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

Andrea Serraino, Leonardo Alberghini, Maria Cristina Fontana, Cosima Annemüller, Christoph Lämmler & Roberto Rosmini

To cite this article: Andrea Serraino, Leonardo Alberghini, Maria Cristina Fontana, Cosima Annemüller, Christoph Lämmler & Roberto Rosmini (2004) Occurrence of enterotoxin genes and macrorestriction analysis of Staphylococcus aureus isolated from bovine mastitis and bulk-tank milk samples in Italy. An epidemiological study, Italian Journal of Animal Science, 3:1, 47-53, DOI: 10.4081/ijas.2004.47

To link to this article: https://doi.org/10.4081/ijas.2004.47
**Occurrence of enterotoxin genes and macrorestriction analysis of *Staphylococcus aureus* isolated from bovine mastitis and bulk-tank milk samples in Italy. An epidemiological study**

Andrea Serraino¹, Leonardo Alberghini¹, Maria Cristina Fontana², Cosima Annemüller³, Christoph Lämmler⁴, Roberto Rosmini¹

¹ Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale. Università di Bologna, Italy.
² Istituto Zooprofilattico della Lombardia e dell’Emilia Romagna. Bologna, Italy.
³ Klinik für Vögel, Reptilien, Amphibien und Fische. Justus-Liebig Universität, Giessen, Germany.
⁴ Institut für Pharmakologie und Toxikologie. Justus-Liebig Universität, Giessen, Germany

Corresponding author: Dr. Andrea Serraino. Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale, Sezione di Igiene e Tecnologia Alimentare. Facoltà di Medicina Veterinaria, Università di Bologna. Via Tolara di Sopra 50, 40064 Ozzano Emilia (BO), Italy - Tel. +39 051 792828 - Fax: +39 051 792842 - Email: serraino@vet.unibo.it

Paper received June 24, 2003; accepted November 19, 2003

**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. Macrogenetic analysis with the restrictions enzyme SmaI revealed 14 pulsed-field gel electrophoresis patterns. These were in part different from each other 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 evidenziare una eventuale correlazione e di valutare la presenza di geni codificanti per la produzione di enterotoxine 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
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). 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 OP 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 7.5) 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 Smal (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 (BioRad 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 PFGE marker and Lambda ladder PFGE 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 phenotypically 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. aureus*.

### 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 et Chen (1992) |
|             | SEA-2   | GTAATTAACCGAAGGTCTCTGTGA | |
| seb         | SEB-1   | TCGCATCAAACGTGACAAACG | Johnson et al. (1991) |
|             | SEB-2   | GCAGGTACTCTATAAGTGCC | |
| sec         | SEC-1   | GACATAAAGCTTAGAAATT | Johnson et al. (1991) |
|             | SEC-2   | AAATCGGATACCTATATCC | |
| sed         | SED-1   | CTAGTTGGTATATCTCTCT | Johnson et al. (1991) |
|             | SED-2   | TAATGTATATCTTTATAGGG | |
| see         | SEE-1   | TAGATAAAAGTTAAAAACAGC | Johnson et al. (1991) |
|             | SEE-2   | TAACTACCGTGGACCTTT | |
| seg         | SEG-1   | ATATTGTGAATGTCACAACCGGATC | Jarraud et al. (1999) |
|             | SEG-2   | AAACCTATGGAACAAAAAGGTACCTAGTTC | |
| seh         | SEH-1   | CAATCACATCATGCGAGAAGCAG | Jarraud et al. (1999) |
|             | SEH-2   | CATCACACACACATAGCACC | |
| sei         | SEI-1   | CTCAAGGGTAGTTAGGTTAGG | Jarraud et al. (1999) |
|             | SEI-2   | AAAAATTCAGGGCAGTCCATC | |

*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 *Sma*I 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 pulstype 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
were negative. On the contrary, all Y pulsotype harbored no SE genes and all X and Z pulsotype strains harbored \textit{seg} + \textit{sei}.

In order to demonstrate the epidemiological relation of strains isolated in cow milk and bulk tank milk the \textit{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 \textit{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

---

**Figure 1.** Typical pulsed-field electrophoretic restriction patterns of chromosomal DNA of \textit{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).
Table 3. Relation between origin, presence of SE genes and PFGE pattern.

| Farm | Origin | n. | Presence of SE genes | PFGE pattern |
|------|--------|----|-----------------------|--------------|
|      |        |    |                       |              |
| 1    | c       | 16 | A D A + D G + I       | negative     |
|      | b       | 5  | 6 10                  | W1           |
| 2    | c       | 4  | A D A + D G + I       | negative     |
|      | b       | 9  | 9                      | W2           |
| 3    | c       | 16 | A D A + D G + I       | negative     |
|      | b       | 4  | 5 1                    | W3(9)*, W4(5), W5(1), W6(1) |
| 4    | c       | 1  | A D A + D G + I       | negative     |
|      | b       | 3  | 3                      | W7(1), W8(3) |
| 5    | c       | 24 | A D A + D G + I       | negative     |
|      | b       | 1  | 24                     | Y2(20), Y3(3), Y4(1) |
| 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 food-borne disease if the milk is mishandled.
REFERENCES

AKINENEN, ō., ANNEMÜLLER, C. A., HASSAN, A., LÄMMLER, C., WOLTER, W., ZSCHÖCK, M., 2001. Toxin genes and other characteristics of Staphylococcus aureus isolated from milk of cows with mastitis. Clin. Diagn. Lab. Immunol. 8: 959-964.

