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ORIGINAL ARTICLE

Prevalence and antibiotic susceptibility of coagulase-negative *Staphylococcus* species from bovine subclinical mastitis in dairy herds in the central region of Argentina

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Subclinical mastitis;
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Abstract Coagulase-negative staphylococci (CNS) are a common cause of bovine subclinical mastitis (SCM). The prevalence of CNS species causing SCM identified by genotyping varies among countries. Overall, the antimicrobial resistance in this group of organisms is increasing worldwide; however, little information exists about a CNS species resistant to antibiotics. The aim of the present study was to genotypically characterize CNS at species level and to determine the prevalence and antibiotic resistance profiles of CNS species isolated from bovine SCM in 51 dairy herds located in the central region of the province of Cordoba, Argentina. In this study, we identified 219 CNS isolates at species level by PCR-restriction fragment length polymorphism of the *groEL* gene. *Staphylococcus chromogenes* (46.6%) and *Staphylococcus haemolyticus* (32%) were the most prevalent species. A minimum of three different CNS species were present in 41.2% of the herds. *S. chromogenes* was isolated from most of the herds (86.3%), whereas *S. haemolyticus* was isolated from 66.7% of them. The broth microdilution method was used to test *in vitro* antimicrobial susceptibility. Resistance to a single compound or two related

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compounds was expressed in 43.8% of the isolates. *S. chromogenes* and *S. haemolyticus* showed a very high proportion of isolates resistant to penicillin. Resistance to two or more non-related antimicrobials was found in 30.6% of all CNS. *S. haemolyticus* exhibited a higher frequency of resistance to two or more non-related antimicrobials than *S. chromogenes*.

PALABRAS CLAVE
Mastitis subclínica;
Bovinos;
Especies de estafilococos coagulasa negativos;
Resistencia a antimicrobianos

Prevalencia y sensibilidad a antibióticos de especies de estafilococos coagulasa negativos provenientes de mastitis subclínica en bovinos de tambos de la región central de Argentina

Resumen Los estafilococos coagulasa negativos (ECN) son una causa frecuente de mastitis subclínica (MSC) en bovinos. La prevalencia de especies de ECN causantes de MSC identificadas por métodos genotípicos varía entre países. La resistencia antimicrobiana en este grupo de organismos se está incrementando en el mundo; sin embargo, existe poca información acerca de las especies de ECN resistentes a antibióticos. Los objetivos del presente estudio fueron caracterizar genotípicamente los ECN a nivel de especie y determinar la prevalencia y los perfiles de resistencia a antibióticos de las especies de ECN aisladas de MSC en bovinos de 51 rodeos situados en la provincia de Córdoba, Argentina. Mediante polimorfismos de los fragmentos de restricción del gen *groEL* identificamos 219 aislamientos de ECN a nivel de especie. *Staphylococcus chromogenes* (46,6%) y *Staphylococcus haemolyticus* (32%) fueron las especies más prevalentes. Un mínimo de 3 especies diferentes de ECN estuvieron presentes en el 41,2% de los tambos. *S. chromogenes* fue aislado en la mayoría de los tambos (86,3%), mientras que *S. haemolyticus* fue aislado en el 66,7% de aquellos. Para el análisis de sensibilidad a los antimicrobianos *in vitro* se usó el método de microdilución en caldo. La resistencia a un único compuesto o a 2 compuestos relacionados fue expresada en el 43,8% de los aislamientos. *S. chromogenes* y *S. haemolyticus* mostraron una muy elevada proporción de aislamientos resistentes a penicilina. La resistencia a 2 o más antimicrobianos no relacionados fue hallada en el 30,6% de los ECN. *S. haemolyticus* exhibió una frecuencia de resistencia a 2 o más antimicrobianos no relacionados más elevada que *S. chromogenes*.

