rep-PCR of *Staphylococcus* aureus strains isolated from bovine mastitis in Argentina

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Abstract

The aim of this study was to assess by rep-PCR the genetic relationship of 52 *S. aureus* strains isolated from mammary infections collected in four herds located in the central dairy region of Argentina. Results were compared with the *in vitro* activity of antimicrobial drugs frequently used for treating bovine mastitis. Twelve different antimicrobials patterns were observed. Forty eight percent of the strains were susceptible to all antimicrobials tested. rep-PCR typing could successfully differentiate *S. aureus* strains of bovine origin. At a first level of similarity (50%), it could be defined 5 clusters namely I to V. Most of the strains (75%) were grouped in cluster I. The results may suggest that genotypes were similar in the different herds. Agreement between antibiotic patterns and rep-profiles was not observed for most isolates. The present report describes the genotypes responsible for the mastitis cases in the central dairy region of Argentina. A better knowledge of infective strains distribution in dairy herds might help in formulating strategies to control of infection. Furthermore, antimicrobial susceptibility of *S. aureus* should be used as guide to select effective drugs to therapy in intramammary infections.

Introduction

Bovine mastitis caused by *Staphylococcus aureus* is an infectious disease that affects both the quality and the quantity of milk production.[1] Monitoring and limiting of the intra-and inter-herd spread of *S. aureus* strains requires the use of efficient and accurate epidemiologic typing systems. The ability to identify specific strains of a causative bacterial species is very important for epidemiological investigations. DNA-based methods have been used for epidemiological identification and comparison of *S. aureus* isolates from human and animal staphylococcal infections. [2,3,4] Several PCR-based methods have shown limitations, either in inter-center pattern reproducibility, as described for AP-PCR analysis[5], or in discrimination, as found with RFLP analysis of the coagulase or protein A gene.[6] Compared to the latter assays, rep-PCR analysis based on multicopy elements of the staphylococcal genome has shown good reproducibility and discriminatory power in single-center studies.[7,8,9]

Different studies on bovine *S. aureus* isolates have been carried out in Argentina[10,11,12,13,14] and in others countries.[2,3] However, little is known about the distribution and genotypes of isolates that predominate within dairy herds of Argentina.[4, 14] The aim of this study was to assess the genetic relationship by rep-PCR among 52 *S. aureus* strains isolated from mammary infections collected from four herds located in the central dairy region of Argentina with the *in vitro* activity of antimicrobial drugs frequently used for treating bovine mastitis.

Material and Method

A total of 52 *Staphylococcus aureus*

strains collected from 4 herds, named A (n=15), B (n=5), C (n=8) and D (n=24), located in the central dairy region of Argentina were used in this study. The isolates were obtained from milk of cows with subclinical mastitis between 1999-2002. Milk samples (10 ml) were taken aseptically from individual quarters of bovine infected udders and 0.01 ml of each sample was cultured on agar plates with 5% sheep blood. Bacterial isolation was performed by standard procedures according to the National Mastitis Council methods.[15] All the isolates were biochemically confirmed to be *S. aureus* by the standard biochemical methods.[16]

For susceptibility testing, isolates were suspended in tripticase soy broth and the suspension was adjusted to a turbidity equivalent to 0.5 McFarland standards. Drug susceptibility testing was performed by agar diffusion method on Mueller Hinton agar. The following disks were used: penicillin (10 IU), ampicillin (10 µg), tetracycline (30 µg), erythromycin (15 µg), gentamicin (40 µg), cephalothin (30 µg), spiramicin (40 µg) and streptomicin (10 µg). The antimicrobials tested are the ones most commonly used in veterinary medicine in Argentina. Isolates were categorized as susceptible, intermediate and resistant based upon interpretative criteria developed by the National Committee of Clinical Laboratory Standards.[17] Oxacillin was included for detection of methicillin-resistant *S. aureus*. Resistance to methicillin was determined according to the test recommended by the NCCLS, using an agar plate containing 6 µg/ml of oxacillin and Mueller Hinton agar supplemented with NaCl (4% w/v; 0.68 mol/L). *S. aureus* ATCC 25923 was used as a recommended quality control reference organism to be run with each group of unknowns.

rep-PCR assay was carried out as described by van Belkum et al.[5] Amplifications were performed in a buffer solution containing 3.0 µM of oligonucleotide, 200 µM of each deoxynucleoside triphosphates, 3.5 µM MgCl$_2$ and 2.5 U of DNA *Taq* polymerase.

