Effect on quarter milk somatic cell count and antimicrobial susceptibility of *Staphylococcus rostri* causing intramammary infection in dairy water buffaloes

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ABSTRACT

In many parts of the world, coagulase-negative staphylococci (CNS) are the predominant cause of intramammary infections (IMI) in dairy cows and in water buffaloes, as well. A longitudinal field study was carried out on one well-managed dairy water buffalo herd to determine the prevalence and distribution of CNS and a recently described CNS-species, *Staphylococcus rostri*, in milk samples to explore its relevance for buffaloes' udder health throughout lactation, and to gain insight into the susceptibility of the latter species toward commonly used antimicrobials. Twice weekly quarter milk samples from a cohort of 11 lactating water buffaloes were collected over an 8-mo period. The CNS ($n = 109$; 76.2% of all culture-positive samples) were the predominant pathogens causing IMI, followed by *Corynebacterium bovis* ($n = 11$; 7.6%) and *Streptococcus* spp. ($n = 9$; 6.2%) other than *Streptococcus uberis* ($n = 2$; 1.4%). Thirty-seven hemolytic staphylococci suspected to be *Staphylococcus aureus* were further differentiated using transfer DNA-intergenic spacer-PCR and *rpoB*-gene sequencing because they were coagulase-negative. Thirty-three of those isolates were identified as *Staph. rostri*, whereas 2 others were identified as *Staphylococcus epidermidis*. None of the *Staph. rostri* isolates displayed resistance to the antimicrobial agents tested. Mean quarter milk somatic cell count (qSCC) of all samples collected throughout lactation was 20,970 cells/mL. The qSCC at sampling of quarters infected with *Staph. rostri* (34,466 cells/mL) and CNS other than *Staph. rostri* (34,813 cells/mL) were significantly higher than the qSCC of noninfected quarters (20,287

cells/mL), yet not significantly different from each other. These findings provide novel insight into the prevalence and distribution, antimicrobial susceptibility, and relevance of *Staph. rostri* compared with other CNS species causing IMI in water buffaloes. Further studies are needed to pinpoint the relevance, niches, and transmission routes of *Staph. rostri*, as well as other CNS in water buffaloes.

Key words: antimicrobial susceptibility, intramammary infection, *Staphylococcus rostri*, water buffalo

INTRODUCTION

World milk production has increased over the last few decades and it is noteworthy that in the last couple of years water buffaloes have taken a share of about 12% of the global milk production. India and Pakistan have been producing 60 and 30% of the world's buffalo milk, respectively, with buffalo milk contributing to 55 and 75% of the total milk produced in those countries (FAO, 2005–2006). Production of buffalo milk has been a tradition in many parts of the world, such as the Caucasian countries, Asia, and Egypt. In Europe, Italy is the main producer of buffalo milk, and reasons for increasing interest in buffalo breeding during recent years can be attributed to the popularity of buffalo mozzarella cheese together with the absence of quotas in the European Community for this type of production. In addition, buffalo milk is more valuable for farmers, as they typically receive at least twice the price compared with bovine milk. Italian river buffaloes (*Bubalus bubalis*), typically reared in central and southern Italy, have recently been introduced in northern Italy as well. Despite the growing market for dairy buffalo products, information on milk quality parameters, including the SCC, and the causes of IMI is needed. Coagulase-negative staphylococci seem to be the most

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frequently isolated bacteria causing IMI in buffaloes, present in up to 78% of the cases (Moroni et al., 2006), followed by *Corynebacterium* spp. and *Streptococcus* spp. (Naiknaware et al., 1998).

Coagulase-negative staphylococci are the most commonly found pathogens in both healthy cows and cows with subclinical mastitis in many countries (Piepers et al., 2007; Supré et al., 2009; Zadoks and Watts, 2009). They are generally considered as minor pathogens, although some species seem to be more relevant for dairy cows' udder health than others (Supré et al., 2011). The different species can be accurately identified using molecular diagnostic techniques such as gene-sequencing (Zadoks and Watts, 2009), transfer DNA-PCR (Supré et al., 2009), (GTG)5-PCR fingerprinting (Braem et al., 2011), amplified fragment-length polymorphism (Piessens et al., 2010), and gap PCR-RFLP (Park et al., 2011), a prerequisite to scrutinize their role for udder health in dairy cows and other animal species such as water buffaloes.

