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Clinical outcomes and molecular genotyping of *Staphylococcus aureus* isolated from milk samples of dairy primiparous Mediterranean buffaloes (*Bubalus bubalis*)

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ABSTRACT

Staphylococcus aureus is one of the most important pathogens causing mastitis in dairy cows and in Mediterranean buffaloes. Genotype B (GTB) is contagious in dairy cows and may occur in up to 87% of cows of a dairy herd. It was the aim of this study to evaluate genotypes present, clinical outcomes, and prevalence of *Staph. aureus* in milk samples of primiparous Mediterranean dairy buffaloes. Two hundred composite milk samples originating from 40 primiparous buffaloes were collected from May to June 2012, at d 10, 30, 60, 90, and 150 d in milk (DIM) to perform somatic cell counts and bacteriological cultures. Daily milk yields were recorded. Before parturition until 40 to 50 DIM, all primiparous animals were housed separated from the pluriparous animals. Milking was performed in the same milking parlor, but the primiparous animals were milked first. After 50 DIM, the primiparous were mixed with the pluriparous animals, including the milking procedure. Individual quarter samples were collected from each animal, and aliquots of 1 mL were mixed and used for molecular identification and genotyping of *Staph. aureus*. The identification of *Staph. aureus* was performed verifying the presence of *nuc* gene by *nuc* gene PCR. All the *nuc*-positive isolates were subjected to genotype analysis by means of PCR amplification of the 16S-23S rRNA intergenic spacer region and analyzed by a miniaturized electrophoresis system. Of all 200 composite samples, 41 (20.5%) were positive for *Staph. aureus,* and no genotype other than GTB was identified. The prevalence of samples positive for *Staph. aureus* was 0% at 10 DIM and increased to a maximum of 22/40 (55%) at 90 DIM. During the period of interest, 14 buffaloes tested positive for *Staph. aureus* once, 6 were positive twice, and 5 were positive 3 times, whereas 15 animals were negative at every sampling. At 90 and 150 DIM, 7 (17.5%) and 3 buffaloes (7.5%), respectively, showed clinical mastitis (CM), and only 1 (2.5%) showed CM at both samplings. At 60, 90, and 150 DIM, 1 buffalo was found with subclinical mastitis at each sampling. At 30, 60, 90, and 150 DIM, 2.5 (1/40), 22.5 (9/40), 35 (14/40), and 10% (4/40) were considered affected by intramammary infection, respectively. Buffaloes with CM caused by *Staph. aureus* had statistically significantly higher mean somatic cell count values (6.06 ± 0.29, Log_{10} cells/mL ± standard deviation) and statistically significantly lower mean daily milk yields (7.15 ± 1.49, liters/animal per day) than healthy animals (4.69 ± 0.23 and 13.87 ± 2.64, respectively), buffaloes with IMI (4.82 ± 0.23 and 11.16 ± 1.80, respectively), or with subclinical mastitis (5.47 ± 0.10 and 10.33 ± 0.68, respectively). Based on our knowledge, this is the first time that *Staph. aureus* GTB has been identified in milk samples of dairy Mediterranean buffaloes.

Key words: *Staphylococcus aureus*, genotype B, mastitis, Mediterranean buffalo, udder health

INTRODUCTION

Staphylococcus aureus is one of the most important pathogens, causing chronic, clinical, and mainly subclinical mastitis in dairy cows all over the world (Sears and McCarthy, 2003). Considering the negative effect of *Staph. aureus* on milk quality and yield, it is recognized as a cause of great economic loss for the dairy industry (Barkema et al., 2006, 2009; Keefe, 2012). It has been demonstrated that *Staph. aureus* isolated from mastitis milk of dairy cows represents a genetically heterogeneous group of bacteria (Fournier et al., 2008). By means of PCR amplification of the 16S-23S rRNA

