinery, other equipment and air. Using PFGE in a dairy plant, Tondo et al. (2000) were able to correlate, isolates collected from contaminated final products with isolates collected from raw milk and from food handlers. Moreover, Zadoks et al. (2002) demonstrated by PFGE that isolates from mastitic bovine milk were different from isolates collected from bovine and human skin.

In different situations different sources of contamination may be predominant and, if the milk is mishandled, the contamination and growth of staphylococci may become a hazard for human consumption.

Conclusions

PCR amplification of the gene encoding for SE production showed a high proportion of strains harboring these genes, with the exception of isolates from farm 5. The macrorestriction analysis of PFGE patterns showed that identical or closely related clones seemed to be responsible for the cases of bovine mastitis in the farms investigated and that a few clones seemed to be responsible for the infection of different herds. In farms in which the prevalence of infection is higher the infected cows are the main source of *S. aureus* contamination, of milk thereby increasing the risk of foodborne disease if the milk is mishandled.

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