The oligonucleotide RW3A 5¢ TCGCTCAAAACAACGACACC 3¢ was used for DNA amplifications. Amplification consisted of a cycle of predenaturation at 94°C for 5 min, followed by 40 cycles of 1 min at 93°C, 1.30 min at 37°C and 1 min at 72C. A final extension step of 72°C for 8 min was included. A negative and a positive control were also included. Each isolate was tested under the same conditions at least twice with the selected oligonucleotide. Amplified products were separated by electrophoresis in a 1.5% agarose gel in 0.5X TBE buffer at a constant voltage of 4 V/cm and stained using ethidium bromide (0.5 µg/ml). PCR pattern analysis was performed as was described previously.[4]

Result and Discussion

The present report describes the genetic relationship of *S. aureus* strains obtained from the central dairy region of Argentina. The strains were tested to antimicrobial resistance. Twelve different drugs patterns were observed (Table 1). Sixteen strains (31%) were resistant to one antimicrobial, eight strains (15%) were resistant to two antimicrobials and three strains (6%) to three drugs. Our results indicated that 28% of *S. aureus* strains collected from the central dairy region of Argentina exhibited resistance to penicillin. The level of resistance was similar to those reported by Frigerio et al.[18] (30%) and lower reported by Calvinho et al.[19] in Argentina. On the other hand, studies carried out in different countries of Europe[20,21] reported a 37% of resistance. Although ß-lactamic antimicrobials are frequently used in intramammary infections in Argentina, our data shown a low percentage of *S. aureus* strains resistant to penicillin. In this study, we did not find resistance against oxacillin. This data are according with others studies[19,22] and indicates that resistance against oxacillin is not a problem in dairy herds in Argentina. No resistance to cephalothin was observed. Recently, studies carried out by Acuña et al.[23] have shown low or null resistance against first

Table 1 - Antimicrobial resistance patterns among bovine *S. aureus* strains

Antibiogram pattern[a]	Strains	Susceptibility to[b]								
		Pn	Em	Tc	Sm	Cf	Amp	Sp	Gn	Ox
0	47, 51, 54, 57, 64, 65, 67, 72, 73, 74, 75, 76, 77, 79, 80, 81, 83, 85, 87, 90, 92, 97, 99, 100, 101	S	S	S	S	S	S	S	S	S
1	48, 53, 55, 59, 61, 69, 78, 108	R	S	S	S	S	S	S	S	S
2	66, 106	S	S	S	S	S	R	S	S	S
3	50, 82, 94, 96, 109	S	S	S	R	S	S	S	S	S
4	93	S	S	R	S	S	S	S	S	S
5	56	R	S	S	S	S	R	S	S	S
6	91, 107	S	S	S	R	S	R	S	S	S
7	52, 84, 89	R	S	S	R	S	S	S	S	S
8	98	S	R	S	R	S	S	S	S	S
9	88	S	S	R	R	S	S	S	S	S
10	62	R	R	S	R	S	S	S	S	S
11	60	R	S	R	R	S	S	S	S	S
12	58	R	R	R	S	S	S	S	S	S

[a] 0: lack of *in vitro* susceptibility to antimicrobials tested; resistance against 1: Pn; 2: Amp; 3: Sm; 4: Tc; 5: Pn/Amp; 6: Sm/Amp; 7: Pn/Sm; 8: Em/Sm; 9: Tc/Sm; 10: Pn/Em/Sm; 11: Pn/Tc/Sm; 12: Pn/Em/Tc.
[b] Amp: ampicillin, Cf: cephalothin, Em: erythromycin, Gn: gentamicin, Pn: penicillin, Sm: streptomycin, Sp: spiramicin, Tc: tetracycline, Ox: oxacillin.

generation cephalosporins.

It is important to remark that different antimicrobial patterns were found within each herd. Forty eight percent of the strains were susceptible to all drugs tested. No resistance to antimicrobials was observed in the isolates collected from herd D, except for strain 78. Twenty five percent of the strains isolated from herd D showed resistance to two antimicrobials and twenty percent of the strains isolated from herd A showed resistance to three drugs. These results may indicate differences in management and treatment practices among these herds, particularly, differences in the type and frequency of drugs use, and thus of exposure to antimicrobials among herds.