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intergenic spacer region (ribosomal spacer-PCR), an accurate and repeatable method to subtype *Staph. aureus*, a total of 17 genotypes were detected in 101 epidemiologically independent isolates (Graber et al., 2009). Genotype B (**GTB**) and C were the most common (80.2%), whereas the other 15 genotypes occurred rarely. It was further demonstrated that the genotypes were highly associated with their virulence gene pattern (Fournier et al., 2008; Graber et al., 2009). Actually, more than 20 different genotypes of *Staph. aureus* with different virulence and pathogenicity factors were identified in mastitis milk of dairy cows, originating from a variety of European countries (Anderson et al., 2012; Cosandey and Graber, 2013; Lundberg et al., 2014). Some *Staph. aureus* strains were recognized as contagious pathogens because they can spread from infected to noninfected cows at milking (Bartlett and Miller, 1993; Fox and Gay, 1993; Sears and McCarthy, 2003). Genotype B was related to contagiousness and increased pathogenicity, causing herd problems with a within-herd prevalence of up to 87%, and often 2 or more quarters were affected per cow. By contrast, other genotypes were found in samples of single cows (ranging from 4 to 33.3%) with only 1 quarter per cow infected (Michel et al., 2011).

As in dairy cows, bacterial mastitis is 1 of the most costly diseases also in dairy Mediterranean buffaloes (Galiero, 2002; Fagiolo and Lai, 2007), mainly in primiparous animals, where the highest incidence of mastitis was reported during the first 2 mo of milking (Dhakal et al., 2008). In dairy buffaloes, *Staph. aureus* is the most problematic and significant contagious pathogen exhibiting strains particularly resistant to antibiotics (Fagiolo and Lai, 2007; Guccione et al., 2014). *Staphylococcus aureus* typically colonizes the injured skin. Damage of the teat end and faulty milking encourages migration of bacteria into the udder, and *Staph. aureus* may remain viable in affected buffalo farms for an extended period of time (Fagiolo and Lai, 2007). According to Trinidad et al. (1990), even mild IMI due to *Staph. aureus* can cause damage to the developing secretory tissue that can lead to decreased milk yield followed by increased SCC. Somatic cell count is usually used as indicator of inflammation to diagnose mastitis (Dhakal et al., 1992; Singh and Ludri, 2001; Moroni et al., 2006), and, according to Cerón-Muñoz et al. (2002), elevated SCC values were related to reduced lactose concentration and milk yield. According to several studies in buffaloes, quarters producing milk with an SCC below the threshold of 200×10^3 cells/mL and associated with negative bacteriological cultures (**BC**) are considered healthy; quarters with analogous SCC values and positive BC caused by udder-specific pathogens are considered affected by IMI. Furthermore, buffaloes producing milk with an SCC upon the threshold of 200×10^3 and positive BC are defined as suffering from subclinical mastitis (**SM**), whereas in cases of clinical mastitis (**CM**), obvious signs of disease involving the appearance of the milk or udder are evident (Moroni et al., 2006; Tripaldi et al., 2010). For commercial purposes, the European Union Directives (46/92 and 71/94) set an upper limit of 400×10^3 cells/mL for the SCC of buffalo milk to be used for products made from raw milk intended for human consumption (Moroni et al., 2006).

In both buffaloes and cattle, clinical suspicion for the presence of mastitis due to *Staph. aureus* is based on a combination of the following findings: visible changes of milk, atrophy or presence of indurations of the affected quarter (Radostits et al., 2007), presence of certain risk factors such as teat lesions or callused teats (Sieber and Farnsworth, 1984; Zadoks et al., 2001), positive result of the California Mastitis Test (**CMT**), and elevation of the SCC (Schukken et al., 2003). Confirmation or exclusion is based on BC of one or, preferably, several consecutive milk samples (Sears and McCarthy, 2003). The *nuc* gene is highly specific for *Staph. aureus* (Brakstad et al., 1992; Graber et al., 2007) and may, therefore, be used for definitive identification of suspicious colonies (Syring et al., 2012).

Although *Staph. aureus* is considered one of the most important udder-specific pathogens in Mediterranean buffaloes (Fagiolo and Lai, 2007), published studies exploring the role of *Staph. aureus* as udder pathogen in first-lactation dairy buffaloes are rare. Therefore, the aims of the present study were to (1) evaluate the clinical outcomes and prevalence of *Staph. aureus* IMI in the primiparous animals of a Mediterranean buffalo herd, (2) identify the genotypes present, (3) describe the dynamics of *Staph. aureus* IMI over repeated samplings within the first 150 DIM, (4) follow the prevalence of udder-specific bacteria other than *Staph. aureus*, and (5) elucidate the potential pathway of infection of primiparous animals.

