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Research Letter

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Biofilm formation and antimicrobial resistance genes of coagulase-negative staphylococci isolated from cows with mastitis in Argentina

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One sentence summary: Biofilm formation, biofilm-associated genes and antimicrobial resistance genes of coagulase-negative staphylococci isolated from cows with mastitis in Argentina.

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ABSTRACT

Mastitis affects the health and welfare of dairy cows worldwide. Coagulase-negative staphylococci (CNS) are known to form biofilms and are increasingly recognized as a cause of persistent bovine intramammary infections. A total of 90 CNS isolated from cows with clinical and subclinical mastitis in Argentina from 2008 to 2014 were identified by PCR-RFLP using the *gap* gene. Standard microtiter plate assays were used to assess CNS biofilm formation, and *Staphylococcus haemolyticus* species formed the strongest biofilms. The presence of biofilm-associated genes *ica*A, *bap* and *aap* was detected in a few isolates, while *emb*P, *fbe*, *atl*E and *eno* were present in the majority of isolates. Genes encoding resistance to β-lactams were detected among the isolates; *bla*Z, *mec*A and *mec*C were detected in 21, 4 and 1 isolate, respectively. Resistance to macrolides and lincosamides ($n = 6$) was attributable to *erm*B, *erm*C, *mph*C or *mrs*A or a combination of those genes. In this study, we identified CNS species involved in mastitis and provide information about pathogenicity and antimicrobial resistance, which is essential to design efficient strategies to control mastitis caused by CNS.

Keywords: biofilm; antimicrobial resistance genes; coagulase-negative staphylococci; bovine mastitis; Argentina

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INTRODUCTION

Bovine mastitis is a multifactorial disease that affects the health of dairy cows and is a major cause of economic losses in dairy herds worldwide (Bradley 2002). Staphylococci are a major cause of mastitis in dairy cows around the world, with some coagulase-positive species (e.g. *Staphylococcus aureus*) associated with more severe illness than coagulase-negative staphylococci (CNS) (Salmon *et al.* 1998; Zhang and Maddox 2000; Gentilini *et al.* 2002; Bradley *et al.* 2007). CNS are a heterogeneous group of more than 50 species and subspecies that are traditionally considered to be minor pathogens. However, CNS are increasingly recognized as an important cause of bovine mastitis worldwide (Pyörälä and Taponen 2009). CNS form part of the normal flora and are found on healthy skin of the udder teat and on milkers' hands. Their presence as part of normal skin flora provides easy access to the teat canal and, under the right conditions, CNS can penetrate secretory tissues resulting in opportunistic infection. CNS are frequently isolated from milk samples collected from cows with clinical and subclinical mastitis in several countries (Salmon *et al.* 1998; Gentilini *et al.* 2002; Bradley *et al.* 2007). In some cases, infections have been associated with tissue damage and decreased milk production (Schukken *et al.* 2009). Furthermore, CNS can cause persistent bovine intramammary infections that last for several months in the absence of intervention (Gillespie *et al.* 2009).

Although CNS are traditionally treated as a homogenous group, pathogenic potential and antimicrobial resistance vary among CNS. For example, differences between the numerous CNS species in epidemiological behavior (Piessens *et al.* 2011) and impact on udder health (Supré *et al.* 2011) have been reported. Many questions remain regarding the impact of CNS on bovine udder health and the contribution of specific species to infection. Given that biofilms play a major role during human infections caused by CNS, the possibility that biofilm formation may be associated with virulence has recently garnered attention from udder health researchers (Piessens *et al.* 2012; Simojoki *et al.* 2012; Tremblay *et al.* 2013; Oliveira *et al.* 2015; Osman *et al.* 2015). Although biofilms do not appear to affect disease severity (Simojoki *et al.* 2012; Tremblay *et al.* 2013; Osman *et al.* 2015), they may play a role in CNS persistence in the intramammary environment (Tremblay *et al.* 2013). Additionally, CNS isolates growing within biofilms are less susceptible to antimicrobials commonly used on farms, including penicillin G/novobiocin combinations and ceftiofur (Tremblay *et al.* 2014). Therefore, biofilm formation by CNS species could possibly impede antimicrobial therapy.