Recently, Riesen and Perreten (2010) described a new hemolytic *Staphylococcus* species cultured from the nasal cavity of healthy pigs sampled at the slaughterhouse in Switzerland named *Staphylococcus rostri*. This species was isolated from almost 50% of the nasal samples and in 22% of the cases it was in association with *Staphylococcus aureus*. Until now, pigs are the only animal species in which *Staph. rostri* has been reported. Besides studies on the antimicrobial resistance patterns (Stegmann and Perreten, 2010) and on detection of *mecA* gene (Vanderhaeghen et al., 2012) within the porcine isolates, no information is available on the prevalence, distribution, and relevance of this novel CNS species in animal species other than swine.

This paper is the first to report on *Staph. rostri* causing IMI in water buffaloes. The relationship between *Staph. rostri* in milk and the quarter milk somatic cell count (qSCC) was studied and compared with the effect of other CNS and major mastitis pathogens on the udder health of water buffaloes in a longitudinal setting. Moreover, data on the *Staph. rostri* antimicrobial susceptibility are provided.

MATERIALS AND METHODS

Sample Collection

A farm located in northern Italy consisting of 270 lactating water buffaloes was used in the study. Lactating animals were housed in cubicles and divided into 5 different groups (i.e., fresh buffaloes, midlactating primiparous buffaloes, midlactating multiparous buffaloes, end-lactating buffaloes, and animals in the sick pen). Buffaloes were fed a TMR and were milked twice

a day in a double herringbone milking parlor (8 × 2). Overall, clinical mastitis was very rare and necessity of treatment was insignificant. Blanket-dry buffalo treatment was not applied. No other treatments, except from those related to postpartum uterine diseases (Excenel, Pfizer s.r.l., Latina, Italy), were administered. Seven primiparous and 4 multiparous animals, ranging between the second and seventh calving, were randomly selected and followed throughout 1 lactation in terms of quarter milk culturing and SCC. Quarter milk samples and data were collected from April 2008 to February 2009, for a total of 17 farm visits. Ten milliliters of milk from each udder quarter was aseptically collected after disinfection of the teat ends and discarding the first streams of milk. The milk samples were collected in sterile vials that were submitted to the laboratory (Department of Health, Animal Science and Food Safety, University of Milan, Italy) at 4°C within 24 h after collection.

Bacteriological Culturing and Identification of Hemolytic CNS

The milk samples (n = 648) were cultured as recommended by National Mastitis Council (NMC, 1999). Ten microliters of each milk sample was plated onto 5% sheep blood agar plates (one plate per buffalo). After incubation at 37°C for 48 h, single isolated colonies were picked up, subcultured, and presumptive identification was performed on the basis of colonies appearance, Gram staining, catalase- and coagulase-testing. Hemolytic staphylococci suspected to be *Staphylococcus aureus* but yielding negative results in the coagulase-test were frozen in nutrient broth with 15% glycerol added before further speciation. Quarter milk SCC was determined by an automated somatic cell counter (Bentley Somacount 150, Bentley Instrument, Chaska, MN). A total of 37 hemolytic CNS isolates were further analyzed using transfer RNA-intergenic spacer-PCR (tDNA-PCR; Supré et al., 2009) with capillary electrophoresis and sequencing of the *rpoB*-housekeeping gene as described by Supré et al. (2011).