MATERIALS AND METHODS

Study Design, Animals, and Farm Management

For our study, 40 primiparous and 20 pluriparous Mediterranean buffaloes were used. They were all reared in a breeding farm of 600 dairy buffaloes, located in the Caserta district in southern Italy, and were chosen by convenience sampling between May and June 2012. Before parturition until 40 to 50 DIM, all primiparous animals were housed separated from the pluriparous animals. Milking was performed in the same milking parlor, but the primiparous animals were milked first.

After 50 DIM, the primiparous were mixed with the pluriparous animals, including the milking procedure.

Milk Samples

Milk samples were collected aseptically in sterile test tubes immediately before regular evening milking, as described by National Mastitis Council (2004) for dairy cows. At 10, 30, 60, 90, and 150 DIM, 2 samples from each quarter of the 40 primiparous were collected. Milk samples were created by mixing equal amounts of milk from all 4 quarters into a sterile test tube (BD Vacutainer, Oxford, UK). The first sample (4 mL) was immediately frozen (−20°C) until further processing for molecular genotyping of *Staph. aureus*, whereas the second sample (50 mL) was used to perform BC and determine the SCC. From the 20 pluriparous animals, 1 composite milk sample of 10 mL was collected once aseptically and frozen at −20°C for BC and genotyping of *Staph. aureus* later.

Clinical Examination, CMT, and Electrical Conductivity

On the days of sampling, each individual primiparous buffalo enrolled was individually submitted to a complete clinical examination before evening milking, with a particular focus on udder health status. Local and systemic signs and changes in milk appearance were recorded during each sampling. Buffaloes showing abnormal macroscopic appearance of the milk were also submitted to complete hemato-biochemical analysis. A complete ultrasonographic examination of the 4 teats and mammary glands was also performed in buffaloes with SM or CM, using a 12-MHz linear probe (General Electrics, Logiq E, Milano, Italy). The teats were dipped in warm water to improve image quality as described for the cow by Sendag and Dinc (1999). California Mastitis Test was routinely performed from each composite milk sample, with values $\geq$1 interpreted as positive. Electrical conductivity (**EC**) and daily milk yields were recorded using automatic dedicated software (Afifarm, Afimilk, Kibbutz Afikim, Israel). Based on this classification, the animals enrolled were defined as healthy (**H**) or affected by IMI, SM, or CM, considering the presence of clinical signs, the SCC values, and the microbiological status (Moroni et al., 2006; Fagiolo and Lai, 2007; Tripaldi et al., 2010).

SCC and Bacteriological Milk Cultures

The composite milk samples were placed in a cool box (4°C) and brought to the reference laboratory within 1 h of collection, where they were submitted to SCC analysis and BC within 2 h of collection. Somatic cell count was determined using an automatic and dedicated analyzer, approved for buffalo milk (Fossomatic 5000, Foss Electric, Hillerød, Denmark). The BC was performed according to guidelines of the National Mastitis Council (1999). Briefly, 10 μL of each milk sample was streaked on one quarter of a blood-agar plate (Merck KGaA, Darmstadt, Germany), incubated at 37°C for up to 48 h, and examined after 24 and 48 h of incubation. The identification of *Staph. aureus* and other mastitis pathogens was according to the guidelines of the National Mastitis Council (1999). Bacterial colonies were tentatively identified on gross morphology, and the number and types of colonies were recorded. According to Kreiger et al. (2007), when 3 or more dissimilar colony types were isolated on the plate, the sample was considered contaminated. Appropriate tests were performed with the colonies isolated, where necessary, to identify the pathogens. These included Gram staining, catalase testing to differentiate between streptococci and staphylococci, and tube coagulase testing using rabbit plasma to differentiate between coagulase-positive staphylococci and CNS. A final identification of microorganisms was performed using the colorimetric automated identification system (Vitek 2 XL 120; bioMerieux Inc., Hazelwood, MO), according to the manufacturer's instructions. *Enterobacteriaceae* were grown on MacConkey agar (Oxoid, Basingstoke, UK) and were also identified using the same automated system. Isolates identified with confidence levels greater than 0.90 were considered identified mastitis pathogens at species level. Otherwise, they were identified at genus level.

