Coagulase gene typing of *Staphylococcus aureus* isolated from cows with mastitis in southeastern Brazil

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Abstract

A typing procedure based on polymorphism of the coagulase gene (*coa*) was used to discriminate *Staphylococcus aureus* isolated from Minas Gerais dairy cows with mastitis. Amplification of the gene from the 64 *S. aureus* isolates produced 27 different polymerase chain reaction (PCR) products; 60 isolates showed only 1 amplicon, and 4 showed 2 amplicons. The isolates were grouped into 49 types by analyzing the restriction fragment length polymorphism (RFLP) of the *coa* gene; the 10 most common types accounted for 39% of the isolates. The results demonstrate that many variants of the *coa* gene are present in the studied region, although only a few predominate.

Résumé

*Une procédure de typage utilisant le polymorphisme du gène de la coagulase (*coa*) a été utilisée afin de distinguer les isolats de* Staphylococcus aureus *provenant de vache laitière avec mammite dans la région de Minas Gerais. L'amplification du gène de 64 isolats de* S. aureus *a résulté en 27 produits de réaction d'amplification en chaîne (PCR) différents; 60 isolats n'ont donné qu'un seul amplicon et 4 ont donné deux amplicons. Les isolats ont été groupés en 49 types suite à l'analyse du polymorphisme de taille des fragments de restriction du gène* coa*; les 10 types les plus fréquents représentant 39 % des isolats. Les résultats démontrent que bien que plusieurs variants du gène* coa *soient présents dans la région étudiée seulement quelques uns prédominent.*

(Traduit par Docteur Serge Messier)

Introduction

Bovine mastitis continues to be one of the most significant endemic illnesses in dairy herds in terms of responsibility for economic losses for the producer as well as the dairy industry (1). Although mastitis can be caused by a variety of pathogens, *Staphylococcus aureus* is considered the most frequent (2).

Natural populations of *S. aureus* have shown considerable variability in genome content (3,4). This variability has contributed to the emergence of distinct epidemiologic profiles that are dependent on the strains prevalent in a herd, which suggests the need to identify such strains or subtypes before applying specific measures to control mastitis (5).

In the last few years, numerous molecular techniques have been used to identify and compare *S. aureus* subtypes. Amplification of the coagulase gene *(coa)* has been considered a simple and accurate method for typing *S. aureus* isolated from distinct sources (6–11). The results of epidemiologic research based on analysis of the *coa* gene suggest that few *S. aureus* subtypes are responsible for most cases of bovine mastitis, and these are widespread (8,11,12).

In Brazil, little information is available about the genetic diversity of *S. aureus* isolated from cows with mastitis. The purpose of this study was to use *coa* gene polymorphism to identify *S. aureus* subtypes isolated from mastitis cases in dairy herds in Minas Gerais state.

Materials and methods

Bacterial strains

We used 64 *S. aureus* isolates from the milk of cows with mastitis. The milk samples had been obtained between 1994 and 1997 from different dairy herds in Minas Gerais state, identified by Cardoso (13), and kept frozen at −20°C in tryptic soy broth (TSB; Biobrás, São Paulo, Brazil) containing 15% glycerol until molecular tests were carried out.

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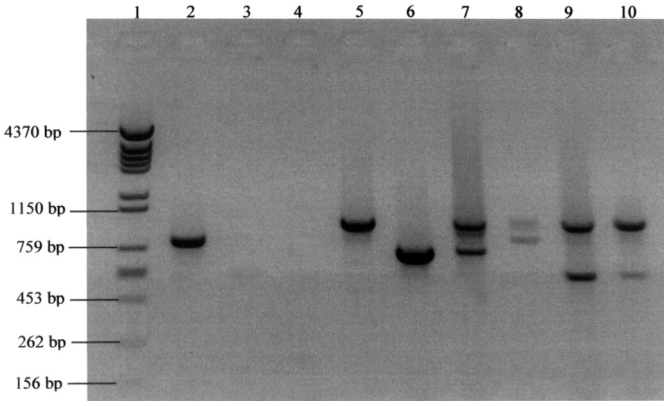

Figure 1. Electrophoretic profile, in 2% agarose gel, of polymerase chain reaction (PCR) products of *Staphylococcus aureus* coagulase gene isolated from cows with mastitis: lane 1 — molecular marker; lane 2 — positive control, *S. aureus* American Type Culture Collection 25923; lanes 3 and 4 — negative controls, *S. epidermidis* and *S. intermedius*; lanes 5 and 6 — isolates with only 1 amplicon; lanes 7 to 10 — isolates with 2 amplicons.

