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Identification of coagulase-negative staphylococci from bovine mastitis using RFLP-PCR of the *groEL* gene

Olinda Cabral da Silva Santos [a], Elaine Menezes Barros [a], Maria Aparecida Vasconcelos Paiva Brito [b], Maria do Carmo de Freire Bastos [a], Kátia Regina Netto dos Santos [a], Marcia Giambiagi-deMarval [a,*]

[a] *Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro, CCS, Bloco I, Cidade Universitária, 21491-590 Rio de Janeiro, RJ, Brazil*

[b] *Embrapa Gado de Leite, Juiz de Fora, MG, Brazil*

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Abstract

Coagulase-negative staphylococci (CNS) have become the predominant pathogens causing bovine mastitis in many countries. CNS infections are associated with damage to milk secretory tissue of the mammary gland by increased connective tissue stroma, moderate increases of somatic cells count in milk and significant production decreases. These consequences impose serious economic losses for the farmers and the dairy industry. Routine veterinary laboratories do not usually identify CNS at the species level. Thereby, the aims of this study were to identify the most common staphylococcal pathogens involved in bovine mastitis using PCR-restriction fragment length polymorphism (RFLP) analysis of a partial *groEL* gene sequence and to compare our results with the identification carried out by the conventional method. A total of 54 isolates of *Staphylococcus*, involved in bovine mastitis, were analyzed by this method. The size and number of the fragments obtained by either AluI or HindIII/PvuII digestions made possible to form clear patterns differentiating, among the isolates, 11 of the most common species of animal staphylococcal pathogens. Most of the isolates clustered together with the reference strain of *Staphylococcus chromogenes* (28) and the type strain of *Staphylococcus epidermidis* (8). Besides, some isolates clustered together with the type strain of *Staphylococcus aureus* (5). All patterns were confirmed by the conventional biochemical method, showing concordant results. Thus, the PCR-RFLP of the *groEL* gene constitutes a reliable and reproducible molecular method for identification of CNS species responsible for bovine mastitis.

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Keywords: Coagulase-negative staphylococci; *Staphylococcus*; Bovine mastitis; *groEL* gene; RFLP-PCR

1. Introduction

Bovine mastitis is a multifactorial disease, which results in reduction in milk yield, in changes in milk composition and in discarded milk. It imposes serious

* Corresponding author. Tel.: +55 21 2256 6746; fax: +55 21 2560 8344.

E-mail address: marciagm@micro.ufrj.br (M. Giambiagi-deMarval).

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economic losses for the farmers and the dairy industry (Ribeiro et al., 2003; Pitkälä et al., 2004). Mastitis can be either moderate or severe, and can be caused by many different bacterial species, mainly by *Staphylococcus* and *Streptococcus* strains (Pyörälä, 2002). Coagulase-negative staphylococci (CNS), which were traditionally considered to be minor mastitis pathogens, have become more common (Huxley et al., 2002). The most frequently isolated CNS species vary according to the geographical region under scrutiny. In a recent study, it was reported that *Staphylococcus simulans* and *Staphylococcus chromogenes* were highly prevalent CNS species in Finland (Taponen et al., 2006).

The determination of *Staphylococcus* to the species level through conventional biochemical testing, although being the gold standard for identification, is laborious, costly and time consuming (Couto et al., 2001). There are commercial biochemical kits available but they have mainly been developed for identifying strains of human origin (Heikens et al., 2005) and results obtained with these rapid identification kits for bovine isolates have not been reliable. Matthews et al. (1990) showed that agreements of Vitek and API systems with conventional methods were 44.6% and 80.8%, respectively. Another report has shown that these methods have an accuracy of 70–90%, although additional tests were also required for final identification (Thorberg and Brändström, 2000). Thus, the identification of CNS based on commercial biochemical kits does not identify all CNS isolates of animal origin (Bes et al., 2000), and misidentification may also happen (Couto et al., 2001).