Identification of Staph. aureus and Molecular Genotyping

Samples for PCR analysis were thawed and 10 μL of milk was streaked both on chrom-agar and on blood-agar plates (bioMérieux Suisse, Geneva, Switzerland) and incubated at 37°C overnight to obtain single colonies typical of *Staph. aureus* and to verify the rate of contamination, respectively. Five colonies of each chrom-agar plate were subcultured on blood-agar plates obtaining the growth of pure strains. The final identification of the *Staph. aureus* was performed verifying the presence of *nuc* gene, coding for the thermonuclease. For this reason, the colonies were resuspended in 100 μL of TEL buffer (10 m*M* Tris/HCl, 10 m*M* EDTA; pH = 8.5), incubated at 95°C for 10 min, and immediately placed on ice. The lysates were then diluted 1:100 in H_2O and served as templates for the different types of PCR. The PCR for the *nuc* gene was performed according to (Syring et al., 2012). Isolates lacking this gene

were excluded from the present study. All the positive isolates were then subjected to genotype analysis according to Fournier et al. (2008).

This method is based on PCR amplification of the 16S-23S rRNA intergenic spacer region. Each reaction contained (total volume = 25 μL) 1× HotStarTaq Master Mix (Qiagen AG, Hombrechtikon, Switzerland), 800 n*M* of each primer (G1 and L1 primer), and 7 μL of diluted lysate. The PCR profile was 95°C for 15 min, followed by 27 cycles at 94°C for 1 min, followed by a 2-min ramp, and annealing at 55°C for 7 min. After a further 2-min ramp, extension was done at 72°C for 2 min. The PCR was terminated by a final extension at 72°C for 10 min followed by cooling down to 4°C. Negative and positive controls were included in every run. For the negative control, sterile water was added instead of nucleic acids. For positive control, we used bovine strains positive for *Staph. aureus* GTB.

The PCR products of the 16S-23S rRNA intergenic spacer region were analyzed by the miniaturized electrophoresis system DNA 7500 LabChip (Agilent Technologies, Santa Clara, CA). The electropherograms were monitored online on a personal computer, evaluated and translated into a pseudo-gel by Agilent Technologies software. For interpretation of the 16S-23S rRNA intergenic spacer region results, 2 patterns (genotypes) were considered different if 2 and more peaks of the electropherogram differed in size. To identify the genotypes, a recently developed in-house computer program was applied (Syring et al., 2012).

Statistical Analysis

Different animal health status, SCC, and milk yields were analyzed by standard descriptive statistics, and normality was assessed using histograms, normal probability plots and Shapiro Wilk tests. Data were expressed as absolute numbers, percentage, or mean ± SD. Somatic cell counts were expressed as $\log_{10}$ (cells/mL). Untransformed and log-transformed variables were described using box plots and compared using parametric tests (Student's *t*-test). Probabilities <0.05 were considered significant. All the statistical data were analyzed using dedicated software (Version 17.0, SPSS, Chicago, IL).

RESULTS

Clinical and Ultrasonographic Findings

Overall appearance of depression, udder swelling and pain, off-color and watery appearance of milk, and presence of flakes, clots, or pus were detected during the clinical examination in CM quarters. A slight neutrophilia (data not shown) and obvious ultrasonographic abnormalities were always found in all the examined mastitic buffaloes. Ultrasonographically, partial teat canal occlusions due to fibrin deposits and irregular outlines of the teat cisterns were observed in buffaloes affected by CM.