Gene typing

Extraction and purification of DNA — We extracted and purified bacterial DNA according to previously published methods (14,15). Bacterial cell lysates were prepared from 0.5 mL of overnight TSB cultures. After centrifugation at 12 000 × *g* for 10 min, the bacterial pellets were washed with 500 μL of Tris-hydrochloride-ethylene diamine tetraacetic acid (EDTA) (TE) buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) and centrifuged again. The pellets were resuspended in 200 μL of TE buffer, pH 7.5, with 15 U of lysostaphin (2 mg; Sigma-Aldrich Brasil, São Paulo, Brazil) per milliliter, and incubated at 37°C for 1 h. Next, 15 μL of proteinase K, 20 mg/mL (Invitrogen Brasil, São Paulo, Brazil), was added and the suspension incubated at 56°C for 1 h. The suspension was then heated at 95°C for 15 min to inactivate the proteinase K. An equal volume of phenol-chloroform was added and the mixture centrifuged at 12 000 × *g* for 10 min. The supernatant was extracted with an equal volume of phenol-chloroform and then chloroform. The DNA in the supernatant was mixed with 2 volumes of 95% ethanol and stored overnight at −20°C. The mixture was then centrifuged at 12 000 × *g* for 5 min. The DNA pellet was washed with ice-cold 70% ethanol, recentrifuged, and dried by tube inversion. The DNA was suspended in 100 μL of sterile TE, pH 7.5, quantified in a spectrophotometer (at 260 nm), and kept frozen at −20°C.

Polymerase chain reaction (PCR) amplification — The 3′ end region of the *coa* gene was amplified with use of the internal primer pair previously reported by Aarestrup et al (8): Coag2, 5′-ACCACAAGGT ACTGAATCAACG-3′ (bases 1432 to 1453); and Coag3, 5′-TGCTTTC GATTGTTCGATGC-3′ (bases 2399 to 2418). For PCR, each reaction mixture contained 1 to 2 μL of target DNA (approximately 350 ng/μL), 1 μL of each of the primers (50 pmol), 0.8 μL of a mix of deoxynucleotide triphosphates (200 μM each), 0.2 μL of *Taq* polymerase (1 U), and 3 μL of PCR 10× buffer (500 mM of KCl; 100 mM of Tris-HCl, pH 8.4; 1% Triton X-100; and 15 mM of $MgCl_2$). The volume of this mix was adjusted to 40 μL with sterile water. Evaporation was prevented by the addition of 50 μL of sterile mineral oil. Amplification was carried out in a thermal cycler (MJ MiniCycler; Bio-Rad Laboratories, Hercules, California, USA) as follows: initial denaturation at 94°C for 2 min, 30 cycles of amplification (denaturation at 95°C for 30 s, annealing at 55°C for 2 min, and extension at 72°C for 4 min), and extension at 72°C for 7 min.

Analysis of restriction fragment length polymorphism (RFLP) — Restriction analysis of the PCR products was performed with *Alu*I (Invitrogen), according to the manufacturer's instructions. We incubated a mixture of 10 μL of PCR products and 10 U of *Alu*I in a thermal cycler at 37°C for 1 h (8).

Agarose gel electrophoresis — The PCR products and *Alu*I digests were separated in 2% and 5% agarose gel, respectively, with 10 mg/mL of an aqueous solution of ethidium bromide (Pharmacia Brasil, São Paulo, Brazil), and photographed under ultraviolet illumination. We used bacteriophage DNA of Phi29 digested with *Hind*III as a molecular marker.

Specificity testing

To test the specificity of the primer pair, we analyzed the DNA of *S. epidermidis* American Type Culture Collection (ATCC) 12228, *S. intermedius* 08/96PE-FUNED, and *S. aureus* ATCC 25923.

Reproducibility testing

We tested PCR reproducibility by interassay analysis of 5 randomly chosen isolates, tested for 5 consecutive d. We tested RFLP-PCR reproducibility by twice submitting 4 different PCR products to *Alu*I digestion.

