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Antimicrobial resistance studies in staphylococci and streptococci isolated from cows with mastitis in Argentina

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ABSTRACT

Background: *Staphylococcus aureus* and *Streptococcus agalactiae* are the main cause of clinical mastitis in dairy cattle in Argentina, whereas coagulase-negative staphylococci (CNS) and environmental streptococci are the main cause of subclinical mastitis. Bacteria isolated from infected animals show increasing antimicrobial resistance.

Objectives: This study aims to determine the antimicrobial resistance of staphylococci and streptococci isolated from milk with mastitis, and to genotypically characterize the methicillin-resistant (MR) staphylococci.

Methods: Isolation was performed on blood agar and identification was based on biochemical reactions. Antimicrobial susceptibility was according to the Clinical and Laboratory Standards Institute guidelines. The antimicrobial resistance genes, SCC*mec* type and *spa* type were detected by the polymerase chain reaction method.

Results: We isolated a total of 185 staphylococci and 28 streptococci from 148 milk samples. Among the staphylococcal isolates, 154 were identified as CNS and 31 as *S. aureus*. Among the 154 CNS, 24.6% (n = 38) were resistant to penicillin, 14.9% (n = 23) to erythromycin, 17.5% (n = 27) to clindamycin, 6.5% (n = 10) to cefoxitin and oxacillin. Among the *S. aureus* isolates, 16.1% (n = 5) were resistant to penicillin, 3.2% (n = 1) to cefoxitin and oxacillin (MRSA). Six MR isolates (5 CNS and 1 MRSA) were positive to the *mecA* gene, and presented the SCC*mec* IVa. The MRSA strain presented the sequence type 83 and the *spa* type 002. Among the 28 streptococcal isolates, 14.3% (n = 4) were resistant to penicillin, 10.7% (n = 3) to erythromycin and 14.3% (n = 4) to clindamycin.

Conclusions: The present findings of this study indicate a development of antimicrobial resistance in main bacteria isolated from cows with mastitis in Argentina.

Keywords: Bovine mastitis; antimicrobial resistance; staphylococci; streptococci; wmolecular typing

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INTRODUCTION

Bovine mastitis, which is the inflammation of the mammary gland tissue, usually has an infectious etiology. The cost of the cow's treatment and the decrease in milk production makes mastitis the most costly disease to the dairy industry [1]. The main microorganisms causing bovine mastitis in Argentina and many other countries around the world are Gram-positive cocci, mainly streptococci and staphylococci [2]. Among streptococci, *Streptococcus agalactiae* is one of the main causes of subclinical mastitis in dairy cattle, despite its antimicrobial susceptibility [3], while *Streptococcus dysgalactiae* and *Streptococcus uberis* are environmental pathogens [4]. Among staphylococci, *Staphylococcus aureus* is the most important pathogen in bovine clinical mastitis, while coagulase-negative staphylococci (CNS) are more frequent in subclinical mastitis cases [5]. These microorganisms currently show increasing antimicrobial resistance and are considered reservoirs of resistance genes [1]. Antimicrobial resistance has become a concern for many reasons, including therapeutic failure, economic losses and public health repercussions. In addition, resistant pathogens may spread and become a serious problem in healthcare institutions and communities [6]. Thus, to select an appropriate antimicrobial therapy, it is important to correctly identify the pathogens involved [7]. Furthermore, to determine strategies to prevent the development of antimicrobial resistance and minimize the risk of spreading, it is important to consider the individual susceptibility of the microorganism, the pharmacokinetics of the drug, the time of action in the site of infection, and the animal toxicity [8].