Nevertheless, antimicrobial therapy remains one of the most effective treatments for intramammary infections. Treatment success relies on proper identification of the pathogen and selection of the most appropriate antimicrobial agents. In Argentina, the most common antimicrobials used to treat mastitis are β-lactams, macrolides and lincosamides (Gentilini *et al.* 2002). In addition to biofilm formation, several antibiotic resistance mechanisms have been described, including β-lactamase production, encoded by *blaZ*, and production of low-affinity penicillin-binding proteins (PBP2a), encoded *mec*A or *mec*C (Zhang *et al.* 2005; Cuny *et al.* 2011). The *mec*A gene is located on a mobile genetic element called staphylococcal cassette chromosome (SCC*mec*), which provides methicillin resistance. This resistance precludes therapy with any of the currently available β-lactam antimicrobials, and may predict resistance to several classes of antimicrobials besides β-lactams (Brakstad and Maeland 1997). Furthermore, resistance to lincosamides and macrolides can be conferred by genes encoding inactivating enzymes, efflux pumps or methylation of the antibiotic's ribosomal target site (Lina *et al.* 1999). CNS associated with the udder may act as a reservoir of antimicrobial resistance genes, including methicillin resistance genes that could be transferred to other important veterinary or zoonotic pathogens such as *S. aureus*. Therefore, this reservoir can be seen as a potential public health threat with respect to antimicrobial resistance and the development of multiple resistance (MR) (Hanssen, Kjeldsen and Sollid 2004; Sawant, Gillespie and Oliver 2009; Febler *et al.* 2010; Sampimon *et al.* 2011). The emersion of methicillin-resistant phenotypes will limit therapeutic options, and successful antimicrobial therapy often depends on accurate minimum inhibitory concentration data. Genotypic methods to detect antimicrobial resistance genes have the advantage of being more rapid than conventional susceptibility tests that depend on culturing bacteria (Asfour and Darwish 2011).

The aim of this study were to identify CNS species isolated from the milk of cows with mastitis in Argentina, to evaluate the isolates for biofilm forming ability and to investigate the presence and frequency of biofilm-associated and antimicrobial resistance-associated genes.

MATERIALS AND METHODS

CNS isolates

A total number of 90 CNS were selected randomly for this study. CNS were isolated from milk samples from individual quarter of cows and heifers with clinical and subclinical mastitis from nine establishments in four provinces in Argentina (Buenos Aires, Córdoba, Entre Ríos and Santa Fé). The numbers of cows in each farm were between 200 and 300 animals, and as part of dry cow therapy, all cows received intrammamary infusion with antibiotic. Only one sample was collected from each animal and animals were not resampled. A total of 8 to 14 isolates were recovered from each per farm, and each isolate came from a different animal in lactation (i.e. 1 isolate/cow). All staphylococci were isolated in the Microbiology laboratory at Facultad de Ciencias Veterinarias, Universidad de Buenos Aires between 2008 and 2014 and maintained frozen at −20°C in trypticase soy broth (Britania Lab. S.R.L, Argentina) containing 20% glycerol. Reference strains *Staphylococcus capitis* ATCC 35661, *S. cohnii* subsp *cohnii* ATCC 35662, *S. epidermidis* ATCC 12228, *S. haemolyticus* ATCC 29970, *S. saprophyticus* ATCC 15305, *S. sciuri* subsp. *sciuri* ATCC 29060, *S. simulans* ATCC 27851, *S. warneri* ATCC 49954, *S. xylosus* ATCC 29972 were used in this study. CNS were routinely cultured in brain–heart infusion (BHI) broth and incubated for 24 h at 37°C.

DNA isolation

Genomic DNA was isolated as described by Tremblay *et al.* (2013). Briefly, 1.5 mL of bacteria from an overnight culture was harvested by centrifugation. The pellets were resuspended in lysostaphin solution (0.1 mg mL^{-1}) and incubated 30 min at 37°C. A proteinase K solution (0.1 mg mL^{-1}) was added, and the mixture was incubated for 10 min at 37°C. The samples were then heated for 5 min at 100°C. Cells and insoluble debris were settled by centrifugation and supernatants were transferred to new tubes.

Table 1. Primers and PCR conditions used for RFLP-PCR identification and for detection of biofilm-associated genes in CNS from bovine milk.