The development of molecular biological methods, such as nucleic acid analysis or protein patterns, may circumvent some of the problems associated with conventional microbiological procedures and add possibilities for the rapid identification of bacteria (Busse et al., 1996). The DNA-based identification systems are targeted for specific pathogens, allows for rapid screening of a large number of pathogens simultaneously, and provides definitive confirmation of pathogens (Gillespie and Oliver, 2005; Taponen et al., 2006). The *groEL* gene, which encodes a 60-kDa polypeptide (known as GroEL, 60-kDa chaperonin, or HSP60 for heat shock protein 60) has the potential to serve as a general phylogenic marker because of its ubiquity and conservation in nature (Segal and Ron, 1996). This gene was proven to be an ideal universal DNA target for identification to the species level because it has well-conserved DNA sequences within a given species, but with sufficient sequence variations to allow for species-specific identification (Goh et al., 1996).

We have successfully used the *groEL* gene as a tool for the identification of the main *Staphylococcus* species involved in human infections by PCR-restriction fragment length polymorphism (RFLP) (Barros et al., 2007). The aim of the present study was to extend the identification using the same approach, PCR-restriction fragment length polymorphism of the *groEL* gene, to the most common staphylococcal strains isolated from cows with mastitis.

2. Materials and methods

2.1. Bacterial isolates and control strains

Staphylococci isolates were obtained from 54 cows and 23 dairy herds located in the Brazilian States of Minas Gerais, Rio de Janeiro and São Paulo, between 1995 and 2003. Individual mammary quarter milk samples were aseptically collected into sterile vials immediately before milking, after discarding the first three milking streams. The milk samples were transported to the mastitis diagnostic laboratory and cultured following National Mastitis Council recommendations (NMC, 1990). CNS isolated in pure culture with a minimum of six colonies were stored in the laboratory culture collection. They were initially identified by the Gram staining, catalase test, acid production from glucose in anaerobiosis and susceptibility to 0.04 U bacitracin (CECON, São Paulo, Brazil) to characterize the genus *Staphylococcus* (Bannerman, 2003). The following type strains were included in the study: *Staphylococcus capitis* ATCC 27840, *Staphylococcus cohnii* ATCC 29974, *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus haemolyticus* ATCC 29970, *Staphylococcus hominis* ATCC 27844, *Staphylococcus xylosus* ATCC 29971 and *Staphylococcus aureus* ATCC 12600. In addition, four clinical strains, all identified to the species level by the biochemical conventional method, were used as positive control strains of *S. chromogenes*, *Staphylococcus hyicus*, *Staphylococcus caprae* and *Staphylococcus sciuri*

species. All strains were grown overnight on blood agar plates at 37 °C for examination of purity and colony characteristics.

2.2. Phenotypic identification

Phenotypic identification of bacterial isolates was achieved by using the reference method according to Bannerman (2003) and Mac Faddin (1977). Twenty-two tests were used as follows: coagulase, hemolysis, clumping factor (“Slidex Staph Plus”, bioMérieux S/A, Inc., Durham, NC, EUA), pyrolidonyl arylamidase (PYR), urease, alkaline phosphatase, ornithine and arginine decarboxylases, nitrate reduction, acetoin production, susceptibility to 5 μg novobiocin (CECON) and acid production from D-trehalose, sucrose, D-ribose, D-cellobiose, D-xylose, α-lactose, D-mannitol, maltose and D-mannose. Susceptibility to 100 μg desferioxamine (Sigma Chemical Co., St. Louis, MO, USA) and 300 UI polymyxin B (CECON) was determined according to Monsen et al. (1998).

2.3. Extraction of genomic DNA

DNA was isolated by the guanidine isothiocyanate extraction method as previously described (Aires de Souza et al., 1996).