The antibiotics frequently used in intramammary infusion therapy are β-lactam antibiotics. However, methicillin-resistant (MR) staphylococci are resistant to all β-lactam antibiotics, because these bacteria acquire a penicillin-binding protein called PBP2a, which has low affinity for these drugs. This low-affinity protein is encoded by the *mec*A/C genes located on a mobile genetic element called staphylococcal chromosomal cassette (SCC*mec*). SCC*mec* has been classified into 13 different types (types I-XIII) and subtypes because of differences in the structural organization and genetic content [9]. Originally, *mec*A$_{LGA251}$ was described as an allele of *mec*A and has been identified in MR *Staphylococcus aureus* (MRSA) from both humans and animals. This *mec*A homolog has been renamed as *mec*C because it shares 70% nucleotide identity with *mec*A [10]. In South America, we detected the first *mec*C gene in a CNS isolated from bovine mastitis [11].

Molecular typing methods are used to find out the clonal relatedness among isolates to monitor and control outbreaks and to determine routes of transmission. Among these, the method with the most discriminatory power is Whole-genome sequencing (WGS) followed by Next-generation sequencing (NGS), pulsed-field gel electrophoresis (PFGE), *spa* typing and multilocus sequence typing (MLST) [12].

The aim of this study was to identify and detect antimicrobial susceptibility in staphylococci and streptococci isolated from milk samples from mastitic cows in several herds in Argentina, and to determine the occurrence and epidemiology of MR strains based on their antimicrobial resistance patterns and molecular typing.

MATERIALS AND METHODS

Isolation and identification of staphylococci and streptococci

A total of 148 milk samples were collected from cows with subclinical and clinical mastitis between June 2016 and December 2017 in the dairy region of Buenos Aires and Córdoba, Argentina. Briefly, 10 μl of each milk sample was spread over a Columbia blood agar plate and incubated at 37°C for 24-48 h. *S. aureus* isolates were identified by Gram-staining, catalase, fermentation of glucose, coagulase, fermentation of mannitol, maltose and trehalose, and Voges-Proskauer. To identify the CNS group, we also evaluated oxidase, beta galactosidase and resistance to novobiocin. Streptococcal isolates were identified by catalase, pattern of hemolysis, growth in broth with 4% and 6.5% of sodium chloride, hydrolysis of esculin, the Pyroglutamic acid β-naphthylamide reaction (PYR) and the CAMP test.

Antimicrobial susceptibility testing

Staphylococcal isolates were tested for antimicrobial susceptibility using five antibiotics: penicillin, clindamycin, erythromycin, cefoxitin and oxacillin, and for streptococcal isolates, three antibiotics were tested: penicillin, clindamycin and erythromycin, using the disk diffusion method by Kirby-Bauer and categorized in accordance with the Clinical and Laboratory Standards Institute criteria [13]. The MICs for erythromycin, clindamycin and penicillin were determined by the agar dilution method [13].

Genotyping of MR-CNS and MRSA

The DNA was extracted using the Wizard Genomic DNA purification kit (Promega, USA). The polymerase chain reaction (PCR) mixture for each reaction was: 200 ng of DNA, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 5 μL of buffer 10X, 1.25 U Taq DNA polymerase (BioLabs) and distilled water to a final volume of 25 μL. The cycles and conditions used were described by Vannuffel et al. [14] and Cuny and Witte [15]. SCC*mec* types were determined by PCR [16,17]. The MRSA identified was analyzed as described by Enright et al. [18]. *Spa* typing was performed as described by Harmsen et al. [19] and Hallin et al. [20].

Statistical analysis

To compare the results obtained with the data from previous studies carried out by this working group, χ^2 statistic and Fisher's exact test were performed using the SPSS software (SPSS Inc., USA) and the values of $p < 0.05$ were considered statistically significant.

RESULTS

Identification

A total of 185 staphylococci and 28 streptococci were isolated from 148 milk samples from eight farms. Among staphylococcal isolates, 154 were identified as CNS (59.8% *S. epidermidis*, 20.2% *S. simulans*, 1.9% *S. sciuri* and 17.6% *S. saprophyticus*) and 31 as *S. aureus*. Among streptococci, eleven were identified as *Streptococcus dysgalactiae*, four as *Streptococcus uberis*, six as belonging to the *Streptococcus bovis* group and seven could not be identified by biochemical tests.