Gene	Primers (5′-3′)	Amplicon size (bp)	No. of PCR cycles (conditions)	Reference	Control strain
gap	ATGGTTTTGGTAGAATTGGTCGTTTA GACATTTCGTTATCATACCAACGTG	933	40 (20 s at 94°C; 30 s at 60°C; 40s at 72°C)	Yugueros *et al.* (2000)	
bap	ACT TAY TRC CHT ATA TCG AAR TAG GCT GTT GAA GTT AAT ACT GTA CCT GC	900	30 (30 s at 94°C; 30 s at 57°C; 30 s at 72°C)	Tremblay *et al.* (2013)	*a*
icaA	CTG TTT CAT GGA AAC TCC TCG ATG CGA TTT GTT CAA ACA	200	30 (30 s at 94°C; 30 s at 57°C; 30 s at 72°C)	Tremblay *et al.* (2013)	*b*
aap	GAA GCA CCG AAT GTT CCA ACT ATC AGT TGG CGG TAT ATC TAT TGT A	289	30 (30 s at 94°C; 30 s at 52°C; 30 s at 72°C)	Tremblay *et al.* (2013)	*b*
fbe	CTA CAA GTT CAG GTC AAG GAC AAG G GCG TCG GCG TAT ATC CTT CAG	273	30 (30 s at 94°C; 30 s at 45°C; 30 s at 72°C)	Tremblay *et al.* (2013)	*b*
atlE	CAA CTG CTC AAC CGA GAA CA TTT GTA GAT GTT GTG CCC CA	682	30 (30 s at 94°C; 30 s at 45°C; 30 s at 72°C)	Tremblay *et al.* (2013)	*b*
embP	AGC GGT ACA AAT GTC AAT AGA AGT GCT CTA GCA TCA TCC	455	30 (30 s at 94°C; 30 s at 47°C; 30 s at 72°C)	Tremblay *et al.* (2013)	*b*
eno	ACG TGC AGC AGC TGA CT CAA CAG CAT CTT CAG TAC CTT C	302	25 (1 min at 94°C; 1 min at 57°C; 1 min at 72°C)	Simojoki *et al.* (2012)	*c*

a. *Staphylococcus aureus* V329 (Cucarella *et al.* 2004).
b. *Staphylococcus epidermidis* ATCC 35984.
c. *Staphylococcus aureus* ATCC 25923.

Species identification by the gap PCR-RFLP

Species identification was achieved by using RFLP-PCR on the *gap* gene as described by Onni *et al.* (2010), with some modifications described elsewhere (Srednik *et al.* 2015).

Biofilm formation assay

Biofilms were formed in 96-well microtiter plates as described by Tremblay *et al.* (2013). Colonies were suspended into fresh BHI supplemented with glucose (0.25%) to 0.5 Mc Farland standard and 200 μl was deposited in the microtiter plate in triplicates. After incubation for 24 h at 37°C, microtiter plates were washed three times with PBS, liquids were removed by aspiration and air-dried. Bacterial biomass was stained with 0.1% safranin, washed once with distilled water and then dried. The destaining solution (50% ethanol–50% glacial acetic acid) was added, and the absorbance at 490 nm of released stain was measured with a microplate reader. Each CNS isolate was tested on three independent days and the following controls were included every time: strong positive control (*S. epidermidis* strain ATCC 35984; $A_{490} > 1.500$), weak positive control (*S. epidermidis* strain ATCC 12228; $0.110 < A_{490} < 0.500$) and contamination control (sterile medium). The ability of a CNS isolate to form a biofilm was classified as negative ($A_{490} < 0.110$), weak ($0.110 < A_{490} < 0.500$), moderate ($0.500 < A_{490} < 1.500$) or strong ($A_{490} > 1.500$). To characterize the composition of the biofilm matrix, enzymatic treatment was used to disperse the pre-formed biofilms as previously described (Tremblay *et al.* 2013).

Detection of biofilm-associated and antimicrobial resistance genes

The biofilm-associated genes tested and nucleotide sequences of the primers are presented in Table 1. Gene amplification was performed as described by Cucarella *et al.* (2004), Rohde *et al.* (2005, 2007) and Simojoki *et al.* (2012). Seven genes were PCR amplified: *bap*, encoding a biofilm-associated protein; *ica*A, encoding intercellular adhesion protein A; *aap*, encoding an accumulation-associated protein; *fbe*, encoding a fibrinogen adhesion; *atl*E, encoding an adhesin and autolysin; *emb*P, encoding a fibronectin adhesin; and *eno*, encoding a laminin-binding protein. The antimicrobial resistance-associated genes detected are listed in Table 2. The PCR mixtures (25 μl) were composed of 200 ng genomic DNA, 0.2 mM deoxynucleotide 5′-triphosphate, 1.5 mM $MgCl_2$, 0.5 μM of each forward and reverse primers, 5 μl 10X PCR reaction buffer, 1.25 U Taq DNA polymerase (BioLabs) and water (to 25 μl). Nine single PCR reactions were performed with different PCR cycle conditions (Tables 1 and 2). The DNA was denatured at 94°C for 6 min and after the final cycle, the reaction was completed with an additional cycle at 72°C for 10 min.