CMT, SCC, and EC

During the current study, the CMT was always positive in animals with CM and SM due to *Staph. aureus*; it was negative in 62.2% and positive in 37.8% of animals with IMI, whereas it was negative in 82.4% and positive in 17.6% of H buffaloes. The SCC values ranged from 4.28 to 5.24 Log_{10} cells/mL (mean = 4.69 ± 0.23 cells/mL) in H buffaloes and from 4.38 to 6.53 Log_{10} cells/mL (mean = 5.10 ± 0.55 cells/mL) in buffaloes that tested positive for *Staph. aureus*. Statistically significant differences were found between the 2 groups ($P < 0.001$). The EC values ranged from 6.90 to 10.40 mS/cm (mean = 8.29 ± 0.61 mS/cm) in H buffaloes and from 7.80 to 14.00 mS/cm (mean = 8.69 ± 1.30 mS/cm) in those positive for *Staph. aureus*.

BC and Staph. aureus Genotyping

The prevalence of the bacteria isolated during the present study is indicated in Table 1. Of all samples, 41/200 (20.5%) primiparous and 12/20 (60%) pluriparous animals were positive for *Staph. aureus*. Without any exception, all *Staph. aureus* strains isolated were identified as GTB. The prevalence of *Staph. aureus* was 0% (0/40) at 10 DIM and increased to 2.5 (1/40), 25 (10/40), 55 (22/40), and 20% (8/40) at 30, 60, 90, and 150 DIM, respectively (Table 1). During the period of interest, 14 buffaloes tested positive for *Staph. aureus* once, 6 were positive twice, and 5 were positive 3 times, whereas 15 animals were always negative for *Staph. aureus*. No co-infection between *Staph. aureus* and other udder-specific pathogens was recorded at 30 and 150 DIM; 1/10 (10%) of samples showed a co-infection (*Streptococcus uberis*) at 60 DIM, and 4/22 (18.2%) samples at 90 DIM [*Staphylococcus* spp. (1); *Streptococcus dysgalactiae* (1); other bacteria (2)]. All of these co-infected samples (5/32) became mono-infected by *Staph. aureus* in the consecutive sampling. Regarding pluriparous buffaloes, no co-infection between *Staph. aureus* and other udder specific bacteria were detected.

Of all samples of primiparous buffaloes, 79/200 (39.5%) were positive for other udder-specific bacteria except *Staph. aureus*. The prevalence of these positive samples increased from 8/40 (20%) at 10 DIM to 28/40 (70%) at 30 DIM and then steadily decreased to 25/40 (62.5%) at 60 DIM to 15/40 (37.5%) at 90 DIM and

Table 1. Prevalence (%; no. in parentheses) of udder-specific pathogens isolated from 4 quarter composite milk samples of 40 primiparous buffaloes at 10, 30, 60, 90, and 150 DIM

	DIM				
Bacteria	10	30	60	90	150
Staphylococcus aureus	0	2.5 (1)	25 (10)	55 (22)	20 (8)
Staphylococci	7.5 (3)	22.5 (9)	25 (10)	22.5 (9)	2.5 (1)
Streptococcus uberis	—	7.5 (3)	10 (4)	—	—
Streptococcus dysgalactiae	2.5 (1)	15 (6)	—	2.5 (1)	—
Escherichia coli	5 (2)	2.5 (1)	10 (4)	5 (2)	—
Other bacteria	5 (2)	22.5 (9)	17.5 (7)	7.5 (3)	5 (2)

3/40 (7.5%) at 150 DIM. *Staphylococcus* spp. and other bacteria were identified at every sampling date (Table 1). The number of negative samples recorded during the whole study was 80/200 (40%).

Classification of Udder Health Status

Clinical mastitis due to *Staph. aureus* was present in 17.5% (7/40) of buffaloes at 90 DIM, and 7.5% (3/40) at 150 DIM; 1 buffalo showed CM both at 90 and 150 DIM. One buffalo showed SM at 60, 90, and 150 DIM. In all CM and SM due to *Staph. aureus*, no co-infections with other udder-specific pathogens were found. Intramammary infection was diagnosed in 2.5 (1/40), 25 (10/40), 35 (14/40), and 10% (4/40) of buffaloes at 30, 60, 90, and 150 DIM, respectively. No IMI was diagnosed at 10 DIM.