2.4. PCR amplification

The *groEL* degenerate primers H279A (5′-GAIIIIGCIGGIGA(TC)GGIACIACIAC-3′) and H280A (5′-(TC)(TG)I(TC)(TG)ITCICC(AG)AAICCIG-GIGC(TC)TT-3′), previously described (Goh et al., 1997), were used to amplify a 550-bp *groEL* gene fragment. The PCR reactions were performed in a PTC-100™ Thermal Cycler (MJ Research, Inc.) in a final volume of 100 μl containing 100 ng DNA, 0.5 μg of each primer, 250 μM concentration of each deoxyribonucleoside triphosphate, 1.5-mM $MgCl_2$ (Biotools) and 2 U of *Taq* DNA-polymerase (Biotools) in 1× amplification buffer (KCl 50 mM, Tris–HCl 20 mM, pH 8.4—Biotools). The PCR mixtures were denatured (3 min at 95 °C), then subjected to 40 cycles of amplification (1 min denaturation at 94 °C, 2 min annealing at 37 °C and 1 min elongation at 72 °C) and completed with 1 cycle of 10 min at 72 °C. The presence of a PCR product was confirmed by agarose gel electrophoresis.

2.5. PCR-RFLP

The restriction fragment length polymorphism, using AluI restriction endonuclease, was carried out as described by Barros et al. (2007). Briefly, digestions were performed with 500 ng of the PCR products in a total volume of 30 μl with 1× reaction buffer, 2 μg of acetylated BSA and 5 U of AluI (Promega) for 2 h at 37 °C. The resulting fragments were separated by electrophoresis for 1 h and 40 min at 150 V in Tris–Borate–EDTA buffer (pH 8.2) through a 10% polyacrylamide gel. The gels were stained with ethidium bromide and visualized on a UV transilluminator. A double digestion with HindIII (10 U) and PvuII (10 U) was also performed for the identification of the species *S. chromogenes*, *S. hyicus* and *S. capitis*, following the same protocol. The size of the fragments was determined by comparison with the 25-bp DNA ladder (Invitrogen).

3. Results

3.1. Identification of Staphylococcus reference strains

The primer pair H279A and H280A successfully primed the synthesis of the expected 550-bp fragment of the *groEL* gene (Fig. 1A). The 550-bp amplicons were AluI digested and the RFLP pattern was obtained for every species analyzed by polyacrylamide electrophoresis (Fig. 1B). Each pattern consisted of one to four DNA fragments ranging in size from approximately 40 bp to 530 bp. All reference strains revealed a pattern which correlated with the computational restriction fragment length analysis of the *groEL* 550-bp fragment digested with AluI, using the Restriction Mapper program, and according to our previous results (Barros et al., 2007) (Table 1). Eleven species-specific restriction patterns were observed. However, the species *S. chromogenes*, *S. hyicus* and *S. capitis* presented similar RFLP patterns (Figs. 1B and 2 and Table 1). For these three species, a double digestion with HindIII and PvuII was then performed and clearly distinguished different RFLP patterns (Table 1 and Fig. 2). All strains revealed

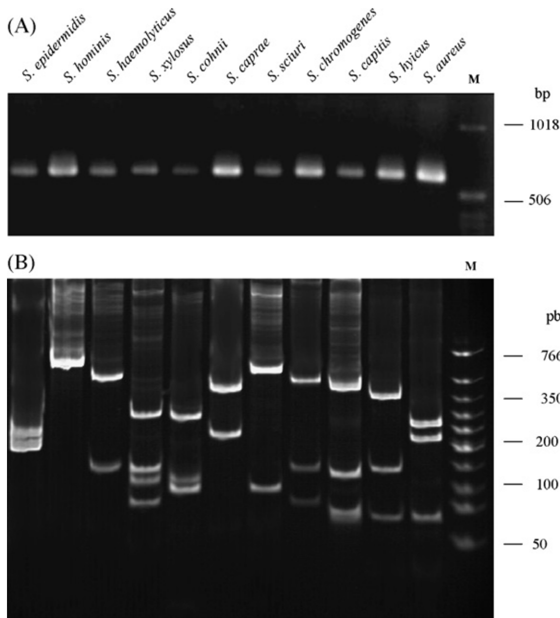

Fig. 1. (A) Agarose gel electrophoresis of 550-bp amplicons of the *groEL* gene from *Staphylococcus* reference strains using primers H279A and H280A. M, 1-kb DNA ladder (Invitrogen). (B) Polyacrylamide gel electrophoresis of the fragments produced by the AluI digestion of the staphylococcal amplicons. Lanes are the same as in panel A. M, low molecular weight DNA ladder (BioLabs).