Statistical analyses

Kruskal–Wallis analyses with Dunn's multiple-comparison post test were performed using GraphPad Prism, version 6.0 (GraphPad Software, San Diego, CA, USA) to compare biofilm formation between CNS species and enzymatic digestions.

RESULTS AND DISCUSSION

CNS are the most commonly isolated bacteria from intramammary infections in several countries that have important dairy production (Gentilini *et al.* 2002; Sampimon *et al.* 2009a). In this study, 90 CNS isolates from milk samples collected from cows suffering from clinical and subclinical mastitis were identified using the *gap* gene. This provides a good discriminatory target to differentiate closely related staphylococci species since phenotypic commercial tests (e.g. API Staph) frequently misidentify CNS species, require additional testing and are not considered to be suited for the identification of CNS isolated from milk samples (Sampimon *et al.* 2009b; Onni *et al.* 2010). In this study (Table 3), the most frequently isolated species were *Staphylococcus chromogenes* (40/90; 44.4%) followed by *S. epidermidis* (12/90; 13.3%) and *S. devriesei* (9/90; 10%). Compared to other countries, the dominant species identified is in agreement with those reported in Belgium (46.4%; Supré *et al.* 2011), USA (36.3%; Sawant, Gillespie and Oliver 2009) and the Netherlands (36%; Sampimon

Table 2. Primers and PCR conditions used to detect antimicrobial resistance genes in CNS from bovine milk.

Gene	Primers (5′-3′)	Amplicon size (bp)	No. of PCR cycles (conditions)	Reference	Control strain
mecA	GTG AAG ATA TAC CAA GTG ATT ATG CGC TAT AGA TTG AAA GGA T	147	30 (30 s at 94°C; 30 s at 57°C; 1 min at 72°C)	Zhang *et al.* (2005)	*a*
mecC	GCT CCT AAT GCT AAT GCA TAA GCA ATA ATG ACT ACC	304	30 (30 s at 94°C; 30 s at 52°C; 30 s at 72°C)	Cuny *et al.* (2011)	*b*
blaZ	ACT TCA ACA CCT GCT GCT TTC GA CCA CTT TTA TCA GCA ACC	173	30 (30 s at 94°C; 30 s at 55°C; 30 s at 72°C)	Martineau *et al.* (2000)	*c*
ermA	CTT CGA TAG TTT ATT AAT ATT AGT TCT AAA AAG CAT GTA AAA GAA	645	30 (1 min at 94°C; 1 min at 54°C; 2 min at 72°C)	Chung *et al.* (1999)	*a*
ermB	AGT AAC GGT ACT TAA ATT GTT TAC GAA AAG GTA CTC AAC CAA ATA	639	35 (1 min at 94°C; 1 min at 50°C; 1 min at 72°C)	Chung *et al.* (1999)	*d*
ermC	GCT AAT ATT GTT TAA ATC GTC AAT TCA AAA CAT AAT ATA GAT AAA	642	Same as *ermA*	Chung *et al.* (1999)	*g*
lnuA	GGT GGC TGG GGG GTA GAT GTA TTA GCT TCT TTT GAA ATA CAT GGT ATT TTT CGA ACT GG	323	30 (30 s at 94°C; 30 s at 57°C; 1 min at 72°C)	Lina *et al.* (1999)	*h*
mefA	ATG CAG ACC AAA AGC GCG AT CGG TAT CTG TTC TGG TAG CG	253	35 (1 min at 94°C; 1 min at 37°C; 2 min at 72°C)	Luna *et al.* (2000)	*i*
mrsA	GGC ACA ATA AGA GTG TTT AAA GG AAG TTA TAT CAT GAA TAG ATT GTC CTG TT	940	25 (1 min at 94°C; 1 min at 50°C; 1 m 30 s at 72°C)	Lina *et al.* (1999)	*e*
mphC	GAG ACT ACC AAG AAG ACC TGA CG CAT ACG CCG ATT CTC CTG AT	772	35 (1 min at 94°C; 1 min at 59°C; 2 min at 72°C)	Lüthje and Schwarz (2006)	*f*

