JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Antimicrobial resistance levels amongst staphylococci isolated from clinical cases of bovine mastitis in Kosovo

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Abstract

Introduction: Mastitis is one of the most frequent and costly disease in cattle. We studied milk samples from cattle with mastitis from farms in Kosovo to identify mastitis-causing pathogens and possible effective antibiotics. Our ultimate goal is to help implement adequate antibiotic management and treatment practices in Kosovo

Methodology: A total of 152 milk samples were collected from cows with clinical mastitis from different farms in Kosovo. After identification of microorganisms, antibiotic susceptibility and the occurrence of enterotoxins was investigated.

Results: Staphylococci were found in 89 samples, of which 58 were coagulase negative and 31 coagulase positive. *S. aureus* was isolated from 27 samples, *S. epidermidis* from 25, and *S. chromogenes* from 15, while other species of staphylococci were isolated from the remaining 22 isolates. Interestingly, the bacterial diversity was different between cows in different periods of lactation and among different breeds. Most of the isolates (76/89) were resistant to two or more antibiotics. The highest resistance was to penicillin and ampicillin (> 65%), followed by tetracycline, oxacillin, streptomycin, chloramphenicol (> 23%), and less than 3% to erythromycin. Of the 89 isolates, 40 produced enterotoxins that were most frequently typed as A and C.

Conclusions: We detected human bacterial pathogens in the cultures of milk samples from cows with mastitis. The isolates demonstrated resistance to two or more antibiotics, some of which are frequently used to treat animal and human infections. We recommend increased control and more stringent use of antibiotics in veterinary as well as human medicine.

Key words: bovine mastitis; staphylococci; molecular identification; antibiotic resistance; enterotoxins.

J Infect Dev Ctries 2016; 10(10):1081-1087. doi:10.3855/jidc.7912

(Received 14 November 2015 – Accepted 26 March 2016)

Introduction

Mastitis is one of the most common diseases in dairy cattle that causes substantial economic losses. Bacterial pathogens (more than 150 species), hygiene, and chemical contaminants have a negative influence on the udder, leading to mastitis, which negatively affects milk quality [1-3]. Streptococci, staphylococci, *Escherichia coli*, as well as other members of the family Enterobacteriaceae are the most common agents of mastitis [4,5]. The toxins producing staphylococci and streptococci are considered the most common bovine mastitis pathogens in many countries [6]. The route of infection is often through intra-mammary penetration by pathogens that originate from the surrounding environment [7]. Staphylococci are grouped into two groups based on their ability to coagulate plasma: coagulase-positive staphylococci (CPS) and coagulase-negative staphylococci (CNS). The first group contains *S. aureus* while the second group contains more than ten different species, including *S. chromogenes*, *S. simulans*, *S. epidermidis*, and *S. saprophyticus*, some of which are considered major mastitis pathogens [6].

Staphylococci, especially *S. aureus*, produce many enterotoxins that are known to cause food poisoning in humans and may be involved in mastitis as well. Genes for the production of these enterotoxins are present in both CNS and CPS [8,9]. Antibiotic treatment is normally used to treat mastitis during lactation and dry periods [10].

In recent years, antibiotic-resistant mastitis-causing pathogens have been a growing concern worldwide [11]. These problematic pathogens have not been thoroughly studied in cows with mastitis in Kosovo. In order to implement adequate management practices to prevent and treat mastitis, it is important to know not only which pathogens cause mastitis, but also which antibiotics the pathogens are sensitive to.

A variety of bacteria have been shown to be involved in mastitis, and the specific infectious agent is influenced by cattle breed, stage of lactation, milk yield, and number of previous lactations [12]. Milk-producing breeds are reported to be more sensitive to mastitis-causing pathogens than breeds intended for meat production [13]. In addition, it has previously been reported that cows in different stages of lactation show different susceptibility to mastitis. Some works have shown that in the early lactation period and at the time of the peak of lactation, cows are more susceptible to infection than animals in mid and late lactation stages. After the third lactation, cows have higher prevalence than those at the first or second lactation [14,15]. Other factors that influence the prevalence of mastitis include feed quality and regime, living conditions, milking process, ruminating, farm facility hygiene, and cow breed [16].

The main objective of this study was to determine the prevalence of intra-mammary pathogens in dairy cows in Kosovo with a special focus on staphylococci (their antimicrobial susceptibility and ability to produce enterotoxins).

Methodology

Sample collection

During the period from January 2013 to May 2014, milk samples (milk and other mammary gland secretion due to udder inflammation) from 152 cows with clinical mastitis were collected from different farms all around Kosovo. Collection of samples was performed according procedures outlined previously [17]. Samples were collected (through milking) into sterile tubes after washing and disinfection of the ostium with iodized alcohol (2.5%), and they were transported under refrigeration (4°C–8°C) in cool boxes with ice packs to the laboratory. In addition, farmers completed a set of questionnaires about cow breed, lactation period, and anamnesis, and the samples were then grouped according to cow breed and lactation period. Milk samples from 152 mastitis-affected cows were collected (one sample from each cow) and then divided into three groups based on the timing of lactation: (i) early lactation period, which starts from the beginning of lactation until the end of week 8 (29 cows); (ii) mid lactation period, which is from week 9 to 22 (82 cows); and (iii) late lactation period, which is from week 23 to the dry period (41 cows) (Supplementary Table 1).

