Short communication: Detection of antibiotic resistance, *mecA*, and virulence genes in coagulase-negative *Staphylococcus* spp. from buffalo milk and the milking environment

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ABSTRACT

The aim of this study was to determinate whether coagulase-negative staphylococci (CNS) from buffalo milk or the milking environment possess virulence factors that are associated with intramammary infections or antimicrobial resistance. Milk samples ($n = 320$) from 80 lactating buffalo were evaluated for clinical and subclinical mastitis by physical examination, the strip cup test, California Mastitis Test (CMT), and somatic cell count (SCC) over a 4-mo period. In addition, swabs were obtained from the hands of consenting milkers (16), liners (64), and from the mouths (15) and nostrils (15) of buffalo calves. No clinical cases of mastitis were observed; however, CMT together with SCC results indicated that 8 animals had subclinical mastitis. Eighty-four CNS isolates were identified by MALDI-TOF MS and *cydB* real-time PCR (qPCR) and then evaluated by qPCR for presence of the *eta*, *etb*, *sea*, *sec*, *cna*, *seb*, *sei*, *seq*, *sem*, *seg*, *see*, and *tst* toxin genes, adhesion- and biofilm-associated genes (*eno*, *ebps*, *fib*, *fnbA*, *coa*), and the methicillin resistance gene (*mecA*). Resistance to antibiotics commonly used for mastitis treatment in Brazil was determined using the Kirby-Bauer test. Two strains were positive for the *see* and *eta* toxin genes; and *mecA* (1), *eno* (27), *ebps* (10), *fnbA* (10), and *coa* (5) genes were also detected. A notable number of isolates were resistant to erythromycin (30), penicillin (26), and cotrimoxazole (18); importantly, 10 vancomycin-resistant isolates were also detected. A

smaller number of isolates were resistant to rifampicin (8), oxacillin (7), clindamycin (5), cefepime (4), tetracycline (3), ciprofloxacin (2), and chloramphenicol (1), and none were resistant to gentamicin or ciprofloxacin. Isolates with resistance to 2 (13 isolates), 3 (3), 4 (3), 5 (1), and 6 (1) antibiotics were detected. Taken together, our findings suggest that CNS isolates may not be a significant cause of clinical or even subclinical mastitis in buffaloes, but they may be a reservoir of virulence and antibiotic resistance genes.

Key words: dairy buffalo, antibiotic resistance, virulence genes, biofilm

Short Communication

Milk and dairy products from cattle and buffalo are often the main sources of protein in developing countries (Preethirani et al., 2015). Mastitis, one of the costliest diseases in these animals, can compromise milk quality and pose public health risks (Pyörälä and Taponen, 2009). In dairy cattle, the CNS are often considered contaminants that cause little or no variation in milk SCC (Taponen and Pyörälä, 2009). That said, some CNS species can affect the udder of dairy cows (Supré et al., 2011). Also, CNS are the most important agents of both clinical and subclinical mastitis in sheep (Martins et al., 2017) and possibly an important mastitis agent in buffalo (El-Jakee et al., 2013).

The CNS can form biofilms (Aguilar and Iturralde, 2001), which protects them from the action of immune system components (Fox et al., 2005) and the penetration of antimicrobial agents (Stewart, 1996). The microbial surface components recognizing adhesive matrix molecules (MSCRAMM), such as collagen binding protein (*cna*), laminin binding protein (*eno*), elastin

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binding protein (*ebpS*), and fibronectin binding protein (*fnbA*), are often associated with CNS attachment and biofilm formation in bovines (Zuniga et al., 2015). The CNS can also produce toxins such as enterotoxin, toxic shock syndrome, and exfoliative toxin (Zuniga et al., 2015). They are typically resistant to a greater number of antimicrobials than other mastitis agents such as *Staphylococcus aureus* and can act as a reservoir of antimicrobial resistance genes (Schoenfelder et al., 2017).

Thus, the aim of this study was to determinate if CNS from buffalo milk and the milking environment possess virulence factors that are associated with IMI or antimicrobial resistance.