Data analysis

The sizes, in base pairs (bp), of the PCR and RFLP products were estimated with the LabImage gel-analysis software program (version 2.7.0; Kapelan Bio-Imaging Solutions, Halle-Saale, Germany). The within-gel standard error ($s_{\bar{x}}$) was calculated by estimated-size analysis from the PCR products of 2 isolates run electrophoretically 4 times. The discriminatory power of the typing method was determined according to the numerical index described by Hunter and Gaston (16). The D-value indicates the probability that 2 isolates randomly selected from the test population will be assigned to different typing groups. The following formula was used:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} nj\,(nj-1)$$

with D = discriminatory index, s = total number of different types, *n*j = number of isolates representing each type, and *N* = total number of isolates in the sample population.

Results

Electrophoresis $s_{\bar{x}}$

The within-gel error was calculated as 8 bp. Thus, PCR products or restriction fragments with this difference were considered the same.

Gene amplification products

The Coag2 and Coag3 primer pair produced 27 amplicons, which ranged from approximately 579 to approximately 1442 bp. Sizes 790,

Table I. Numbers of distinct patterns of restriction fragment length polymorphism (RFLP), determined by *Alu*I testing of polymerase chain reaction (PCR) products of the coagulase gene of *Staphylococcus aureus*, isolated from cows with mastitis

PCR products (approximate number of base pairs [bp])	Number of isolates	Number of distinct RFLP patterns
1442	1	1
1262	1	1
1181	1	1
1165	1	1
1129	1	1
1113	1	1
1080	1	1
1042	1	1
1026	2	1
1006	2	2
994	1	1
972-579	2	1
972-739	1	1
950-800	1	1
950	2	2
900	1	1
880	2	2
850	1	1
838	3	2
824	1	1
800	1	1
790	10	4
759	13	11
725	5	4
684	1	1
602	2	2
579	5	2
Total	64	49

759, 725, and 579 bp were the most frequent, accounting for 52% of the studied isolates; 60 isolates had only 1 PCR product, and the other 4 had 2 PCR products, with sizes of approximately 972 and 579 (2 isolates), 972 and 739, and 950 and 800 bp (Figure 1). There was no amplification product of the DNA from the other *Staphylococcus* species. However, amplicons were observed for all *S. aureus* isolates investigated (100% typability).

Reproducibility of the PCR products was demonstrated with 100% of the tested isolates. Although there was some variation in intensity, the bands were always present and their sizes reproducible. The discriminatory index for the PCR-based typing method was 0.92.

Restriction-fragment patterns

The numbers of *Alu*I RFLP patterns, by PCR product and genotype frequency, are shown in Tables I and II, respectively. Most of the PCR products generated a single pattern, but 9 products (those of 1006,

Table II. Frequency of coagulase genotypes in the isolated *Staphylococcus aureus*

Type code	Genotype: PCR products (bp)	Genotype: RFLP pattern (bp)	Frequency (%)
1	1442	453-301-162-80	1.6
2	1262	453-301-162	1.6
3	1181	453-301-162-80	1.6
4	1165	453-301-162-80	1.6
5	1129	453-301-162-80	1.6
6	1113	552-229-80	1.6
7	1080	453-301-162-80	1.6
8	1042	453-301-162-80	1.6
9	1026	453-301-162	3.1
10	1006	453-301-162-80	1.6
11	1006	453-301-162	1.6
12	994	453-301-162-80	1.6
13	972-579	453-377-301-215-162-80	3.1
14	972-739	453-301-215-162-80	1.6
15	950-800	453-301-184-162-80	1.6
16	950	453-301-162-80	1.6
17	950	453-229-80	1.6
18	900	453-301-162-80	1.6
19	880	453-301-162-80	1.6
20	880	715-80	1.6
21	850	301-184-162-80	1.6
22	838	667-80	1.6
23	838	602-80	3.1
24	824	552-244	1.6
25	800	301-244-184-80	1.6
26	790	508-229-80	1.6
27	790	715-80	3.1
28	790	636-80	6.3
29	790	602-80	4.7
30	759	301-184-162-80	1.6
31	759	229-184-162-80	3.1
32	759	453-215-80	1.6
33	759	453-229-80	3.1
34	759	453-184-80	1.6
35	759	730-80	1.6
36	759	667-80	1.6
37	759	636-100	1.6
38	759	579-229	1.6
39	759	579-100	1.6
40	759	579-80	1.6
41	725	301-244-184-80	1.6
42	725	636-80	3.1
43	725	602-80	1.6
44	725	552-244	1.6
45	684	334-229	1.6
46	602	334-229	1.6
47	602[a]		1.6
48	579	334-229	6.3
49	579[a]		1.6

[a] Not digested by *Alu*I

PCR — polymerase chain reaction; RFLP — restriction fragment length polymorphism; bp — base pairs