Introduction

Bovine mastitis is one of the most costly and complex diseases of the dairy industry[3,9]. The complexity is reflected in the numerous causative pathogens, the variety and magnitude of the physiological responses to these pathogens and the variation in efficacy of control measures for different causative organisms[4,7,17,18,23,32]. Coagulase-negative staphylococci (CNS) have been traditionally considered minor pathogens. However, their importance has increased because they have become the most frequently isolated group of species from bovine milk in many areas around the world[16,29,33]. CNS usually cause subclinical mastitis (SCM), resulting in an increase in the somatic cell count (SCC) and a decrease in milk quality with the resulting economic losses that this implies[30]. The understanding and control of CNS mastitis are complicated by the heterogeneity of this group of bacteria. So far, based on Jean Euzéby's List of Procaryotic names with Standing in Nomenclature (http://www.bacterio.net/s/staphylococcus.html), the genus *Staphylococcus* contains 47 species and 24 subspecies. Even though, the prevalence of CNS species varies among the studies; six species have been reported to be commonly involved in intramammary infections (IMI)[20,30]. Recently, it has been found that CNS speciation based on the biochemical profile is not accurate enough for bovine CNS identification[26]. Hence, molecular methods have become an important diagnostic tool to improve CNS species differentiation[5,15,39]. CNS tend to be more resistant to antibiotics than *Staphylococcus aureus* and easily develop multiresistance[2,27,32]. In addition, limited information is available regarding differences in antimicrobial susceptibility among CNS species identified by genotyping[27,38]. As yet, nothing has been reported about the prevalence and susceptibility antibiotics of CNS species of dairy cows in Argentina. Recently, a research conducted in our laboratory[8] showed that CNS represent the most prevalent bacterial group of minor pathogens isolated from the subclinical infection of dairy cows in Córdoba province, Argentina. The aim of the present study was to determine the prevalence and antibiotic resistance profiles of CNS species isolated from bovine subclinical mastitis in 51 dairy herds in the central region of Argentina.

Materials and methods

Species identification of CNS isolates by PCR-RFLP analysis of a *groEL* gene sequence

In this study, we included 219 (49.6%) CNS randomly selected isolates from a total of 441 isolates in pure cultures from composite milk samples with a SCC ≥200 000 cells/ml in a previous study[8]. The isolates were obtained from a previous cross-sectional study conducted on a sample randomly selected from 51 dairy farms located in Córdoba province, Argentina, with a herd size of around 100 and 250 cows[37]. The intramammary therapy of clinical cases was administered in 96.1% of the herds. Antibiotics used for dry cow therapy were applied to all cows in 58.8% of herds for at least one year. Among the milking hygienic practices, udder cleaning before milking was implemented in 94.1% of herds, whereas other practices such as using individual paper towels and, pre- and post-test dipping were not or were less frequently applied. On average, 45 lactating cows in each herd were sampled by the same investigator. In total, 2296 composite milk samples were collected. A volume of 0.01 ml of milk was streaked on trypticase soy agar plates (Sigma–Aldrich, USA) containing 5% sheep blood and incubated at 37 °C; plates were examined for growth at 24 and 48 h. A mammary gland was considered infected by CNS when growth of ≥500 CFU/ml of a particular organism and only one colony type on the plate were isolated[8]. Genomic DNA was extracted from all isolates and all type strains according to Aires-de-Sousa et al.[1]. PCR-RFLP analysis of a partial *groEL* gene sequence was performed as described by Santos et al.[25]. The CNS isolates were genotyped at species level as follows: (1) the presence of a DNA fragment (approximately 550 bp) obtained by PCR of the partial *groEL* gene sequence was identified, (2) the banding patterns obtained after digestion by *Alu*I restriction enzyme of the *groEL* gene of type strains were determined, (3) these patterns were compared with the known sequences from the NCBI GenBank database, using the NEB cutter program (version 2.0)[36], (4) those CNS isolates with the same pattern of reference or type-T strains were assigned to a specific species. To differentiate *S. chromogenes* from *S. hyicus* and *S. capitis*, PCR products amplified from reference and type-T strains and/or IMI isolates were digested with *Hind*III and *Pvu*II[25]. The following reference and type-T strains were included in this study: *S. aureus* ATCC 29740, *S. capitis* subsp. *capitis* ATCC 35661, *S. caprae* ATCC 35538^T, *S. chromogenes* ATCC 43764^T, *S. epidermidis* ATCC 12228, *S. haemolyticus* ATCC 29970^T, *S. hyicus* ATCC 11249^T, *S. saprophyticus* ATCC 49453, *S. sciuri* subsp. *sciuri* ATCC 29060, *S. sciuri* subsp. *carnaticus* ATCC700058^T, *S. simulans* ATCC 11631, *S. warneri* ATCC 49454, *S. xylosus* ATCC 29971^T.