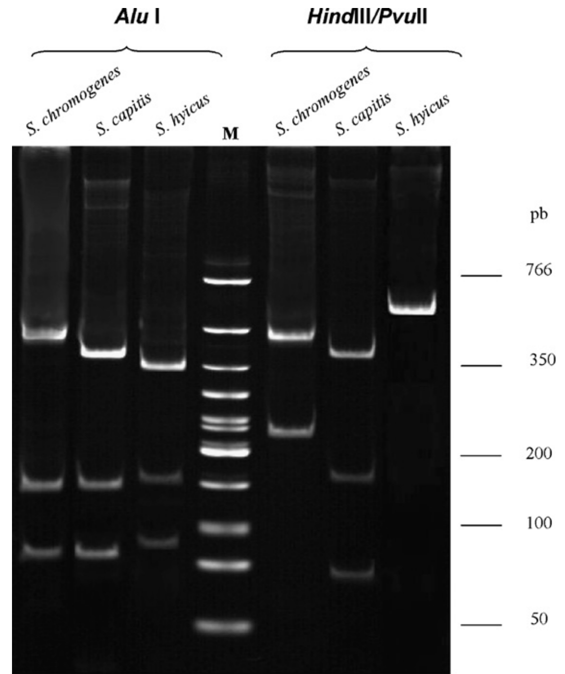

Fig. 2. Polyacrylamide gel electrophoresis of the fragments produced by AluI and HindIII/PvuII digestions of the *groEL* amplicons from *Staphylococcus chromogenes*, *Staphylococcus capitis* and *Staphylococcus hyicus*, respectively. M, low molecular weight DNA ladder (BioLabs).

a pattern which correlated with the computational analysis, however, it should be remarked that a standard deviation of 10% in the fragment size may be observed during electrophoresis of double-stranded DNA (Sambrook and Russell, 2001).

3.2. RFLP patterns and their correlation with the conventional method results

To validate the assay proposed, a total of 54 isolates of *Staphylococcus* were analyzed by this method. The

Table 1
RFLP patterns of the staphylococcal control strains using the Restriction Mapper program[a]

Patterns obtained with the control strains	Size of fragments[b]	
	AluI	HindIII and PvuII
Staphylococcus chromogenes	75, 107, 371	180, 374
Staphylococcus epidermidis	173, 189, 191	–
Staphylococcus aureus	14, 78, 204, 258	–
Staphylococcus capitis	9, 11, 66, 107, 360	15, 66, 114, 360
Staphylococcus haemolyticus	9, 107, 438	–
Staphylococcus sciuri	14, 93, 446	–
Staphylococcus xylosus	20, 75, 78, 123, 258	–
Staphylococcus caprae	182, 372	–
Staphylococcus cohnii	2, 24, 65, 90, 93, 267	–
Staphylococcus hominis	5, 547	–
Staphylococcus hyicus	10, 100, 117, 324	14, 539

[a] Version3; http://www.restrictionmapper.org/.
[b] Only those fragments above 40 bp (bolding numbers) were considered for the PCR-RFLP species-specific patterns.

RFLP patterns using the AluI restriction enzyme allowed us to organize the strains in 11 restriction patterns (Table 1). However, the patterns corresponding to the species *S. chromogenes*, *S. hyicus* and *S. capitis* were analyzed subsequently by double digesting the 550-bp fragments with HindIII and PvuII. By PCR-RFLP using the *groEL* fragment we were able to clearly distinguish, among the 54 staphylococcal isolates, 11 restriction fragments patterns corresponding to *S. chromogenes* (28), *S. epidermidis* (8), *S. aureus* (5), *S. capitis* (3), *S. haemolyticus* (2), *S. sciuri* (2), *S. xylosus* (2), *S. caprae* (1), *S. cohnii* (1), *S. hominis* (1), and *S. hyicus* (1) (data not shown). The identification to species level of each pattern was confirmed by the conventional method as described in Section 2. For each pattern, the control strain (type or reference) of the staphylococcal species was included. Most of the isolates clustered together with the reference strain of *S. chromogenes* (28) and the type strain of *S. epidermidis* (8). Furthermore, some isolates clustered together with the type strain of *S. aureus* (5).