Antimicrobial resistance

Among the 154 CNS, 24.6% (n = 38) were resistant to penicillin, 14.9% (n = 23) to erythromycin, 17.5% (n = 27) to clindamycin, and 6.5% (n = 10) to cefoxitin and oxacillin (MR). Among the 31 *S. aureus* isolates, 16.1% (n = 5) were resistant to penicillin and 3.2%

Table 1. Antimicrobial resistance of staphylococci (n = 185) and streptococci (n = 28) isolated from bovine mastitis.

Antimicrobials	CNS (n = 154)	*S. aureus* (n = 31)	*Streptococcus* spp. (n = 28)
PEN	38 (24.6)	5 (16.1)	4 (14.3)
FOX/OXA	10 (6.5)	1 (3.2)	ND
ERY	23 (14.9)	0	3 (10.7)
CLI	27 (17.5)	0	4 (14.3)

Values are presented as number (%).
CNS, coagulase-negative staphylococci; PEN, penicillin; FOX, cefoxitin; OXA, oxacillin; ERY, erythromycin; CLI, clindamycin; ND, not determined.

(n = 1) to cefoxitin and oxacillin (MRSA). Regarding streptococci, 14.3% were resistant to penicillin and clindamycin and 10.7% were resistant to erythromycin (**Table 1**).

For CNS, the MIC_{50} and MIC_{90} values for penicillin were 0.06 μg/mL and 1 μg/mL respectively, those for erythromycin were 0.5 μg/mL and 128 μg/mL respectively, and those for clindamycin were 0.06 μg/mL and 256 μg/mL respectively (**Table 2**, **Fig. 1**).

For *S. aureus*, the MIC_{50} and MIC_{90} values for penicillin were both 0.12 μg/mL, those for erythromycin were both 0.5 μg/mL, and those for clindamycin were 0.03 μg/mL and 0.06 μg/mL respectively (**Table 3**, **Fig. 2**).

Table 2. MIC values for n = 154 coagulase-negative staphylococci isolated from bovine mastitis

Antimicrobials	MIC		Resistance	
	50	90	Number	%
Penicillin	0.06	1	38	24.6
Erythromycin	0.5	128	23	14.9
Clindamycin	0.06	256	27	17.5