Antibiotic susceptibility testing

The broth microdilution method was used to carry out *in vitro* antimicrobial susceptibility testing according to Clinical Laboratory Standards Institute guidelines (CLSI)[6] document M31-A3. Custom-made microtiter plate panels were used (Trek Diagnostic Systems, Magellan Biosciences, UK-USA) (Sensititre-TREK). The antimicrobial agents and dilution ranges tested for each agent were as follows: penicillin (0.06–8 μg/ml), oxacillin (0.25–4 μg/ml), erythromycin (0.25–4 μg/ml), tetracycline (2–16 μg/ml) and clindamycin (0.5–2 μg/ml). *S. aureus* strain ATCC 29213 was included in each assay as a control. The minimum inhibitory concentrations (MICs) results were evaluated based on veterinary interpretive criteria of the CLSI[6], with resistance breakpoints of 0.25 μg/ml for penicillin, 0.5 μg/ml for oxacillin, 1 μg/ml for erythromycin and 8 μg/ml for tetracycline. Clindamycin is not licensed for the treatment of bovine mastitis; therefore, no approved breakpoints are available for the classification of bovine staphylococcal isolates from mastitis cases as susceptible or resistant. The MIC value required to inhibit 90% of the isolates tested was defined as MIC_{90}. Isolates were categorized as susceptible or resistant, with intermediates classified as resistant. Isolates with a MIC for oxacillin >0.5 μg/ml were additionally examined for the presence of the *mecA* gene by PCR as proposed by Mo and Wang[14].

Results

Distribution of CNS species

Two hundred and nineteen CNS isolates from cows with SCM were identified to the species level by PCR-RFLP of the *groEL* gene. The distribution of different species among identified CNS is shown in Table 1, where *S. chromogenes* and *S. haemolyticus* were the predominant species. A minimum of three different CNS species were present in 41.2% of the herds. *S. chromogenes* was isolated in 86.3% of all farms, whereas *S. haemolyticus* was isolated in 66.7% of them (Table 1).

Antibiotic susceptibility

In the present study 219 CNS isolated from cows with SCM and identified by genotyping were evaluated for susceptibility to five antimicrobials (Table 2). Penicillin was used as the representative of penicillinase-labile penicillins and is the most frequently used in Argentina for bovine mastitis treatment. One hundred and thirteen strains (51.6%) of the 219 strains were resistant to penicillin; The MIC_{90} value for this antibiotic was higher than 8 μg/ml, well above the recommended breakpoint. Thirty (13.7%) of all CNS isolates tested were resistant to oxacillin; the MIC_{90} value for this antibiotic was 0.5 μg/ml. Resistance to oxacillin was attributed to the presence of the *mecA* gene in 2 of 12 (16.7%) of the oxacillin-resistant isolates with a MIC >0.5 μg/ml. Moderate resistance to erythromycin and tetracycline was detected among CNS isolates, 29.2% and 30.1%, respectively. In our study, the MIC_{90} values of erythromycin and tetracycline for CNS were more than one or two dilution steps higher than those of the recommended breakpoint, respectively. Table 3 shows phenotypic resistance profiles of CNS obtained from bovine SCM. Of 219 CNS isolates, 56 (25.6%) were phenotypically susceptible to all antimicrobial agents tested. Eighty-five (38.8%) isolates expressed resistance to a single compound, and 11 isolates (5%) expressed resistance to 2 related compounds (β-lactams antimicrobial agents) and 67 (30.6%) isolates expressed resistance to compounds

Table 1 Species distribution of coagulase-negative staphylococci (CNS) isolates from bovine subclinical mastitis in 51 Argentinean dairy herds

Species	No. of isolates[a]	CNS IMI[b] (%)	No. of herds[c]	Distribution of each CNS species in dairy herds sampled (%)
Staphylococcus chromogenes	102	46.6	44	86.3
Staphylococcus haemolyticus	70	32.0	34	66.7
Staphylococcus warneri	16	7.3	14	27.5
Staphylococcus xylosus	14	6.4	11	21.6
Staphylococcus simulans	8	3.6	8	15.7
Staphylococcus epidermidis	5	2.3	5	9.8
Staphylococcus hyicus	4	1.8	4	7.8
Total	219		51	

[a] Each organisms was isolated from only one cow (composite sample).
[b] IMI: intramammary infections.
[c] Number of dairy herds from which each species was isolated.