Quarter Infection Status

Sampled quarters were considered to have an IMI if the milk cultured ≥ 100 cfu/mL in a pure growth, except for CNS, where the requirement was set to ≥ 200 cfu/mL (Dohoo et al., 2011b). Quarters were allocated into 5 classes based on the bacteriological culture results obtained at the different samplings throughout lactation: (1) noninfected; (2) infected with *Staph. rostri*; (3) infected with CNS other than *Staph. rostri*; (4) infected with *Streptococcus* spp.; and (5) infected with

other pathogens including *E. coli*, other gram-negative bacteria, *Corynebacterium bovis*, other esculin-positive cocci, *Staph. aureus*, and *Proteus* spp. Samples from which 3 or more different pathogens could be isolated were considered as contaminated and excluded from the analyses. Using the previously mentioned classification, IMI status could be defined for 634 of the samples (97.8% of all samples).

Antimicrobial Susceptibility Testing

Minimal inhibitory concentrations of 18 antibiotics (Table 1) were determined for the 33 *Staph. rostri* isolates causing IMI, using broth dilution test, according to the procedure described in CLSI guidelines M31-A3 (CLSI, 2008). A commercially available microdilution MIC system (Sensititer companion1F, Trek Diagnostics System, East Grinstead, UK) was used according to the manufacturer's instructions. The Sensititer plate reading was performed manually, recording the last concentration of antimicrobial without turbidity or deposit of cells at the bottom of the well. The CLSI resistance breakpoints for *Staphylococcus* spp. were used (CLSI, 2008); if breakpoints were differentiated for host species, cattle breakpoints were selected.

Statistical Analysis

The association between the quarter IMI status and qSCC at sampling throughout lactation was determined by fitting a linear mixed regression model with buffalo as random effect and quarter as repeated effect (Proc Mixed, SAS 9.3, SAS Institute Inc., Cary, NC). A first-order autoregressive correlation structure was used to account for the clustering of repeated samplings within a quarter. An ln transformation of SCC was performed to obtain a normalized distribution. The model

included ln SCC at sampling as outcome variable with sampling time (17 levels), DIM (8 levels), quarter IMI status (5 levels, main predictor of interest), parity (2 levels), and season of calving (4 levels) as categorical predictor variables. A Bonferroni correction was used to adjust for pairwise comparisons. Significance was assessed at $P < 0.05$.

RESULTS

Descriptive Statistics

A total of 648 quarter milk samples were processed. The majority of samples ($n = 491$; 76%) were noninfected, whereas 143 (22%) quarter samples had an IMI and 14 (2%) were contaminated. Coagulase-negative staphylococci were the predominant pathogens causing IMI ($n = 109$; 76.2% of all pathogens) followed by *C. bovis* ($n = 11$; 7.7%), and *Streptococcus* spp. other than *Strep. uberis* ($n = 9$; 6.2%; Table 1). In primiparous buffaloes, 1.6 quarters on average per animal were infected. Of those IMI, 22.0% were caused by *Staph. rostri*. A similar pattern was observed in multiparous buffaloes, where 1.4 quarters on average per animal were infected. Of those IMI, 27.9% were caused by *Staph. rostri*.

On at least one sampling, each buffalo yielded hemolytic gram-positive and catalase-positive bacteria in at least one quarter. All these isolates ($n = 37$) were coagulase-negative and were therefore considered to be different from *Staph. aureus*. Identification with API Staph ID 32 test (bioMérieux, Marcy l'Etoile, France) was attempted for 2 of those isolates. Isolate 165 gave a profile ascribed to *Staphylococcus simulans* with a probability of 93%; isolate 168 was identified with wide uncertainty as either *Staph. simulans* or *Staphylococcus chromogenes* with a probability of 48.9 and 42.7%, respectively. The unsatisfactory identification of those

Table 1. Prevalence and distribution of mastitis pathogens isolated from quarters of 11 water buffaloes throughout 1 lactation

Item	Samples (n)	% pos _{tot} ¹	% pos _{inf} ²
Noninfected	491	75.8	—
Infected	143		
CNS (other than <i>Staphylococcus rostri</i>)	76	11.7	53.1
<i>Staph. rostri</i>	33	5.1	23.1
<i>Corynebacterium bovis</i>	11	1.7	7.7
<i>Staphylococcus aureus</i>	1	0.1	0.7
<i>Streptococcus uberis</i>	2	0.3	1.4
<i>Streptococcus</i> spp. (other than <i>Strep. uberis</i>)	9	1.4	6.3
Other esculine-positive cocci	5	0.8	3.5
<i>Escherichia coli</i>	1	0.1	0.7
Other gram-negative bacteria	3	0.5	2.1
<i>Proteus</i>	2	0.3	1.4
Contaminated	14	2.2	—

¹Percentage quarters of all sampled quarters ($n = 648$).