Eighty-four CNS isolates were obtained from milk samples collected from 80 lactating buffaloes in a typical small herd in São Paulo State, Brazil, and swab samples were taken of the hands of consenting milkers (16), liners (64), and the mouths (15) and nostrils (15) of buffalo calves. Following physical examination of the mammary gland (Radostits et al., 2007), milk samples were submitted for strip cup, California Mastitis Test (CMT), and SCC analyses (Schalm and Noorlander, 1957). As suggested by Dhakal (2006), 200,000 cells/mL was used as the cut-off to indicate the presence of subclinical mastitis. Microbiological culture was done according to the National Mastitis Council Guidelines (National Mastitis Council, 2004), and isolates were identified based on colony morphology, Gram staining, and catalase and coagulase reactions. Determination of species of putative CNS was done by MALDI-TOF analysis at the Animal Health Laboratory, University of Guelph (Guelph, ON, Canada) and with species-specific *cydB* quantitative (q)PCR tests (Pizauro et al., 2017; de Almeida et al., 2018).

For qPCR tests, DNA was extracted using the method of Kuramae-Izioka (1997). Real-time PCR primers (Table 1) were used at a concentration of 10 pmol/ μ L in 25- μ L reaction mixtures containing 5 μ L of DNA (50 ng/ μ L) and 20 μ L of Light Cycler 480 (LC480) Probe Master mix (Roche Diagnostics, Indianapolis, IN). The amplification parameters were as follows: 1 cycle of 95°C for 10 min, followed by 40 cycles consisting of 95°C for 10 s, 55°C for 20 s, and 72°C for 25 s. Ramp rates were 4.4°C/s, 2.2°C/s, and 4.4°C/s, respectively. *Streptococcus suis* and water were included with each primer pair as negative controls. *Staphylococcus aureus* COL (*seb*, *mecA*, *eno*), NewMan (*eta*, *etb*, *sea*, *sec*, *seq*), MW2 (*sea*, *cna*, *fnbA*, *tsst-1*, *ebpS*, *sec*, *seq*), Mu50 (*tsst-1*, *see*, *sei*, *sem*, *seg*, *see*), and ATCC 25923 (*Tstag*, *coa*, *fib*) were used as positive controls for virulence genes. These genes were selected because of their contribution to IMI and maintenance of the microorganism (*tsst*, *eta*, *etb*, *sea*, *sec*, *can*, *seb*, *sei* *seq*, *sem*, *seg*, *seh*, *eno*,

ebpS, *fib*, *fnbA*, and *coa*) and for antimicrobial resistance, specifically β -lactam resistance (*mecA*). A melting step (predicted primer melting temperature: 62°C) was done at the end of each run to confirm the presence of a single product.

Antibiotic resistance testing (Kirby-Bauer) was performed according to the manual of the Clinical and Laboratory Standards Institute (CLSI, 2013). Overnight cultures, diluted to McFarland standard 0.5 ($\sim 10^8$ cfu/mL), were spread on Mueller-Hinton agar plates and antibiotic disks of cefepime (30 μ g), ciprofloxacin (5 μ g), chloramphenicol (30 μ g), clindamycin (2 mg), erythromycin (15 μ g), gentamicin (10 mg), oxacillin (1 μ g), penicillin G (10 U), rifampicin (30 μ g), trimethoprim-sulfamethoxazole (25 μ g), tetracycline (30 μ g), and vancomycin (30 μ g) (Sensifar e Multifar-Cefar, São Paulo, Brazil) were added. *Staphylococcus aureus* ATCC 25923 was used as the control. The disk inhibition zones were interpreted according to manufacturer's standards.

Biofilm production was measured using the microtiter assay of Cucarella et al. (2001); sterile TSB was used as the negative control and *Staph. aureus* ATCC 25923 as the positive control. Strains were considered biofilm producers if their optical density at 570 nm (OD_{570}) was ≥ 0.1 (Christensen et al., 1985). The Congo red agar test was performed as described previously (Kaiser et al., 2013).

In milk, calf, and liner samples, the most frequently isolated CNS was *Staph. chromogenes* (n = 47) followed by *Staph. agnetis* (n = 14), *Staph. sciuri* (n = 3), *Staph. equorum* (n = 2), *Staph. haemolyticus* (n = 2), *Staph. caprae* (n = 1), *Staph. hominis* (n = 1), and *Staph. warneri* (n = 1) (Table 2). The most frequently isolated CNS species from milkers' hands was *Staph. epidermidis* (n = 8), followed by *Staph. chromogenes* (n = 3), *Staph. saprophyticus*, and *Staph. pasteurii* (n = 1) (Table 2). During further evaluation based on a screening CMT reaction and confirmed by an SCC >200,000 cells/mL, 8 *Staph. chromogenes*, 1 *Staph. sciuri*, and 4 *Staph. agnetis* were associated with subclinical mastitis.