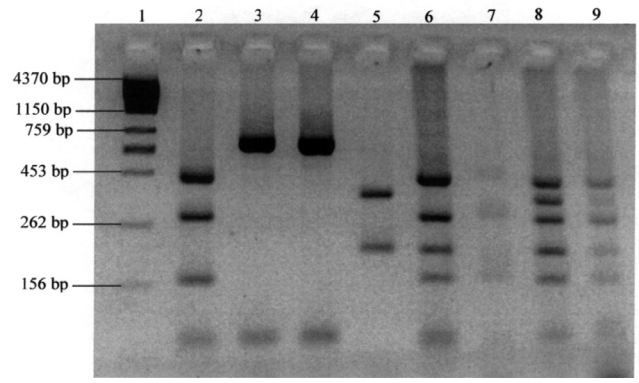

Figure 2. Electrophoretic profile, in 5% agarose gel, of *Alu*I restriction fragments of polymerase chain reaction (PCR) products: lane 1 — molecular marker; lanes 2 to 5 —isolates with only 1 amplicon; lanes 6 to 9 — isolates with 2 amplicons.

950, 880, 838, 790, 759, 725, 602, and 579 bp) generated 2 or more patterns. The isolates with only 1 PCR amplicon produced 2 to 4 restriction fragments, whereas those with 2 amplicons produced 5 to 6 restriction fragments (Figure 2).

The agarose gel analysis of the *Alu*I RFLP patterns showed 49 different types. Types 9, 13, 23, 27, 28, 29, 31, 33, 42, and 48 were the most common and accounted for 39% of the isolates. The remaining 39 types accounted for only 1 isolate each (1.6% of the total).

Reproducibility of the *Alu*I RFLP was observed with all repeatedly tested PCR products. The PCR products of 2 isolates were not digested by the *Alu*I; therefore, this method had 97% typability. The discriminatory index was 0.99.

Discussion

Production of coagulase is an important phenotypic feature, used worldwide to identify *S. aureus*. Although the role of this protein in *S. aureus* infection is not completely understood, the variability of the 3′ end region of the *coa* gene is the basis for a typing method used for isolates from infected humans (6,7,17) and animals (8,18). By this method, we detected many different genotypes among the studied isolates, which suggests that *S. aureus* has considerable heterogeneity in the sampled region. Although many genotypes were detected, only a few predominated. In an earlier study, performed in the south of Brazil (18), 7 *coa* PCR types were observed, and 2 accounted for more than 50% of the isolates. Thus, in some Brazilian regions most cases of mastitis may be caused by *S. aureus* strains with the same *coa* genotype.

According to Su et al (12), the presence of few types may permit greater efficiency in measures to control *S. aureus* mastitis, since the important virulence factors could be specifically targetted. Furthermore, these researchers demonstrated that organisms with the predominant coagulase genotypes were more resistant to neutrophil activities than those with the rare genotypes, which indicated that specific features in the former may help them overcome host defence mechanisms.

Calculation of the discriminatory power of the typing method yielded high values: 0.92 for PCR amplification and 0.99 for RFLP analysis. According to Hunter and Gaston (16), an index greater than 0.90 can be interpreted with confidence and is thus desirable. Although the number of analyzed strains was low, the high number of *Alu*I RFLP patterns and the absence of an epidemiologic relation between the isolates may have contributed to the high RFLP index.

The primer pair amplified more than 1 PCR product in 4 isolates, which suggests the presence of different allelic forms of the *coa* gene. Some *S. aureus* isolates express more than 1 immunologic form of the coagulase protein (6), but this appears to be a very uncommon finding: only Goh et al (6) and Schwarzkopf and Karch (7) had reported it.

With the PCR method, an amplification product was not observed for the DNA of another coagulase-positive species of *Staphylococcus*. According to Aarestrup et al (8), this could indicate that the *S. aureus coa* gene differs from the *coa* gene of other *Staphylococcus* species that have the ability to coagulate mammalian plasma. These authors suggested that specific *coa* gene primers could be used to identify and discriminate between coagulase-positive *Staphylococcus* species.

In conclusion, our results demonstrate that although mastitis in the studied region is caused by *S. aureus* strains that have many variants of the *coa* gene, only a few *coa* gene variants predominate. Further studies are needed to determine the common characteristics of the predominant strains. The information gathered could be used to develop control measures for mastitis caused by *S. aureus*.

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