Isolation and identification of bacteria

Tenfold serial dilutions were made from each milk sample. Each dilution was plated onto Baird-Parker agar plates, bile aesculin agar plates, and blood agar plates for isolation of staphylococci, enterococci, and streptococci, respectively, as well as onto Endo agar and sorbitol MacConkey agar plates for Gram-negative bacteria. All bacteriological media used in the study were purchased from Oxoid (Oxoid, Basingstoke, United Kingdom). The plates were incubated at 37°C for 24–48 hours. Total viable counts of staphylococci, streptococci, enterococci, and *E. coli* were determined. Five to ten colonies from each plate were subjected to Gram staining and tested for catalase and coagulase activity using the Staphylotect Dry Spot TestKit (Oxoid, Basingstoke, United Kingdom). The purity of staphylococcal isolates was ensured by their characteristic morphology on mannitol salt agar. Based on the morphological and biochemical tests, all isolates analyzed from each sample were identical. Therefore, only one staphylococcal isolate from each sample was used for further analysis.

DNA isolation, polymerase chain reaction (PCR), and DNA sequencing

Bacterial genomic DNA was extracted with a DNA purification kit as described previously [18]. Briefly, 5 mL of bacterial culture was centrifuged at 6,000 g at 4°C for 5 minutes. The bacterial pellets were re-suspended in 400 μL lysis solution containing 20 μL of RNAase and proteinase K. Cells were then lysed by using FastPrep (Bio101 Savant, Thermo Electron Corporation, Mitford, United States) at 6 m/s for 20 seconds. In addition, DNA was purified using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, United States) according to the manufacturer's instructions. The DNA concentration was determined with a Nano-Drop spectrophotometer (NanoDrop Technologies, Wilmington, USA). The universal primers 11F (50-TAACACATGCAAGTCGAACG-30) and 5R (50-GGTTACCTTGTTACGACTT-30) were used for DNA amplification by PCR [18,19]. The PCR reaction was performed as follows: 95°C for 1 minute, 30 cycles of 95°C for 15 seconds, 54°C for 30 seconds, followed by final polymerization at 72°C for 90 seconds. The PCR product was analyzed by electrophoresis in agarose gel (1.2%). PCR products were then purified with NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) and sequenced by ABI prism 377 DNA sequencing system (Applied Biosystems, Darmstadt, Germany).

Antibiotic resistance and hemolytic analyses

Susceptibility to nine antibiotics, most of which are commonly used in the Balkan region, was determined

using a microtiter plate assay with series of twofold dilutions of the antibiotics [20]. The antibiotics were penicillin G (testing range 0.25–512 μg/mL), ampicillin (0.25–512 μg/mL), chloramphenicol (0.5–64 μg/mL), erythromycin (0.125–1,024 μg/mL), kanamycin (16–8,192 μg/mL), oxacillin (0.125–64 μg/mL), streptomycin (16–2,048 μg/mL), tetracycline (0.25–32 μg/mL), and vancomycin (0.25–32 μg/mL). The minimum inhibitory concentration (MIC) of the antibiotics was calculated after growth in antibiotic containing Mueller-Hinton broth at 35°C [21]. The cultures were measured using microtiter plate reader (Labsystems Ascent Reader MF, Helsinki, Finland) at 620 nm, as has been described previously [22]. *S. aureus* ATCC 25923 was used as positive control. Criteria for antibiotic susceptibility for bacterial status determined according to the Clinical and Laboratory Standards Institute (CLSI) [23]. Due to the lack of CLSI guidelines about streptomycin, the guidelines and process for Gram-positive bacteria was used in this study [23]. Streptomycin and penicillin are commonly used as combination treatment in Kosovo; based on that, the study was done to determine if there was resistance.

Detection of staphylococcal toxin production

Pure cultures were regrown on tryptic soya broth, and enterotoxins were detected using a reversed passive latex agglutination (SET-RPLA) kit without quantification (Oxoid, Basingstoke, United Kingdom). This kit is capable of detecting staphylococcal enterotoxins A, B, C, and D.