All 84 CNS isolates tested formed biofilms in the microtiter plate assay with an $OD_{570} > 0.1$. In addition, all isolates produced black colonies on Congo red agar. Regardless of source, no differences in the biofilm-forming capabilities of these isolates were observed.

Virulence genes were detected in a small number of the CNS isolates studied (Table 2). One *Staph. chromogenes* and one *Staph. agnetis* (both from milk samples) were positive for the enterotoxin A (*sea*) and enterotoxin E (*see*) genes, respectively. Adhesion genes were detected more frequently. The laminin binding protein (*eno*) gene was present in 27 isolates, whereas 10 iso-

Table 1. Sequence of primers (F, forward; R, reverse) used in PCR assays

Gene	Primer	Sequence (5'-3')	Product (bp)	Reference
<i>coa</i>	F	GTCTTGAAAGTAGCTCATCTAAACTTG	228	Paniagua-Contreras et al., 2012
	R	ATCCAAATGTTCCATCGTTGTATTC		
<i>eta</i>	F	CGCTGCGGACATTCCCTACATGG	676	Paniagua-Contreras et al., 2012
	R	TACATGCCCCGCCACTTGCTTGT		
<i>etb</i>	F	GAAGCAGCCAAAAACCCATCGAA	419	Paniagua-Contreras et al., 2012
	R	TGTTGTCCGCCCTTTACCACTGTGAA		
<i>sea</i>	F	TTGCAGGGAACAGCTTTAGGCAATC	252	Paniagua-Contreras et al., 2012
	R	TGGTGTACCACCCGCACATTGA		
<i>sec</i>	F	CCCTACGCCAGATGAGTTGCACA	602	Paniagua-Contreras et al., 2012
	R	CGCCTGGTGCAGGCATCATATC		
<i>cna</i>	F	AAAGCGTTGCCTAGTGGAGA	192	Arciola et al., 2005
	R	AGTGCCTTCCCAAACCTTTT		
<i>seb</i>	F	GACATGATGCTGCACCAGGAGA	355	Paniagua-Contreras et al., 2012
	R	AACAAATCGTTAAAAACGGCGACACAG		
<i>sei</i>	F	GGCCACTTTATCAGGACA	328	Paniagua-Contreras et al., 2012
	R	AACTTACAGGCAGTCCA		
<i>seq</i>	F	GGAATTACGTTGGCGAA	330	Paniagua-Contreras et al., 2012
	R	AACTCTCTGCTTGACCA		
<i>sem</i>	F	CATATCGCAACCGCTGA	148	Paniagua-Contreras et al., 2012
	R	TCAGCTGTTACTGTGCGA		
<i>seg</i>	F	GTTAGAGGAGGTTTTATG	198	Paniagua-Contreras et al., 2012
	R	TTCTTCAACAGGTGGAGA		
<i>see</i>	F	TGCCCTAACGTTGACAACAAGTCCA	170	Paniagua-Contreras et al., 2012
	R	TCCGTGTAAATAATGCCTTGCCTGAA		
<i>eno</i>	F	ACGTGCAGCAGCTGACT	302	Munro et al., 2011
	R	CAACAGCATCTTCAGTACCTTC		
<i>ebpS</i>	F	CATCCAGAACCAATCGAAGAC	186	Munro et al., 2011
	R	CTTAACAGTTACATCATCATGTTTATCTTTTG		
<i>fib</i>	F	CTACAACTACAATTGCCGTC AACAG	404	Munro et al., 2011
	R	GCTCTTGTAAGACCATTTTTCTTCAC		
<i>fnbA</i>	F	GATACAAAACCCAGGTGGTG	191	Arciola et al., 2005
	R	AAAGCGTTGCCTAGTGGAGA		
<i>tst</i>	F	AGCCCTGCTTTTACAAAAGGGGAAAA	306	Paniagua-Contreras et al., 2012
	R	CCAATAACCACCCGTTTTATCGCTTG		
<i>mecA</i>	F	GCGGAGATCAAAGACAA	310	Paniagua-Contreras et al., 2012
	R	CCATCTATAGCTGTGTGG		

lates were positive for the fibronectin binding protein A (*fnbA*) gene, and 10 isolates possessed the elastin binding protein (*ebpS*) gene.