a. MRSA 97, *g*. MRSA 154N, *i*. SCN-3 (Dr. Marie Archambault, Université de Montréal, Québec), *b*. *S. aureus* LGA_{251} (García-Álvarez *et al.* 2011), *c*. *S. aureus* 29213, *d*. *S. aureus* CCRI-1317, *e*. CCRI-9330, *f*. CCRI-8926 (Centre de Recherche en Infectiologie de l'Université de Laval, Québec), *h*. M979-11 (MAPAQ Diagnostic laboratory, Québec).

Table 3. Biofilm-associated genes patterns.

Biofilm-associated gene combinations	N° isolates (%)
icaA	1 (1.2%)
icaA-eno	5 (6.2%)
icaA- embP-eno	1 (1.2%)
bap-embP	1 (1.2%)
bap-eno	2 (2.5%)
bap-altE-fbe-embP-eno	1 (1.2%)
aap-altE-fbe-embP-eno	3 (3.7%)
altE-embP-eno	10 (12.4%)
altE-eno	8 (9.9%)
fbe-eno	6 (7.4%)
embP-eno	5 (6.2%)
eno	37 (45.7%)
Total CNS positives	81 (90%)

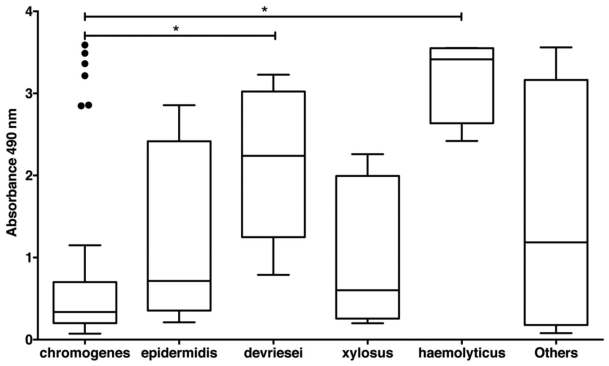

Figure 1. Biofilm formation by the CNS species studied. *Staphylococcus chromogenes* (n = 40), *S. epidermidis* (n = 12), *S. devriesei* (n = 9), *S. xylosus* (n = 5), *S. haemolyticus* (n = 4) and other species (n = 20).

et al. 2009a). *Staphylococcus epidermidis* was also reported as the second most common species in the USA (22%; Sawant, Gillespie and Oliver 2009) and the Netherlands (13%; Sampimon *et al.* 2009a). However, the third species most frequently identified varied in each country.

Biofilm formation assay

Biofilms play an important role in the virulence of *Staphylococcus* spp (Tormo *et al.* 2005). There are only a few studies on biofilm formation and genes associated with CNS causing bovine mastitis (Tormo *et al.* 2005; Piessens *et al.* 2012; Simojoki *et al.* 2012; Tremblay *et al.* 2013; da Costa Krewer *et al.* 2015; Osman *et al.* 2015). The majority (96.7%) of isolates were able to form biofilms, whereas a minority of isolates (3.3%) were considered biofilm negative. Among the biofilm-positive isolates, 47.8%, 13.3% and 35.6% were weak, moderate and strong biofilm formers, respectively. The ability to form biofilms varied among CNS species. We found that *S. haemolyticus* and *S. devriesei* isolates form significantly more biofilms ($P < 0.05$) than *S. chromogenes* isolates (Fig. 1).

As previously reported (Tremblay *et al.* 2013) using a similar assay, the majority of our isolates were biofilm positive (97.8% vs 85.1%). In contrast, Simojoki *et al.* (2012) observed that most CNS were biofilm negative (68.7%) and a minority of isolates were biofilm positive (31.3%). This may be attributed to the use of trypticase soy broth by Simojoki *et al.* (2012) and BHI used in this study and by Tremblay *et al.* (2013) to culture bacteria. It is well established that the chemical composition of growth media (e.g. plant base vs. animal-organ base) will influence the expression of bacterial genes, such as exopolysaccharides synthesis genes,

Table 4. Biofilm-associated and antimicrobial resistance genes among CNS species.