Table 2 Proportion of resistant isolates and minimum inhibitory concentration (MIC) distribution for 219 isolated coagulase-negative staphylococci (CNS) from bovine subclinical mastitis

Antimicrobial agents	Resistant isolates (%)	Number of CNS isolates for each MIC (μg/ml) values									MIC_{50}	MIC_{90}
		≤ 0.06	0.12	0.25	0.5	1	2	4	8	≥ 16		
Penicillin	51.6	96	10	22	4	20	13	8	11	35	0.25	> 8
Oxacillin	13.7			189	18	6	0	0	6		0.25	0.5
Erythromycin	29.2			83	72	[11]	[4]	[8]	41		0.5	> 4
Tetracycline	30.1						134	19	[8]	58	2	> 16
Clindamycin[a]	NA				162	15	8	34			0.5	> 2

White fields denote the range of dilutions tested for each substance. MICs above the range are given as the concentration closest to the range. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration. Bold vertical lines indicate breakpoints based on veterinary interpretive criteria (CLSI 2008). Numbers in brackets indicate the number of isolates with a MIC in the intermediate resistant range.
[a] Clindamycin is not licensed for the treatment of bovine mastitis. As a consequence, no approved breakpoints are available for the classification of bovine staphylococcal isolates from mastitis cases as susceptible or resistant; NA, not applicable.

belonging to different antimicrobial classes. In *S. chromogenes* and *S. haemolyticus*, the most prevalent isolated CNS species, a large proportion of the strains investigated were resistant to penicillin, 45.1% and 58.6%, respectively. Although resistance to oxacillin was lower than that found in other 2 resistant species to the same antibiotic, the number of isolates of these species was too low to draw conclusions. For *S. warneri*, the third most common CNS species, the proportion of resistant isolates to penicillin was less common than in other species and no resistance to oxacillin was observed. Finally, in *S. xylosus*, penicillin resistance was the most common among the species tested. For other species, *S. xylosus*, *S. epidermidis* and *S. hyicus*, the number of resistant isolates was too low to be analyzed. The percentage of resistant *S. haemolyticus* strains to two or more non-related antimicrobials was higher than that observed in *S. chromogenes*, 42.8% and 24.5%, respectively.

Discussion

Genotypic methods have shown to have higher specificity and sensitivity[39] than other methods for discriminating among species, resulting in a better alternative for the identification of CNS isolates. A number of PCR amplicon sequencing-based methods for the identification of CNS have been reported[5,26,30]. Amplified fragment length polymorphism (AFLP)[20,31] and PCR-restriction fragment length polymorphism (RFLP) applied to the *groEL* or *gap* genes have been used to identify CNS isolated from bovine milk[15,25]. The discrepancy observed in the distribution of CNS species identified by genotyping causing SCM in different countries, might be due to differences in experimental design[10,20,30,31,35,38] however, *S. chromogenes* was one of the most prevalent species both in this work and in previous studies carried out in other countries such as Finland,

Table 3 Phenotypic resistance profiles of coagulase-negative staphylococci (CNS) obtained from bovine subclinical mastitis

Species	Total	Phenotypic resistance to antibiotics[a]								
		0	1				2			>2
			P	O	E	T	PO	PT	PE	ET	
Staphylococcus chromogenes	102	25	13	2	17	12	8	10	3	0	3 POT, 3 POE, 6 PET
Staphylococcus haemolyticus	70	18	14	1	7	0	0	12	3	3	5 POE, 7 PET
Staphylococcus warneri	16	9	3	0	3	0	0	0	0	0	1 PET
Staphylococcus xylosus	14	0	3	0	0	1	2	3	0	0	1 POT, 2 POE, 2 PET
Staphylococcus simulans	8	2	6	0	0	0	0	0	0	0	–
Staphylococcus epidermidis	5	0	0	0	1	1	1	0	0	0	2 POT
Staphylococcus hyicus	4	2	0	0	0	1	0	0	0	1	–
Total	219	56	39	3	28	15	11	25	6	4	32

[a] When phenotypic resistance to 0, 1 or 2 compounds was observed, the number of isolates with the specified resistance profile is shown. For isolates with resistance to >2 tested compounds, resistance profiles are shown. E: erythromycin; O: oxacillin; P: penicillin; T: tetracycline.