Of the 56 isolates from milk, 1 isolate possessed *sea*, 1 *seh*, 17 *eno*, 8 *ebpS*, 8 *fnbA*, and 4 *coa*. Of the 5 isolated from calf nostrils and mouth, only 2 possessed the *eno*

Table 2. Occurrence of virulence genes in *Staphylococcus* species isolated in buffalo milk and milking environment

CNS species	Isolates	Origin					No. of qPCR-positive isolates from milk/environment ¹							
		Milk	Calf	Liners	Milker's hands	Biofilm formation	<i>mecA</i>	<i>sea</i>	<i>see</i>	<i>eno</i>	<i>cna</i>	<i>ebps</i>	<i>fnbA</i>	<i>coa</i>
<i>Staph. agnetis</i>	14	10		4		+			1	3		2	3	1
<i>Staph. caprae</i>	1	1				+				1				
<i>Staph. chromogenes</i>	50	40	3	4	3	+		1		11		4	5	2
<i>Staph. epidermidis</i>	8				8	+				4		1	1	1
<i>Staph. equorum</i>	2	2				+					1	1	1	
<i>Staph. haemolyticus</i>	2		1	1		+	1			2		1		
<i>Staph. hominis</i>	1	1				+				1		1		1
<i>Staph. pasteurii</i>	1				1	+				1				
<i>Staph. saprophyticus</i>	1				1	+				1				
<i>Staph. sciuri</i>	3	1	1	1		+				2				
<i>Staph. warneri</i>	1	1				+				1				
Total	84	56	5	10	13		1	1	1	27	1	10	10	5

¹All isolates were negative for the *tst-1*, *eta*, *etb*, *sec*, *cna*, *seb*, *sei*, *seq*, *sem*, *seg*, and *fib* genes. qPCR = quantitative PCR.

gene. Of the 10 isolates from the liners, 1 was positive for *mecA*, 2 for *eno*, and 1 for *ebpS*. Of the 13 isolated from milkers' hands, 6 were positive for *eno*, 1 for *ebpS*, 1 for *fnbA*, and 1 for *coa*.

All isolates were susceptible to ciprofloxacin and gentamicin, but resistance to penicillin (20.1%), cotrimoxazole (17.8%), erythromycin (15.4%), vancomycin (10.7%), rifampicin (8.33%), oxacillin (8.33%), clindamycin (4.76%), cefepime (4.76%), tetracycline (3.57%), and chloramphenicol (1.19%) was observed (Table 3). Among the resistant isolates, 21 (25.0%) were resistant to more than one antimicrobial agent, including *Staph. chromogenes* (9), *Staph. epidermidis* (7), *Staph. haemolyticus* (2), *Staph. caprae* (1), *Staph. hominis* (1), and *Staph. sciuri* (1). Some differences were seen in the resistance of the isolates based on site. The milk isolates were resistant to vancomycin (6), penicillin (7), erythromycin (8), cotrimoxazole (8), cefepime (3), and oxacillin (4), whereas isolates from liners were resistant to vancomycin (2), rifampicin (1), oxacillin (1), penicillin (2), erythromycin (1), cotrimoxazole (5), and tetracycline (1). Isolates from calf mouth and nostril were resistant to clindamycin (1), vancomycin (1), oxacillin (1), erythromycin (1), and tetracycline (1), whereas isolates from milkers' hands were resistant to chloramphenicol (1), rifampicin (5), cefepime (1), oxacillin (1), penicillin (8), erythromycin (3), and cotrimoxazole (2).

Eight *Staph. chromogenes*, 1 *Staph. sciuri*, and 4 *Staph. agnetis* isolates were associated with subclinical mastitis, as indicated by both positive CMT and SCC results. To our knowledge, this is the first report of *Staph. agnetis* or *Staph. sciuri* being associated with subclinical mastitis in buffalo. Several CNS (i.e., *Staph. chromogenes*, *Staph. hyicus*, *Staph. simulans*, *Staph. epidermidis*, *Staph. warneri*, *Staph. caprae*, and *Staph. xylosus*) associated with udder infections in bovines, sheep, and goats were detected in milk samples that were CMT negative and that had SCC <200,000 cells/mL. Nevertheless, these species could be considered potential mastitis agents and a potential source of virulence and antibiotic resistance genes.