To our knowledge, this is the first study carried out in Argentina which describes the genetic relationships among S. aureus isolates from bovine mastitis by rep-PCR. As shown in previous reports, rep-PCR was proven to be a highly discriminate and rapid screening method to classify a large number of isolates into clusters.[24] Several studies have shown rep-PCR to have good correlation with PFGE results but, in general, with slightly less discriminatory power.[25,26] In this study, rep-PCR typing could successfully

differentiate S. aureus strains of bovine origin. A total of 31 rep-profiles in the range size from 300 to 6000 bp were identified after rep-PCR analysis. Figure 1 shows the results obtained with the oligonucleotide RW3A. A dendrogram that included all profiles was constructed on the basis of the levels of similarity (Figure 2). At a first level of similarity (50%), it could be defined five
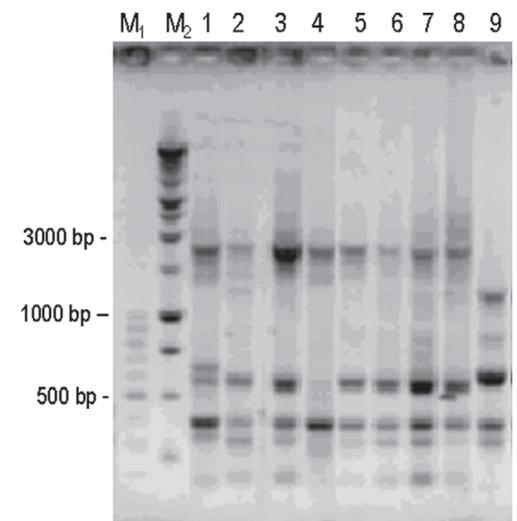
Figure 1 - rep-PCR profiles of nine *S. aureus* bovine isolates from the central dairy region of Argentina. M_1: molecular weight marker 100 bp and M_2: molecular weight marker1 kb

Strain n°, region- herd, antibiogram pattern
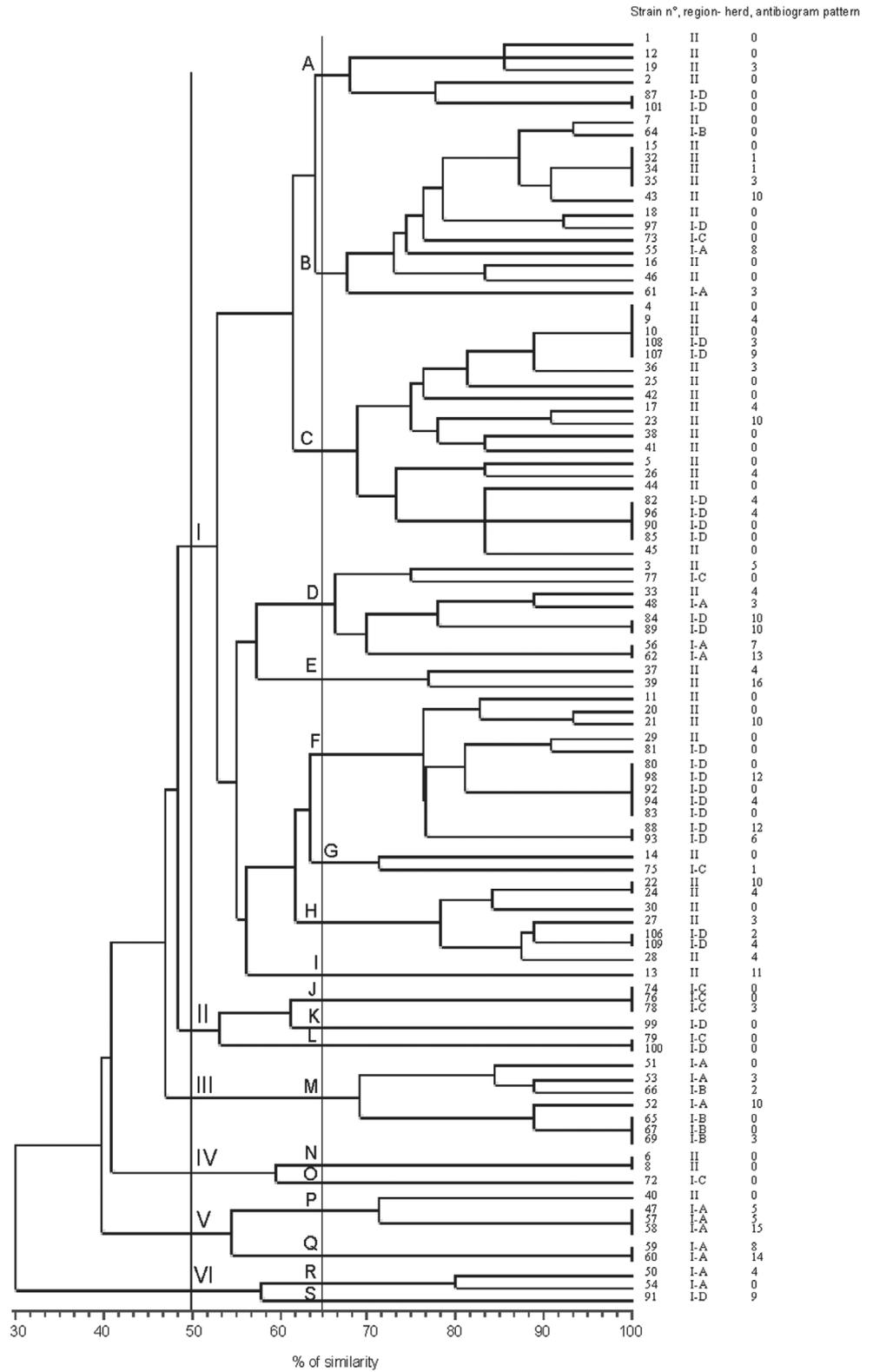
Figure 2 - Genetic relationship among the bovine *S. aureus* strains as estimated by clustering analysis by rep-PCR