Association Between SCC and Udder Health Status

The mean values of SCC detected in buffaloes affected by CM, SM, IMI due to *Staph. aureus*, or H were 6.06 ± 0.29, 5.47 ± 0.10, 4.82 ± 0.23, and 4.69 ± 0.23 Log_{10} cells/mL (±SD), respectively. Statistically significant differences were found between mean values of SCC in CM and SM ($P = 0.0002$), between CM or SM and IMI ($P < 0.0001$), and between CM or SM and H buffaloes ($P < 0.001$; Figure 1).

Association Between EC and Udder Health Status

Regarding the EC, the mean values recorded in animals affected by CM, SM, IMI due to *S. aureus*, or H were 10.49 ± 1.94, 8.37 ± 0.50, 8.21 ± 0.28, and 8.29 ± 0.61 mS/cm, respectively. Statistically significant differences of EC were observed between buffaloes with CM as compared with those with SM, IMI, or H ($P < 0.01$).

Association Between Udder Health Status and Milk Yield

Buffaloes with CM caused by *Staph. aureus* had significantly lower mean daily milk yields (7.15 ± 1.49, L/animal per day) as compared with H animals (13.87 ± 2.64 L/animal per day; $P < 0.0001$), buffaloes with IMI (11.16 ± 1.80 L/animal per day; $P < 0.0001$), and those with SM (10.33 ± 0.68 L/animal per day; $P = 0.001$; Figure 2).

DISCUSSION

The current study evaluated the clinical outcomes and prevalence of *Staph. aureus* in milk samples of dairy primiparous Mediterranean buffaloes, simultaneously performing molecular genotyping of the isolated strains. The importance of *Staph. aureus* as a contagious pathogen has been widely recognized both in dairy cows and buffaloes, but to our knowledge no previous studies have investigated its role in buffalo herds and its genetic characteristics.

The molecular analysis performed in the present study revealed that GTB was the only *Staph. aureus* strain

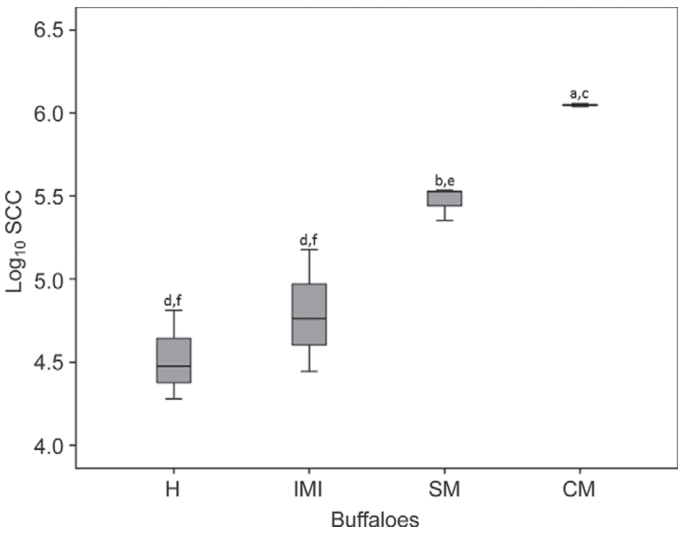

Figure 1. Mean Log_{10} SCC values of milk samples in 40 primiparous buffaloes with 4 different udder health statuses: H = healthy; IMI = intramammary infection; SM = subclinical mastitis; CM = clinical mastitis. Statistically significant differences were found between animals with different udder health statuses. a,b: $P < 0.001$, c,d: $P < 0.0001$; e,f: $P < 0.001$.