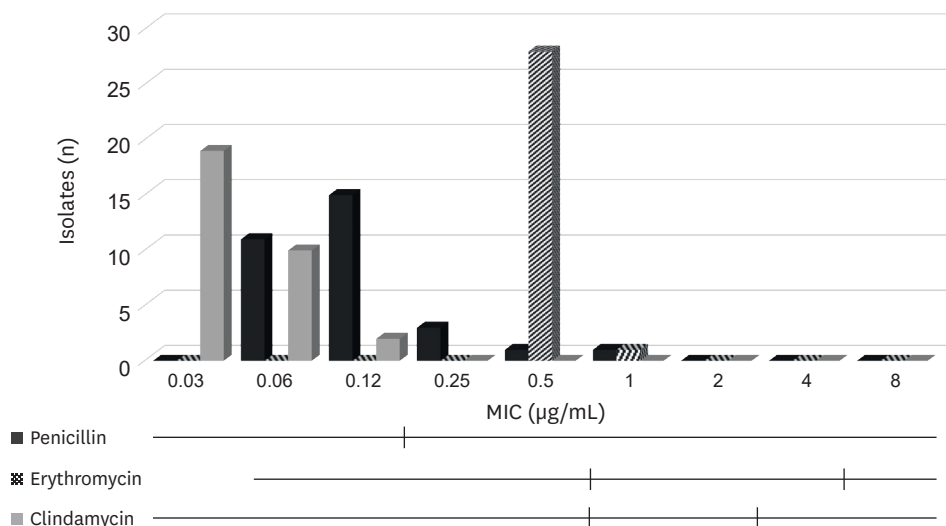MIC, minimum inhibitory concentration.
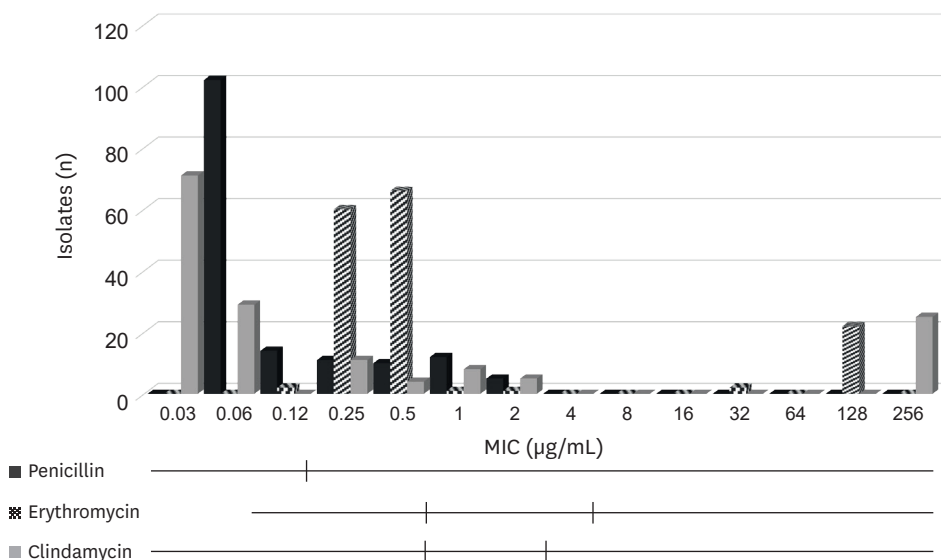
Fig. 1. Distribution of MIC values for n = 154 coagulase-negative staphylococci isolated from bovine mastitis. Black vertical lines indicate the Clinical and Laboratory Standards Institute breakpoints used to classify the isolates as susceptible, intermediate (if available) or resistant.
MIC, minimum inhibitory concentration.

Table 3. MIC values for n = 31 *S. aureus* isolated from bovine mastitis

Antimicrobials	MIC		Resistance	
	50	90	Number	%
Penicillin	0.12	0.12	5	16
Erythromycin	0.5	0.5	0	0
Clindamycin	0.03	0.06	0	0

MIC, minimum inhibitory concentration.

Fig. 2. Distribution of MIC values for n = 31 *S. aureus* isolated from bovine mastitis. Black vertical lines indicate the Clinical and Laboratory Standards Institute breakpoints used to classify the isolates as susceptible, intermediate (if available) or resistant.
MIC, minimum inhibitory concentration.

For streptococci, the MIC_{50} and MIC_{90} values for penicillin were 0.03 µg/mL and 1 µg/mL respectively, those for erythromycin were 0.12 µg/mL and 0.5 µg/mL respectively and those for clindamycin were 0.06 µg/mL and 4 µg/mL respectively (**Table 4**, **Fig. 3**).

Genotypic characterization

Among MR isolates, five CNS and one MRSA were positive to the gene *mec*A by PCR, and all of them presented the SCC*mec* IVa. Methicillin-susceptible isolates were negative for the *mec*A/C genes. The MRSA strain detected in this study belonged to sequence type (ST) 83 and *spa* type 002.

DISCUSSION

CNS are the most frequent bacteria isolated from bovine mastitis in Argentina [21] as well as in other countries such as China (73%) and Switzerland (66%) [22,23].