²Percentage quarters of all infected quarters ($n = 143$).

2 isolates, together with the results from Sampimon et al. (2009) on bovine isolates, made us decide to abandon phenotypic methods and apply genotypic ones, previously proposed as the gold standard (Zadoks and Watts, 2009). Thirty-seven isolates were subjected to tDNA-PCR and compared with its library consisting of both reference strains and field isolates originating from bovine milk and teat apices (Supré et al., 2009). The majority of isolates clustered together, indicating they represented the same species; although no exact identification could be made as the species, *Staph. rostri* was not included in the tDNA-PCR library at that time. Then, sequencing of the *rpoB* gene was performed followed by BLAST analysis (<http://blast.ncbi.nlm.nih.gov>). In the case of one isolate, the *rpoB* amplification failed and another showed a 99% identity with the sequences of 3 *Staphylococcus* species (*Staphylococcus muscae*, *Staphylococcus microti*, and *Staph. rostri*) present in National Center for Biotechnology Information database, precluding identification. Neither isolate was examined further and remained unidentified. In total, 33 isolates were identified as *Staph. rostri*, whereas 2 others were identified as *Staphylococcus epidermidis*. Afterward, the tDNA-PCR library was updated with the species *Staph. rostri*.

Antimicrobial Susceptibility

None of the *Staph. rostri* isolates displayed resistance to the antimicrobial agents. Furthermore, their MIC was below the lowest concentration for 18 antibiotics. For chloramphenicol, the majority of the isolates (n = 28) had an MIC equal to 8 µg/mL, twice the lowest concentration tested. One isolate demonstrated an MIC of 16 µg/mL, which is classified as an intermediate MIC breakpoint (Table 2). For ceftiofur, 6 isolates had an MIC equal to 0.5 µg/mL, which is also twice the lowest concentration tested, but for this antibiotic we should consider that an MIC of 0.5 µg/mL is still very low and far away from the resistance breakpoint of 8 µg/mL (Table 2).

Udder Health

The overall geometric mean qSCC throughout lactation at sampling was 20,970 cells/mL (interquartile range = 7,000 to 54,000). Quarter milk SCC at sampling first increased from 62,177 cells/mL between 15 and 46 DIM to 77,478 cells/mL between 45 and 75 DIM, then steeply decreased to 14,013 cells/mL at 166 to 195 DIM, and again increased up to 46,525 cells/mL

Table 2. Minimum inhibitory concentrations of the 18 antimicrobial agents tested for the 33 *Staphylococcus rostri* isolates

Antimicrobial ¹	Number of <i>Staph. rostri</i> isolates with MIC (µg/mL) of											
	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
Ampicillin			33									
Oxacillin + 2%NaCl			33									
Amoxicillin/clavulanic acid ²							33					
Amikacin							33					
Cefpodoxime						33						
Ticarcillin/clavulanic acid constant 2 ³								33				
Trimethoprim/sulfamethoxazole ²				33								
Cefazolin							33					
Gentamicin					33							
Imipenem					33							
Clindamycin			33									
Penicillin	33											
Doxycycline						33						
Ceftiofur			27		6							
Marbofloxacin			33									
Enrofloxacin			33									
Erythromycin				33								
Chloramphenicol							4		28	1		

¹The dilution ranges tested for each antibiotic are those within the shaded area. Values situated below this range indicate the number of isolates with MIC values below the lowest concentration tested. Resistance breakpoints are indicated with a vertical line to the right of the breakpoint value.

²Concentration given for amoxicillin and trimethoprim with a ratio respectively of 2:1 and 1:19.

³Concentration given for ticarcillin, clavulanic acid concentration is 2 µg/mL in all dilutions.