	S. chromogenes	*S. epidermidis*	*S. devriesei*	*S. xylosus*	*S. haemolyticus*	Others	Total strains (%)
n (%)	40	12	9	5	4	20	90 (100)
Biofilm							
Negative	2					1	3 (3.3)
Weak	26	6		2		9	43 (47.8)
Moderate	6	2	3	1			12 (13.3)
Strong	6	4	6	2	4	10	32 (35.6)
Biofilm genes							
icaA	1	1	2	1	1	3	9 (10.0)
bap		1		1		1	3 (3.3)
aap		3					3 (3.3)
fbe		6	2		4		12 (13.3)
atlE	13	7		1	1	1	24 (26.6)
embP	13	6		1	1	1	23 (25.5)
eno	38	10	5	3	4	11	79 (87.7)
Antibiotic-resistant genes							
mecA	1	2		1			4 (4.4)
mecC						1	1 (1.1)
blaZ	7	5		1	1	7	21 (23.3)
ermA							0
ermB	2	1					3 (3.3)
ermC	2	1				1	4 (4.4)
lnuA							0
mefA							0
mrsA	1						1 (1.1)
mphC	2			1			3 (3.3)

and consequently biofilm formation of staphylococcus species (Christensen *et al.* 1982; Fredheim *et al.* 2009). We found that *S. haemolyticus* isolates had the highest ability to form biofilm whereas Tremblay *et al.* (2013) found that *S. xylosus* had the highest ability followed by *S. haemolyticus*. In this study, *S. chromogenes* isolates had the lowest ability to form biofilms whereas in Canadian isolates (Tremblay *et al.* 2013), *S. epidermidis* isolates had the lowest ability to form biofilms. The differing results may be explained by differences in CNS species distribution in different countries. In addition, intraspecies variations are not uncommon and have been observed for *S. chromogenes*, *S. epidermidis*, *S. haemoloyticus* and *S. hominis* (Tremblay *et al.* 2013; Oliveira *et al.* 2015). Furthermore, a recent study identified three distinct genotypes within *S. chromogenes* (Ajitkumar *et al.* 2013), which may also provide an explanation for intraspecies variations in biofilm formation.

Detection of biofilm-associated genes

Several genes have been associated with biofilm formation by *Staphylococcus* species (Simojoki *et al.* 2012; Tremblay *et al.* 2013). The majority (90%) of the isolates were positive for at least one of the seven genes evaluated, with different combinations (Table 3), and only nine isolates were negative for all seven genes (Table 4). The majority of isolates (n = 79) were positive for the *eno* gene (88%) and this gene was also the dominant gene detected in Finnish isolates (75%) (Simojoki *et al.* 2012). The *eno* gene was found widely distributed, regardless of species and biofilm-forming ability. It is, therefore, likely that *eno* is not a biofilm-specific gene. Very few isolates were positive for *icaA* (n = 9), *bap* (n = 3) or *aap* (n = 3). Furthermore, *aap* was exclusively found in *S. epidermidis*. The *icaA*-positive rate was similar to the rates found by Simojoki *et al.* (2012). However, our biofilm positive/*icaA*-negative rate (96.6%) was higher than the one observed (13.8%) by Osman *et al.* (2015). Again, *bap* was only detected in a few isolates (n = 3), which supports observations that *bap* is rarely found outside of *S. xylosus* isolates (Piessens *et al.* 2012; Tremblay *et al.* 2013). Other genes were found in <30% of the isolates and were frequently found almost exclusively in *S. chromogenes* and *S. epidermidis*. Based on our observations and the literature, the number of positive isolates appears to be dependent on the distribution of species isolated from the milk samples and their geographical region. Overall, there is little evidence that specific genetic markers usually attributed to biofilm formation in *S. aureus* and *S. epidermidis* isolated from humans are applicable to other CNS isolates associated with dairy cows. Therefore, there is a need to identify new genetic determinants for biofilm formation in CNS species.