clusters namely I to V. Most of the strains (75%) were grouped in cluster I, this result may suggests that genotypes were similar in the different herds. A further differentiation was established at 60% was considered to define 10 clusters namely A to J.

Genetic heterogeneity among *S. aureus* isolates was observed within herds. The fifteen *S. aureus* strains from herd A were divided into 11 profiles belonged to clusters A, B, D, F, H and J and the twenty four isolates from herd D were divided into 12 profiles belonged to clusters B, C, E, and I. However, predominant profiles were found within herds. Three predominant profiles were found in herd A (strains 47-57-58; 56-62; 59-60), two predominant profiles were identified in herd D (strains 82-96-90-85; 80-98-92-94-83) and one predominant profile in herds B (strains 65-67-69) and C (isolates 74-76-78).

Furthermore, isolates obtained from the different herds were not identical to each other, except for the strains 79 and 100 belonged to herds C and D, respectively. Interestingly, 50% of the *S. aureus* strains isolated from herd D were genotypically similar to strains isolated from herd C, despite of the nearest distance between herds was approximately 200 Km, suggesting a close genetic relationship between herds, as reported Zadoks et al.[3]. The finding of similar *S. aureus* strains in the different herds suggests, as shown previously, that some strains may be responsible for cases of bovine mastitis.[27,28,29]

Antimicrobial patterns alone were found to be of limited value in differentiating closely related strains. However, in this study, rep-PCR was able to differentiate among many isolates that were indistinguishable by drug susceptibility testing, as reported previously.[29] Agreement between antimicrobial patterns and rep-profiles was not observed for most isolates. Some isolates from different herds that had the same rep-profile had different drug patterns (Figure 2). A low percentage of isolates (7.7%) shared rep-profiles and antimicrobial patterns, isolates 84 and 89 from herd D had the same rep-profile and antimicrobial pattern 7 and isolates 82 and 96 from herd D had the same rep-profile and antimicrobial pattern 3. This finding contrasts with Rivas et al.[30] who reported statistical associations between some *S. aureus* ribotypes and *in vitro* antimicrobial patterns, suggesting that at least some resistance to antibiotics may be predicted by genotypic methods.

The present report describes the genotypes responsible for the mastitis cases in the central dairy region of Argentina. A better knowledge of infective strains distribution in dairy herds might help in formulating strategies to reduce infection spread. Furthermore, drug susceptibility of *S. aureus* should be used as guide to select adequate antimicrobials to therapy of mammary infections.

Acnowlegdment

Rep-pcr de *Staphylococcus* aureus isolados a partir de mastite bovina na Argentina

Resumo

O objetivo do presente estudo foi determinar a relação genética entre rep-PCR de 52 linhagens *Staphylococcus aureus* isoladas de infecções mamárias em quatro fazendas leiteiras da região leiteira central da Argentina. Os resultados foram comparados com a atividade *in vitro* dos antimicrobianos freqüentemente utilizados no tratamento da

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mastite bovina. Foram observados 12 diferentes perfis de antimicrobianos. Do total de linhagens, 45% foram suscetíveis a todos os antibióticos ensaiados. A caracterizacão por rep-PCR pode diferenciar com sucesso as linhagens de *S. aureus* de origem bovina. Num primeiro nível de similaridade (50%) foram definidos cinco grupos denominados de I a V. A maioria das estirpes (75%) agruparam-se no grupo I. Os resultados sugerem que os genotipos foram similares. Os genotipos não foram asociados com os perfis de antimicrobianos na maioria dos isolados. O presente estudo descreve os genotipos responsáveis pelos casos de mastites na região central da Argentina. O melhor conhecimento da distribução das linhagens infectantes em fazendas leiteras poderia auxiliar na formulacão de estratégias para o controle de infecção. Além disso a suscetibilidade aos antimicrobianos de linhagens de *S. aureus* deve ser usada para montear a selecão de drogas efetivas para a terapia intramamária.

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