Table 3. Final linear mixed regression model for the natural log-transformed quarter milk SCC as outcome variable

Independent variable	Observations (no.)	β	SE	LSM	P-value
Sampling (maximum 17 samplings per quarter) ¹	—	—	—	—	0.009
Infection status					0.001
Noninfected	491	Referent	—	3.01	—
CNS (other than <i>Staphylococcus rostri</i>)	76	0.54	0.16	3.55	0.001
<i>Staph. rostri</i>	33	0.53	0.27	3.54	0.020
<i>Streptococcus</i> spp.	11	0.53	0.27	3.54	0.050
Other bacteria	23	0.48	0.38	3.49	0.200
DIM					0.034
<45	59	Referent	—	4.13	—
46–75	92	0.22	0.36	4.35	0.530
76–105	80	−0.56	0.49	3.62	0.257
106–135	80	−1.35	0.61	2.77	0.025
135–165	96	−1.20	0.67	2.92	0.075
166–195	76	−1.50	0.74	2.63	0.043
196–225	75	−0.97	0.79	3.16	0.221
>225	120	−0.23	0.84	3.89	0.785

¹Estimates are not shown.

at the end of the lactation (Table 3). No substantial differences in quarter milk SCC were observed between primiparous (32,459 cells/mL) and multiparous animals (27,938 cells/mL; $P = 0.770$). Quarter milk SCC at sampling of quarters infected with *Staph. rostri* (34,466 cells/mL) and quarters infected with CNS other than *Staph. rostri* (34,813 cells/mL) was significantly higher than in noninfected quarters ($P = 0.001$ and $P = 0.020$, respectively). The difference in qSCC at sampling between quarters that were infected with *Staph. rostri* at a certain sampling and quarters that were infected with CNS other than *Staph. rostri* was not significant (Table 3). No significant differences in qSCC were observed between quarters infected with either CNS or *Staph. rostri*, in particular, and quarters infected with *Streptococci* spp. (34,813 cells/mL). No clinical mastitis was detected during the study period.

DISCUSSION

The CNS group isolated from the milk of dairy cows consists of more than 50 different species and subspecies, of which approximately a dozen are commonly found in milk from cows (Piessens et al., 2011; Supré et al., 2011). The epidemiology and relevance of this group as a cause of bovine mastitis is currently under debate (De Vliegher et al., 2009). However, CNS have become the predominant bacteria found in dairy cows' milk samples in many areas around the world (Makovec and Ruegg, 2003; Pitkälä et al., 2004; Piepers et al., 2007), and are highly prevalent in the milk from fresh heifers (De Vliegher et al., 2012) as well as in milk from water buffaloes (Moroni et al. 2006). This is the first paper that reports on the presence of the newly described CNS species *Staph. rostri* in animals other than pigs and more specifically in the milk of buffaloes. The

precise longitudinal data, including quarter level data together with the molecular identification of 37 isolates, allowed us to estimate the share of *Staph. rostri* as a cause of CNS IMI in buffaloes as well as to explore their relevance for the buffaloes' udder health throughout lactation compared with other CNS. The superiority of molecular tools over phenotypic ones in correctly recognizing pathogens, and CNS in particular, at the species level was again confirmed, although the number of tested isolates was very limited. As was expected based on the findings in dairy cows (Sampimon et al., 2009), a phenotypic approach through API Systems seemed unsuitable for an accurate identification of the different CNS isolates in water buffaloes.

In this study, single quarter milk samples were used to determine the infection status. Previous studies have shown that single samples are often in agreement, and that the sensitivity of a single sample is better than that of duplicate samples interpreted in series and comparable to that of triplicate samples, with only a marginal loss in specificity (Dohoo et al., 2011a). A more stringent definition for CNS IMI compared with IMI caused by other bacteria was selected to avoid false-positive results (i.e., an expected increase of Sp from 87% to more than 95%), as these organisms commonly occur on teat skin (Braem et al., 2012).