Detection of antimicrobial resistance genes

The presence of resistance genes and resulting clinical response to treatment remains unexplored (Sampimon *et al.* 2011). Genotypic methods have several advantages to monitor resistance over conventional culture methods such as disk diffusion test in agar, a standard method (CLSI) (Asfour and Darwish 2011). In addition, CLSI guidelines agree that checking for the presence of *mecA* and *blaZ* genes by PCR is the most reliable method to detect MR (CLSI 2013). However, the ancestor of *mecA* is naturally present in some CNS species (Couto *et al.* 1996; Sawant, Gillespie and Oliver 2009) and as stated in a previous study (Sampimon *et al.* 2011), the use of a *mecA* as a marker to determine the resistance profile of staphylococcal species from bovine milk could result in false positives due to the detection of the ancestral *mecA* gene. Table 4 shows the distribution of antimicrobial resistance genes among CNS species. For β-lactam resistance genes,

21 (23.3%), 4 (4.4%) and 1 (1.1%) isolates were positive for the *bla*Z, the *mec*A and the *mec*C genes, respectively. Only in one isolate, belonging to *S. chromogenes* species, we found both *bla*Z and *mec*A genes. β-Lactam resistance is low and coincides with Sawant, Gillespie and Oliver (2009) in USA but in other countries such as the Netherlands or Korea the resistance is higher (Moon *et al.* 2007; Sampimon *et al.* 2011). MR prevalence among CNS is similar to that found in USA and Switzerland (Sawant, Gillespie and Oliver 2009; Moser *et al.* 2013). However, CNS-MR isolated from bovine mastitis is higher in the Netherlands (Sampimon *et al.* 2011).

Four of the five MR isolates that were resistant to cefoxitin (FOX) carried the *mec*A gene. Resistance can be the result of other genetic determinants, and researchers should try to investigate alternate resistance mechanisms. Specifically, the isolate, identified as *S. saprophyticus,* was resistant to penicillin, oxacillin (OXA) and FOX, but the *mec*A gene was not detected. Further tests were performed to identify the genetic determinant responsible for antimicrobial resistance. This isolate was tested by PCR for the presence of *mec*C gene and it was positive. This gene was recently identified as a novel allele of *mec*A (*mec*A $_{LGA251}$) in methicillin-resistant *S. aureus* (MRSA) from both humans and a range of animal species across Europe (García-Álvarez *et al.* 2011; García-Garrote *et al.* 2014; Harrison *et al.* 2013; Loncaric *et al.* 2013). This gene was renamed by the International Working Group on the Classification of Staphylococcal Cassette Chromosome (SCC) Elements as *mec*C. Detection of macrolide and lincosamide (ML) resistance among CNS isolates from bovine mastitis is important because the majority of ML genes are plasmid borne and the potential for the spread of resistance across pathogens is present (Sampimon *et al.* 2011). Several studies have been performed to identify CNS reservoirs (Pate *et al.* 2011). Some CNS species, such as *S. epidermidis,* are also commonly found in humans (Gatermann, Koschinski and Friedrich 2007), and human and dairy cattle may share *S. epidermidis* strains (Sampimon *et al.* 2011). Such exchange of bacteria can provide new sources of antimicrobial resistance in human health and veterinary medicine.

We found low ML resistance among CNS (6.7%) isolates compared to other countries, with the majority of the isolates testing positive for the *erm*C, *erm*B and *mph*C genes, with the following combinations: *erm*C (one isolate); *erm*B and *erm*C (two isolates); *erm*B, *erm*C and *mph*C (one isolate); *mph*C (one isolate); and *mph*C and *mrs*A (one isolate).

In European countries, ML resistance ranges from 15.6% to 44% and resistance was mostly encoded by *erm*C, *lnu*A, *mrs*A and *mph*C (Lina *et al.* 1999; Lüthje and Schwarz 2006; Aslantas, Öztürk and Ceylan 2011; Kot *et al.* 2012). For example, Kot *et al.* (2012) found 15.6% of isolates in Poland were ML resistant and *erm*C was the dominant gene detected among the resistant isolates (55.5%). These data highlight that antimicrobial resistance is encoded and spread by different mechanisms in Europe and the Americas. Results and additional data of all strains are available in suplementary data.

CONCLUSION

CNS species are the predominant bacteria isolated from cows with mastitis in Argentina and form a heterogeneous group with species-specific antimicrobial-resistant rates. Some CNS species may persist in the mammary gland due to biofilm production and, as a consequence, result in persistent infections. Our study shows that almost all CNS strains are able to form biofilm, and biofilm formation in CNS species is unlikely to occur due to a single component and/or process. Proper identification, characterization of resistance genes in addition to an improved understanding of the contribution of each species to udder disease are required to ensure that appropriate therapeutic and management decisions are taken.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Conflict of interest. None declared.

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