All of the CNS studied produced biofilms and contained one or more MSCRAMM genes, most commonly *eno* (32.1%), *fnbA* (12.0%), and *ebpS* (12.0). These results are in partial agreement with analysis of 67 CNS isolated from bovine milk by Zuniga et al. (2015). Salaberry et al. (2015) also observed a higher frequency of *eno* and *bap* genes in CNS isolates than in coagulase-positive staphylococci from cases of caprine subclinical mastitis. The fact that these genes were also present in CNS from liners, milkers' hands, and the nostrils and mouths of calves is consistent with the idea that these isolates may be a reservoir of some virulence

Table 3. Antimicrobial resistance of CNS isolated from buffalo milk and milking environment

CNS species (no. of samples)	Antibiotic ¹ [no. (%)]											
	Clin	Van	Clo	Rif	Fep	Oxa	Pen	Ery	Cip	Gen	Cot	Tet
<i>Staph. chromogenes</i> (50)	3 (6)	8 (16)	0	1 (2)	4 (8)	6 (12)	5 (10)	6 (12)	0	0	10 (20)	1 (2)
<i>Staph. haemolyticus</i> (2)	0	1 (50)	0	1 (50)	0	1 (50)	1 (50)	2 (100)	0	0	0	1 (50)
<i>Staph. caprae</i> (1)	0	0	0	0	0	0	1 (100)	0	0	0	1 (100)	0
<i>Staph. hominis</i> (1)	0	0	0	0	0	0	1 (100)	1 (100)	0	0	0	0
<i>Staph. sciuri</i> (3)	1 (33.3)	0	0	0	0	0	0	0	0	0	0	1 (33.3)
<i>Staph. epidermidis</i> (8)	0	0	0	5 (62.5)	0	0	7 (87.5)	3 (37.5)	0	0	2 (25.0)	0
<i>Staph. equorum</i> (2)	0	0	0	0	0	0	0	1 (50.0)	0	0	0	0
<i>Staph. warneri</i> (1)	0	0	0	0	0	0	1 (100)	0	0	0	0	0
<i>Staph. agnetis</i> (14)	0	0	0	0	0	0	0	0	0	0	2 (14.2)	0
<i>Staph. saprophyticus</i> (1)	0	0	1 (100)	0	0	0	0	0	0	0	0	0
<i>Staph. pasteurii</i> (1)	0	0	0	0	0	0	1 (100)	0	0	0	0	0

¹Clin = clindamycin, Van = vancomycin, Clo = chloramphenicol, Rif = rifampicin, Fep = cefepime, Oxa = oxacillin, Pen = penicillin, Ery = erythromycin, Cip = ciprofloxacin, Gen = gentamicin, Cot = cotrimoxazole, Tet = tetracycline.

genes (Otto, 2013). That said, it is difficult to compare our gene findings with other buffalo and bovine studies because the same phenotypes or genotypes were not always studied (Sender et al., 2017). In the current study, one *Staph. chromogenes* isolate harbored the *sea* gene, whereas 1 *Staph. agnetis* isolate had the *see* gene. Only a few studies have investigated the toxigenic potential of CNS and its enterotoxins in the development of food poisoning (Martins et al., 2017), so the significance of this finding is not clear at this time.

Although some of the antimicrobial compounds tested may not be legal for use in all countries, the results of this study underscore the need for improvements in antimicrobial stewardship and infection control programs in small dairy buffalo farms (Cheng et al., 2019). This is highlighted by the fact that resistance to penicillin and cotrimoxazole was common and more than 25% of the CNS studied were resistant to more than one antibiotic. Also, a *mecA*-positive *Staph. haemolyticus* isolate (that could be easily transmitted to the milk or other animals) was recovered from a liner. This finding is consistent with reports of the *mecA* gene in *Staph. aureus* from buffalo milk and buffalo dairy products (Saka and Terzi Gulel, 2018). The *mecA* gene encodes penicillin binding protein 2a (PBP2a), which has a low affinity to all β -lactam antibiotics (particularly methicillin), and it is responsible for producing the methicillin-resistant *Staph. aureus* (MRSA) phenomenon. β -Lactam resistance can often be attributed to mutations in the *mecA* gene, although other genetic elements may also be involved (Elhassan et al., 2015). Given the growing use of unpasteurized milk in some countries (e.g., Whitehead and Lake, 2018), knowing about the presence of antibiotic-resistant organisms in buffalo (and other) milk is essential.

In conclusion, more than a quarter of the CNS isolates tested were resistant to one or more of the antibiotics tested, including one *Staph. haemolyticus* isolate that was *mecA* positive. Although only 2 isolates were positive for toxin genes, genes associated with biofilm production and cell attachment were found in almost all isolates tested, including those isolated from healthy animals. It appears that CNS were less likely to be associated with mastitis in buffalo than in bovines, but further studies are needed. Although this study was conducted in a single buffalo herd, the management was typical of small buffalo dairy operations and should provide a good basis for comparison with larger Brazilian farms or small farms in other countries.

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