Similar to previous findings (Moroni et al., 2006), the vast majority of IMI were caused by CNS. *Staphylococcus rostri* accounted for almost one-third of CNS IMI. We attempted to identify the latter with tDNA-PCR, recognized as a highly accurate molecular tool for the identification of CNS in dairy cows (Supré et al., 2009). However, the current library did not include *Staph. rostri*, a recently described species colonizing the noses of healthy pigs. Sequencing of the *rpoB* housekeeping gene was necessary to arrive at the correct identification.

Antimicrobial susceptibility profiles were available as well. Besides the data provided by Stegmann and Perreten (2010), no other information is available on the antimicrobial susceptibility of *Staph. rostri*. The *Staph. rostri* isolates originating from the nasal cavities of healthy pigs showed resistance against multiple commonly used antibiotics (i.e., tetracycline, streptomycin, and trimethoprim). In our study, all *Staph. rostri* isolates were susceptible to the most commonly used antimicrobials. For chloramphenicol, a less commonly used antimicrobial in the water buffalo industry, one isolate was classified as intermediately susceptible with an MIC of 16 µg/mL. An identical MIC was found by Stegmann and Perreten (2010) for 2 isolates from pigs. The reduction of susceptibility to this drug in one *Staph. rostri* isolate was quite peculiar because chloramphenicol has been banned in EU since 1994 in any food-producing animals. Furthermore, other drugs derived from chloramphenicol, such as florfenicol and tiamfenicol, were not used on this farm. Of course, the resistant bacteria might have been introduced via purchased animals or via contact with other species, such as pets. Interestingly, the isolate showed no resistance to any of the other antibiotics. In some bacteria, such as *Salmonella*, multidrug resistance to several antibiotics, including chloramphenicol, frequently occurs (Gebreyes et al., 2004). The discrepancy between our results and the findings on porcine isolates of Stegmann and Perreten (2010) could be due to the higher burden of antimicrobial usage in swine compared with dairy farming, although this needs confirmation. Still, CNS other than *Staph. rostri* isolated from cases of (sub)clinical mastitis in Brazilian buffalo showed varying levels of resistance to antibiotics (de Medeiros et al., 2011). In our study, the antimicrobial resistance patterns from CNS other than *Staph. rostri* were not determined. One should also take into account that the different *Staph. rostri* isolates might have belonged to one subtype, different from the isolates found by de Medeiros et al. (2011). Still, the latter hypothesis could not be confirmed nor refuted as the different isolates were not further strain-typed in our study.

The relation between IMI caused by *Staph. rostri* and qSCC was precisely studied. The hierarchical structure of the longitudinal data was taken into account when estimating the effect of CNS IMI on the qSCC. A random buffalo effect and repeated quarter effect were included in the model to correct for potential differences in qSCC between buffaloes and between quarters within a buffalo, irrespective of the IMI-status. The overall qSCC in our study was lower compared with the findings of a previous study that focused on water buffalo milk (Moroni et al., 2006). The qSCC in infected

quarters was higher than in noninfected quarters, as was expected based on previous findings (Moroni et al., 2006), although the difference was less pronounced than in dairy cattle. Also different from what we are used to in dairy cattle, CNS IMI increased qSCC to a same extent as *Streptococci* spp. No differences in qSCC were observed between *Staph. rostri* IMI and IMI caused by other CNS.

Staphylococcus rostri might be widely spread among buffaloes, although a herd-specific distribution, as has recently been described for CNS species isolated from cows' milk, should be taken into consideration (Piesens et al., 2011; Supré et al., 2011) but needs confirmation. To our knowledge, *Staph. rostri* has previously only been isolated from the nasal cavities from healthy pigs (Riesen and Perreten, 2010; Vanderhaeghen et al., 2012). The buffalo farm included in this study had no epidemiological relationships with pig holdings, making transmission of the *Staph. rostri* species from pigs to buffaloes unlikely.

The findings of the current study provide novel insights into the epidemiology and antimicrobial susceptibility of *Staph. rostri* involved in water buffalo IMI. Further surveys are needed to pinpoint the relevance, niches, and transmission routes of CNS in general and *Staph. rostri* in particular in water buffaloes.

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