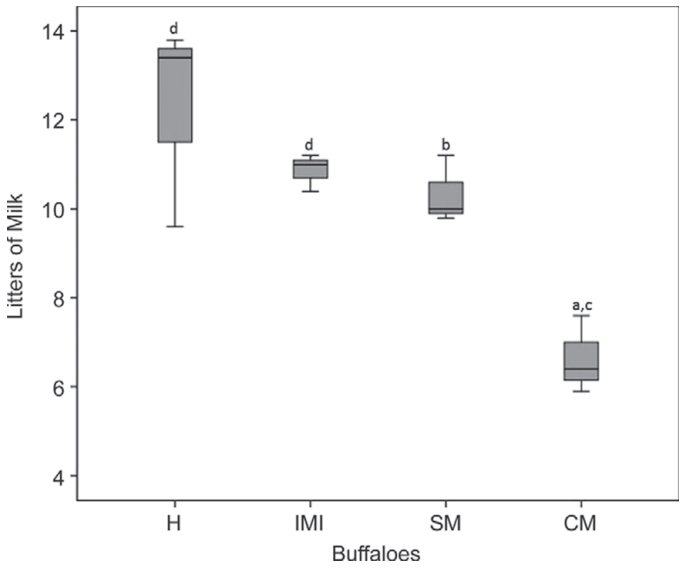

Figure 2. Mean milk yields (L) in 40 primiparous buffaloes with 4 different udder health statuses: H = healthy; IMI = intramammary infection; SM = subclinical mastitis; CM = clinical mastitis. Statistically significant differences were found between animals with different udder health statuses. a,b: $P = 0.001$; c,d: $P < 0.0001$.

isolated from samples collected both in primiparous and pluriparous buffaloes; analogous studies performed in cows confirmed the high prevalence of GTB-positive samples, showing similar within-herd prevalence (up to 96.1%; Graber et al., 2009). To our knowledge, our report is the first in which isolation of *Staph. aureus* GTB from milk samples of dairy primiparous Mediterranean buffaloes was described. Its contagiousness and ability to produce within-herd problems, as well as its virulence due to its gene patterns (enterotoxin genes A and D and polymorphic leucotoxin E gene), were widely described not only in Swiss dairy herds (Fournier et al., 2008; Syring et al., 2012) but also in dairy cows of several European countries (Cosandey and Graber, 2013).

Staphylococcus aureus is one of the most important and best-studied pathogens causing mastitis in dairy cows (Schällibaum, 1999; Zecconi et al., 2005). According to our results, the prevalence of animals with udder health problems (buffaloes affected by IMI, SM, and CM) caused by the bacterium (up to 55%; Table 1) as well as the incidence of positive samples for *Staph. aureus* collected during the whole sampling period (20.4%) were lower than those described for cows (Michel et al., 2011). Nevertheless, considering the prevalence of CM, SM, and IMI due to the bacterium, as well as its relationship with other udder-specific pathogens isolated in the present study (Table 1), *Staph. aureus* confirmed its pathogenicity in Mediterranean buffaloes and it can be considered one of the most important pathogens causing within-herd problems in this dairy species.

Staphylococcus aureus is globally recognized for its relevant economic loss for farmers and the dairy industry due to its negative influence on milk yield (Sears and McCarthy, 2003; Seegers et al., 2003). Once established in the udder, *Staph. aureus* can persist through the precalving period and into the first lactation (Halasa et al., 2007; Hogeveen et al., 2011). According to Trinidad et al. (1990), even mild IMI can lead to decreased milk production followed by increased SCC because of damage to the developing secretory tissue. Secretory tissue is replaced by connective tissue having a deleterious effect on future milk yield (Kreiger et al., 2007). Additional economic losses in dairy cows are also attributed to a compromise of the milk quality due to increased SCC levels (Michel et al., 2011). In the present study, milk production was observed to progressively decline from H to IMI, SM, and CM (Figure 2). The difference was statistically significant between H and CM buffalo ($P < 0.0001$). The results of the current study showed that IMI caused by *Staph. aureus* GTB in Mediterranean buffalo is associated with significant losses in milk and mozzarella cheese production. This is supported by the results of a recent study performed in Mediterranean buffaloes in which IMI due to udder-specific pathogens led to a SCC above the threshold of 200×10^3 cells/mL, which is associated both with significantly decreased milk yields and poor milk quality due to changes in its composition and coagulating properties (Tripaldi et al., 2010).