Table 4. MIC values for n = 28 streptococci isolated from bovine mastitis

Antimicrobials	MIC		Resistance	
	50	90	Number	%
Penicillin	0.03	1	4	14
Erythromycin	0.12	0.5	3	11
Clindamycin	0.06	4	4	14

MIC, minimum inhibitory concentration.
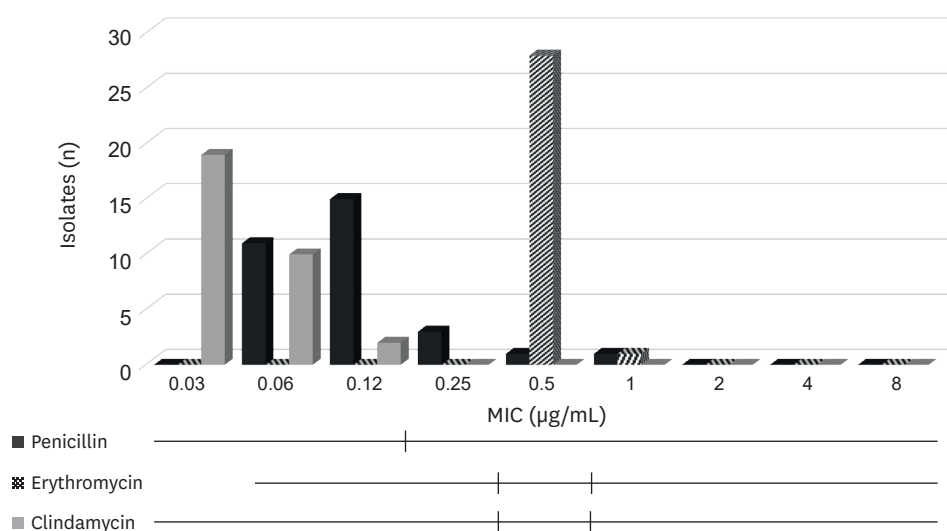
Fig. 3. Distribution of MIC values for n = 28 streptococci isolated from bovine mastitis. Black vertical lines indicate the Clinical and Laboratory Standards Institute breakpoints used to classify the isolates as susceptible, intermediate (if available) or resistant.
MIC, minimum inhibitory concentration.

In this study, staphylococci were the most prevalent bacteria isolated, being *S. epidermidis* and *S. simulans* the species most commonly found, in accordance with that found in Switzerland (26% and 22% respectively) [23]. However, *S. chromogenes* was the most prevalent isolate in Brazil (42.5%) [24] and in previous studies in Argentina (46.6%, 44.4% respectively) [21,25].

In this study, *S. aureus* was found in low percentage (14.5%) as compared with previous studies in our country (60.7%) [26] and other studies carried out in different countries: Colombia (65%) [27] and India (60.87%) [28].

Regarding streptococci, *S. dysgalactiae* was the most frequent isolate of this genus, in agreement with that reported in India [28]. On the other hand, in Mongol, *S. agalactiae* was the most prevalent [4], and *S. uberis* the most prevalent in a previous study in Argentina [29]. The CNS antimicrobial resistance values here found for erythromycin, penicillin and oxacillin were lower than those reported by Raspanti et al. [1] in Argentina (29.2%, 51.6%, and 13.7%, respectively), but higher than those previously reported by us in 2002 (6.5%, 18%, and 4% respectively) [7]. In Poland and China, penicillin resistance has been found to be higher (30% and 55.5% respectively) [30,31]. In Brazil [32], resistance to both penicillin and oxacillin has been found to be higher (79% and 29% respectively), and some reports have shown 100% resistant for penicillin [33], while resistance to erythromycin and clindamycin has been found to be lower (37.5% for both). In Poland, erythromycin resistance has been found to be lower (13.7%) [30].