ANNEMÜLLER, C., LÄMMLER, C., ZSCHÖCK, M., 1999. Genotyping of Staphylococcus aureus isolated from bovine mastitis. Vet. Microbiol. 69: 217-224.

ASPÉRGER, H., 1994. Staphylococcus aureus. In: Monograph on the significance of pathogenic microorganisms in raw milk by IDF group of experts A10/11 – bacteriological quality of raw milk, IDF, Brussels, Belgium, pp 24-42.

BALABAN, N., RASOOLY, A., 2000. Staphylococcal enterotoxins. Int. J. Food Microbiol. 61: 1-10.

BRISSABOIS, A., LAFARGE, V., BROUILLAUD, A., DE BUYSER, M. L., COLLETTE, C., GABIN-BASTU, B., THERIOL, M. F., 1997. Les germes pathogènes dans le lait et les produits laitiers: situation en France et en Europe. Rev. Sci. Tech. Off. Int. Epiz. 16: 452-471.

BRYAN, F. L., 1983. Epidemiology of milk-borne diseases. J. Food Prot. 46: 637-649.

CARDOSO, H. F. T., SILVA, N., SENIA, M. J., CARMO, L. S., 1999. Production of enterotoxins and toxic shock syndrome toxin by Staphylococcus aureus isolated from bovine mastitis in Brazil. Lett. Appl. Microbiol. 29: 347-349.

DE BUYSER, M. L., DUFOUR, B., MURIELLE, M., LAFARGE, V., 2001. Implication of milk and milk products in food-borne diseases in France and in different industrialized countries. Int. J. Food Microbiol. 67: 1-17.

DINGES, M. M., ORWIN, P. M., SCHLIEVERT, P. M., 2000. Exotoxins of Staphylococcus aureus. Clin. Microbiol. Rev. 13: 16-34.

FITZGERALD, J. R., MEANEY, W. J., HARTIGAN, P. J., SMYTH, C. J., KAPUR, V., 1997. Fine structure molecular epidemiological analysis of Staphylococcus aureus recovered from cows. Epidemiol. Infect. 119: 261-269.

JABRAUD, S., COZON, G., VANDEMEesch, F., BES, M., ETIENNE, J., LINA, G., 1999. Involvement of enterotoxins G and I in staphylococcal toxic shock syndrome and staphylococcal scarlet fever. J. Clin. Microbiol. 37: 2446-2459.

JOHNSON, M. W., TYLER, S. D., EWAN, E. P., ASHTON, P. E., POLLARD, D. R., ROZEE, K. R., 1991. Detection of genes for enterotoxins, exfoliative toxins and toxic shock syndrome toxin 1 in S. aureus by polymerase chain reaction. J. Clin. Microbiol. 29: 426-430.

LÄMMLER, C., AKINENEN, O., ANNEMÜLLER, C., WOLTER, W., ZSCHÖCK, M., 2000. Molecular analysis of virulence factors of Staphylococcus aureus isolated from bovine subclinical mastitis. In: A. Zecconi (Ed.), Proc. Symp. on Immunology of Ruminant Mammary Gland, Stresa, Italy, pp 326-330.

MATTHEWS, K. R., KUMAR, S. J., O'CONNOR, S. A., HARMON, R. J., FOX, L. K., OLIVER, S. P., 1994. Genomic fingerpints of Staphylococcus aureus of bovine origin by polymerase chain reaction-based DNA fingerprinting. Epidemiol. Infect. 112: 177-186.

MONDAY, S. R., BOHACH, G. A., 1999. Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal isolates. J. Clin. Microbiol. 37: 3411-3414.

STEPHAN, R., ANNEMÜLLER, C., HASSAN, A. A., LÄMMLER, C., 2001. Characterization of enterotoxigenic Staphylococcus aureus strains isolated from bovine mastitis in north-east Switzerland. Vet. Microbiol. 78: 373-382.

STRAUB, J. A., HERTEL, C., HAMMES, W. P., 1999. A 23S rDNA-targeted polymerase chain reaction-based system for detection of Staphylococcus aureus in meat starter cultures and dairy products. J. Food Prot. 62: 1150-1156.

TENOVER, F. C., ABREIT, R. D., GOERING, R. V., MICKELSEN, P. A., MURRAY, B. E., PERSING, D. H., SWAMNATHAN, B., 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. 33: 2233-2239.

TONDO, E. C., GUIMARAES, M. C. M., HENRIQUES, J. A. P., AYUB, M. A. Z., 2000. Assessing and analysing contamination of a dairy products processing plant by Staphylococcus aureus using antibiotic resistance and PFGE. Can. J. Microbiol. 46: 1108-1114.

TSEN, H. Y., CHEN, T. R., 1992. Use of the polymerase chain reaction for the specific detection of type A, D and E enterotoxigenic Staphylococcus aureus in foods. Appl. Microbiol. Biotechnol. 37: 686-690.

VANDERLINDEN, P. B., FEGAN, N., MILLS, L., DESMARCHELIER, P. M., 1999. Use of pulse field gel electrophoresis for the epidemiological characterization of coagulase positive Staphylococcus aureus isolated from meat workers and beef carcasses. Int. J. Food Microbiol. 48: 81-85.

ZADOKS, R. N., VAN LEEUWEN, W. B., KREFT, D., FOX, L. K., BARKEMA, H. W., SCHUKKEN, Y. H., VAN BELKUM, A., 2002. Comparison of Staphylococcus aureus isolates from bovine and human skin, milking equipment, and bovine milk by phage typing, pulsed-field gel electrophoresis, and binary typing. J. Clin. Microbiol. 40: 3894-3902.