Regarding the mean SCC values observed in buffaloes, IMI due to *Staph. aureus* mono-infection revealed lower SCC mean values in Mediterranean buffaloes (4.82 ± 0.23 Log_{10} cells/mL) as compared with those detected for cows (5.97 ± 0.49 Log_{10} cells/mL; Graber et al., 2009). Interactions among infection, inflammatory processes, and immune responses in individual udder quarters are still controversially discussed in dairy cows (Schwarz et al., 2011), whereas in dairy buffalo no complete studies are present to explain the relationship. Different leukocyte behavior during the initial phase of the inflammation and stronger pressure from udder pathogenic microorganisms of the environment or stressful events (i.e., kicking during premilking preparation of the udder or during taking the quarter foremilk samples) might have triggered the elevation of the SCC and may explain the difference observed between the 2 species.

Regarding the epidemiological properties of *Staph. aureus*, the prevalence of IMI at several DIM ranged between 2.5 (1) and 35% (14), with peaks of infections detected between 60 and 90 DIM; CM and SM were recorded between 60 and 150 DIM. The higher prevalence of IMI due to *Staph. aureus* GTB observed could contribute to a significant increase of clinically evident

udder disorders between 90 (17.5%) and 150 (7.5%) DIM. These results confirm that *Staph. aureus* GTB is not only contagious, but it is often associated with a clear pathological state. Indeed, abnormal SCC values were recorded in animals affected by SM (5.47 ± 0.10 Log_{10} cells/mL) and CM (6.06 ± 0.29 Log_{10} cells/mL; Figure 1); moderate swelling and visible clots (chunks) in the milk, especially in forestripping, represented additional macroscopic evidence associated with a pathological state caused by the bacterium.

According to the current study, the prevalence of other bacteria than *Staph. aureus* strongly declined in the primiparous animals (Table 1) when *Staph. aureus* GTB appeared. A similar phenomenon was observed in herds of dairy cows with IMI due to GTB (Michel et al., 2011). Indeed, cow and quarter prevalence of CNS and streptococci were considerably and significantly lower in herds with GTB IMI than in herds characterized by IMI due to other *Staph. aureus* genotypes (genotype C or the remaining genotypes, GTOG). These results suggest that GTB, as a contagious pathogen, protects the mammary gland from infections due to other mastitis pathogens.

No IMI due to *Staph. aureus* was detected at 10 DIM, whereas a higher prevalence of infection and, consequently, SM and CM were observed between 60 and 150 DIM. These effects may have been caused by mixing the primiparous with the pluriparous animals in the herds, as initiated by the farmer between 40 and 50 DIM. We hypothesized a reservoir role of *Staph. aureus* GTB for the pluriparous animals. The supplementary analysis of 20 pluriparous buffaloes supported this hypothesis, revealing positive results for *Staph. aureus* in 60% of the samples and the presence of the GTB as the only single strain detected in the herd.

Considering the findings of our study and the epidemiological properties of GTB in Mediterranean buffaloes, the following management strategies might be considered. Similar to dairy herds, primiparous and all pluriparous buffaloes that test negative ought to be milked first and those that test positive milked last or with a separate milking unit to avoid further transmission within the herd (Voelk et al., 2014). Additional management strategies might be beneficial, including (1) treating or culling of affected animals, (2) initiating biosecurity measures and testing of buffaloes originating from other herds (Berchtold et al., 2014; Voelk et al., 2014), and (3) implementing and maintaining hygienic milking practices, such as wearing gloves, forestripping, teat cleaning, and postmilking teat disinfection (Barkema et al., 2009; Dufour and Dohoo, 2013). Complete sanitation of the herd might be very costly and time consuming, lasting for up to 1 yr, as reported in dairy cattle herds (Kirchhofer et al., 2007).

CONCLUSIONS

The findings of the current study demonstrate for the first time the presence of *Staph. aureus* GTB in dairy Mediterranean buffaloes, and GTB can be considered one of the most important bacteria causing IMI, SM, and CM in this species. The clinical outcomes, the high within-herd prevalence and the negative effect on milk quality and milk yield confirmed that this pathogen is responsible for contagious mastitis and great economic losses in the dairy buffalo industry. Strategies of prevention and control of udder health problems due to *Staph. aureus* GTB are proposed in this study. However, more complete studies about the effectiveness of the proposed biosecurity measures should be performed both in primiparous and pluriparous buffalo herds to improve scientific knowledge focused on dairy buffalo.

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