All *S. aureus* isolates detected in this study were susceptible to erythromycin and clindamycin, and showed lower resistance to penicillin resistance than in previous years (23.1%) [34]. We reported the first MRSA from milk samples in Argentina [35], which would indicate a change or evolution in antimicrobial resistance. The emergence of MR is due to the acquisition and insertion of the SCC*mec* into the chromosome. This acquisition of antimicrobial resistance has presented a challenge to the medical world in terms of limits of treatment and control of staphylococcal infections. As *S. aureus* is known to carry an arsenal of virulence factors, is one of the major causes of hospital and community-acquired infections, resulting in serious

consequences [12]. There are reports showing that humans in contact with livestock are at high risk of becoming colonized and infected with livestock-associated MRSA (LA-MRSA). These studies suggest that livestock and other animals may become a permanent reservoir for human MRSA infections [12].

The streptococcal isolates showed higher antimicrobial resistance for the drugs tested than that previously reported in Argentina (0% for penicillin, 1.9% for erythromycin, 1.9% for clindamycin) [36], but lower than that reported in China [4] (95% for penicillin, 66.7% for erythromycin, 65.4% for clindamycin).

Epidemiological cutoff values (ECOFFs) distinguish between organisms with and without phenotypically expressed resistance mechanisms for a bacterial species and a corresponding antibiotic. These two groups are termed "non-wild-type" and "wild-type" respectively. ECOFFs are only used to detect isolates with acquires resistance to an antibiotic. A publicly available database for identifying ECOFFs is available on the EUCAST website (www.eucast. org). Epidemiological cutoffs are microbiological parameters used by EUCAST in the process of clinical breakpoints setting and correspond to the upper MIC/lower inhibition zone size of a wild-type distribution. The upper MIC value of the wild-type distribution separates microorganisms without (wild-type) and with acquired resistance mechanisms (non-wild-type) to the agent in question. The majority of isolates in this study showed a wild-type distribution. In **Table 1** we can observe the number of isolates showing a non-wild-type distribution. Acquired resistance mechanisms can be detected by PCR or using modern molecular techniques as WGS, and can be used to validate ECOFFs that have been estimated from phenotypic data [37]. In our study six MR staphylococci were positive to *mec*A gene. Other resistance genes that can be detected in staphylococcal specie were reported in a previous publication [25]. Several mechanisms of antibiotic resistance can also be detected in streptococci [38].

The SCC*mec* type IVa detected in MRSA and MR-CNS isolates is frequent in isolates recovered from milk [22,30]. However, in China, the most frequent has been found to be the SCC*mec*XII [39], and in Italy the SCC*mec*V [40]. In contrast, the sequence type of the MRSA found in this study is not the most frequently found in milk from mastitic cows [22,41]. In fact, there is not much information of ST83, except that in human cases located in Chile, Poland, Spain and the Gambia reported in the database www.mslt.net.com.

In China, the most prevalent ST found in MRSA are ST71 and ST97 [22] and in Italy the most prevalent are ST152 and ST398 [40]. Interestingly, in Argentina, we have previously reported ST97, ST705, ST746, ST2102 and ST2187 in methicillin-sensitive *S. aureus* [34]. Regarding *spa* typing, Asadollahi et al., found that *spa* types t002 and t008 were the most frequently repeated *spa* type in 16 countries of different continents [42].

CNS were the most frequent bacteria isolated in this study. The resistance profile for CNS was higher than that found in previous studies in our country, but lower than that found in other countries. *S. aureus* susceptibility was high and streptococci showed higher resistance than that previously reported in Argentina but lower than that previously reported in other countries. Five MR-CNS were negative for the *mec*A/C genes, probably because they carry some other resistance mechanism. SCC*mec*VIa remains the most frequent in mastitic milk. There is no information of this ST in animals. The report of an MRSA isolate from milk from cows with mastitis in Argentina indicates the development of antimicrobial resistance and the necessity for a

continuous monitoring for a rational management of drugs in mastitis therapy. Monitoring antimicrobial profiles in animals is important to collect data for trends in antimicrobial resistance phenotypes and genotypes to identify new or emerging resistance profiles.

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