4. Discussion

Worldwide, mastitis is the most common infectious disease affecting dairy cows and the most economically important disease of the dairy industry. CNS has been considered a minor pathogen of bovine mastitis; however, recently, many studies have shown the importance of CNS infection in the bovine mammary gland. The high percentage of CNS-infected quarters indicates that CNS have the ability to elude the immune system, persisting in the mammary gland for a long time (Zhang and Maddox, 2000), and can be the cause of substantial economic losses due to tissue damage and to decrease in milk production. The reduction in milk production attributed to sub-clinical mastitis may account for 66% of the total losses (Bramley et al., 1996). Despite variations between herds and countries, *S. chromogenes*, *S. simulans* and *S. epidermidis*, in general, appear to be the most frequently isolated CNS from mammary secretion samples worldwide (De Vliegher et al., 2003; Taponen et al., 2006).

Most of the commercial identification systems (e.g. API-Staph, Staph-Zym, and ID 32 Staph) are not designed to identify important veterinary pathogens. These methods were primarily developed for human strains and their poor performance for identifying CNS strains of animal origin is most likely due to a limited number of veterinary strains in databases (Bes et al., 2000). For example, around 63% of the *S. chromogenes* isolates were identified incorrectly as *S. simulans* (Matthews et al., 1990). Additionally, the reference method of Bannerman (2003) is costly and time consuming to be used in a clinical laboratory. Genotypic methods may provide a better alternative for the identification of clinical and animal staphylococcal isolates, because of their higher specificity and sensitivity (Goh et al., 1997; Forsman et al., 1997; Taponen et al., 2006). Several molecular targets have been exploited for the molecular identification of *Staphylococcus* species, including the 16S rRNA gene, *femA* gene, *sodA* gene, *rpoB* gene and *groEL* gene (Bialkowska-Hobrzanska et al., 1990; Goh et al., 1996; Vannuelfel et al., 1999; Poyart et al., 2001; Drancourt and Raoult, 2002). The *groEL* gene is ubiquitous and encodes a highly conserved housekeeping protein, which is essential for cell survival. It is more variable than the 16S rRNA gene sequence and, therefore, potentially useful for the identification of genetically related species (Heikens et al., 2005). The *groEL* gene was successfully used for staphylococcal speciation by Southern blot (Goh et al., 1997) and sequencing (Kwok et al., 1999). However, PCR-RFLP molecular approach is easier to perform than Southern blot and less expensive and less equipment-dependent than sequencing.

In this study, we used the PCR-RFLP of the *groEL* gene with the AluI, previously described by our group for the identification of nosocomial isolates of CNS (Barros et al., 2007), to identify staphylococcal isolates involved in bovine mastitis. We have found similar results with those obtained by Barros et al. (2007), indicating that CNS isolates from bovine mastitis could be identified by the same approach. This reinforce that the PCR-RFLP of the *groEL* gene is a potentially valuable tool for accurate identification of *Staphylococcus* independent of the origin of the isolate. In this work, the size and number of the fragments generated by either AluI or HindIII/PvuII digestions made possible to obtain clear clusters differentiating, among the isolates, 11 of the most common species of animal staphylococci. Although the RFLP patterns were

consistent, the species *S. chromogenes*, *S. hyicus* and *S. capitis* presented similar three-fragments AluI patterns, and, in this case, we recommend a double digestion with HindIII and PvuII to obtain distinctive RFLP patterns among these species. It should be remarked that previously to RFLP analysis, basic phenotypic methodology should be performed on the samples to exclude non-*Staphylococcus* strains that could present cross-profile with *Staphylococcus* strains once the *groEL* gene is ubiquitous.

Considering that correct species identification is important for mastitis control and in epidemiological investigations, as well as in understanding of the significance of infections caused by different CNS species, PCR-RFLP of the *groEL* gene proved to be an adequate tool for the identification of the most common animal staphylococcal pathogens, independent of their phenotypic characteristics.

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