Sweden, Belgium, Canada and Brazil, ranging between 23.3% and 78.8% of CNS isolates. To our knowledge, only Piessens *et al.*[20] revealed that *S. haemolyticus* was the second most prevalent CNS species as in our study causing 27.6% of cases. Special attention should be given to both species, considering that the udder was found to be their main reservoir[20].

In our study, a minimum of three different species of CNS were present in 41.2% of the herds. *S. chromogenes* and *S. haemolyticus* were isolated in most of the farms. This is in line with other studies, where these species have been isolated from all analyzed herds[20,30]. Although the reasons for the increased prevalence of some CNS species are not known, these might be attributed to the ability of each species to adapt to the mammary gland[10,20] or to the different management patterns of mastitis control schemes[30].

One limitation to the present study is the fact that we cannot be sure that the CNS were isolated from the diseased quarter since other non-infectious factors could cause an inflammatory response. However, CNS in pure culture allow to ensure that they are the only etiological agents causing subclinical mastitis.

The direct comparison of studies on antimicrobial susceptibility of CNS is often difficult mainly because of the use of different methodologies and breakpoints for testing susceptibility. A great variability has been reported in the MIC_{90} values for penicillin in CNS, ranging from 0.12 μg/ml to 32 μg/ml[2,11,12,19,22,24,34]. The MIC_{90} value for penicillin found in our study was well above the recommended breakpoint (CLSI, 2008). Moreover, the penicillin resistance percentage was higher than that described by Gentilini *et al.*[11] in Argentina and those reported in other countries such as Uruguay[12], The Netherlands[27], USA[22] and Sweden[2]. In the last decade, Gentilini *et al.*[11] reported methicillin resistance among CNS isolated from bovine mastitis in our country. Since continuous surveillance is needed for the early detection of this kind of resistance, oxacillin has been used in this study to test susceptibility to methicillin. The MIC_{90} value for oxacillin was similar to those reported by different authors[11–13,24,28]. The percentage of resistant strains found was higher than that reported by other authors[2,11–13], and similar to that reported in studies from Finland, Germany, USA, Sweden and The Netherlands[19,21,22,27,28]. The variability in the MIC_{90} values of the antimicrobial activity of penicillin and oxacillin against CNS highlights the relevance of determining β-lactams susceptibility patterns for this bacterial group. Macrolides are frequently used in Argentina for bovine mastitis treatment, since high concentrations in milk are obtained following parenteral administration. Although erythromycin was evaluated as representative of this group, tylosin is used in the therapy. Tetracycline was included in this study for epidemiological purposes as representative of tetracyclines; however, they are not recommended for mastitis treatment in Argentina because of the risk of an extended residue presence in milk. The MIC_{90} value for erythromycin and tetracycline was higher than that observed by several authors in Argentina, Finland, Germany, Sweden and USA[2,11,13,19,21,28]. Although clindamycin is not used in the treatment of bovine mastitis, the determination of the MIC for this antibiotic was conducted in order to test susceptibility to lincomycin; however, specific interpretative breakpoints are defined for pirlimicyn[6]. Susceptibility to pirlimycin could not be inferred from clindamycin results because some mechanism (enzymatic modification) does not exhibit cross-resistance. The MIC_{90} value for clindamycin obtained in this study was similar to that previously described by Gentilini *et al.*[11] in our country and higher than that reported by other investigators in different countries[2,13,19,21]. The proportion of CNS resistant to a single compound or two related compounds obtained in our study was found to be similar to that described by Sampimon *et al.*[27] *S. haemolyticus* and *S. chromogenes* were the most frequent resistant species; therefore, it would also be hypothesized that they should be more ''at risk'' of developing antibiotic resistance or acquiring resistance determinants. Conversely, the absence or lower percentages of resistant isolates to multiple drugs for both species were found by Sampimon *et al.*[27] We conclude that CNS species from bovine subclinical mastitis differ in prevalence and antimicrobial resistance profiles, which may have implications for treatment and management decisions when CNS are the predominant bacterial group on dairy farms.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

The authors declare that they have no conflicts of interest.

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