Results

Bacterial identification

A total of 152 milk samples were obtained from mastitis-diagnosed cows from different farms. From each sample, 5 to 10 individual bacterial colonies were obtained from the highest dilutions where there was growth. Staphylococci, enterococci, and streptococci were isolated using selective media. Before the selection of representative staphylococcal isolates from each of the milk samples, approximately 5 to 10 different colonies from each sample were Gram stained and tested for hemolysis and catalase production. In the end, one Gram-positive and one Gram-negative isolate was selected from each milk sample, for a total of 167 isolates. Staphylococci were isolated from 89 samples ranging in concentration from 2×10^4 to 3×10^6 CFU/mL. In 81 samples, staphylococci were the only type of bacteria isolated, while in the remaining samples, staphylococci occurred in conjunction with other bacteria (Table 1). Enterococci were isolated in 33 samples at concentrations ranging from 3×10^3 to 6.2×10^6 CFU/mL, and all were esculin positive. Enterococci were isolated in pure culture from 22 milk samples and in mixed cultures with other species in 11 samples (5 samples with Gram-negative, 4 with staphylococci, and 2 with both staphylococci and Gram-negative bacteria) (Table 1). Streptococci were isolated in 14 samples at concentrations ranging from 4×10^3 to 5×10^5 CFU/mL; in 2 samples, they were found in conjunction with staphylococci. Gram-negative bacteria were isolated in 31 samples at concentrations ranging from 22×10^2 to 67×10^5 CFU/mL; in 7 samples, they were found in conjunction with other potential pathogens (Table 1 and Supplementary Table 1).

Enterococci were the dominant isolates (22/29) in samples taken in the early lactation period; 1 isolate of *Staphylococcus* was found in a sample taken in this period (Supplementary Table 1). Unlike the early lactation period, the mid and the late period samples were dominated by staphylococci (in mid lactation, 36 isolates from 62 samples; in late lactation, 50 isolates from 61 samples). Only in a few late and mid lactation samples were enterococci found (5 in early lactation and 6 in mid lactation). Streptococci and Gram-negative bacteria were less abundant and almost equally distributed in all three lactation stages (Table 1 and Supplementary Table 1). The range of total viable counts in the samples of different cows from the three different lactation stages is more or less the same

Table 1. Distribution of bacterial isolates found in milk samples from 152 mastitis-diagnosed cows.

Species	Infected cows (n)	%
Only staphylococci	81	53.3
Only enterococci	22	14.5
Only streptococci	12	7.9
Only Gram-negative	24	15.8
Staphylococci & enterococci	4	
Staphylococci & enterococci & Gram-negative	2	
Enterococci & Gram-negative	5	
Staphylococci & streptococci	2	
Total	**152**	

(Supplementary Table 2). The composition of the bacterial flora in the milk samples differed between the lactation stages and cows of different breeds (Supplementary Table 1). Staphylococci were the most frequent isolates from clinical cases of mastitis. Based on this finding, testing of antimicrobial resistance was confined to the staphylococci that were isolated from cows with mastitis.

16s RNA gene sequencing, coagulase-positive and -negative staphylococci

The majority of isolates from infected cows were staphylococci (89/167 isolates). The diversity of staphylococci found in this study is summarized in Table 2. The 16S rRNA DNA gene sequence analysis is well known for distinguishing staphylococci species [24]. Both 16s rRNA DNA gene sequence analysis and coagulase tests were used to distinguish the staphylococci species. The results showed that among the staphylococcal isolates, 58 were coagulase negative (CNS) and the remaining 31 were coagulase positive (CPS), which is in agreement with a previous work that showed that CNS was the dominant group [24]. The molecular results of the present study showed that *S. aureus* was the most common species among CPS (27/31; 87.1%), while the remaining 4 CPS isolates were identified as *S. hyicus*. Among the 58 CNS isolates, *S. epidermidis* (n = 25) was the dominant species, followed by *S. chromogenes* (n = 15), *S. capitis* (n = 7), *S. saprophyticus* (n = 7), *S. cohnii* (n = 2), and *S. warneri* (n = 2) (Table 2).

Antibiotic susceptibility

In Kosovo, little information is available about antibiotic susceptibility of staphylococci obtained from cows with mastitis. The antibiotic resistance of the staphylococcal isolates against nine different antibiotics was tested, and a high prevalence of resistance was found. Among the 89 staphylococcal isolates, 81 and 61 were resistant to penicillin and ampicillin, respectively, followed by tetracycline (44 isolates), streptomycin (29 isolates), oxacillin (22 isolates), chloramphenicol (21 isolates), kanamycin (12 isolates), and erythromycin (3 isolates). Resistance to vancomycin was not detected. It should be noted that 74 out of 89 isolates were resistant to two or more antibiotics (53 CNS and 18 CPS) (Figure 1 and Supplementary Table 2).

Staphylococcus toxin production

Staphylococci are able to produce different heat-stable enterotoxins (A, B, C, D, and E) that might result in foodborne infections [25]. According to food safety standards, staphylococci can produce toxins at high numbers (> 10^5 CFU/mL), which poses a health risk to consumers [26]. Using the SET-RPLA kit, 40 out of 89 (45%) staphylococcal isolates were found to produce at least one enterotoxin (Figure 2). Enterotoxin A and C were the dominant entities. Enterotoxin A was produced by 23 isolates, C by 18 isolates, B by 8 isolates, and D by 2 isolates. As shown in Supplementary Table 2, 11 of the staphylococci were shown to produce 2 enterotoxins.

Figure 1. Antibiotic susceptibility of staphylococci isolated from cows with clinical mastitis.

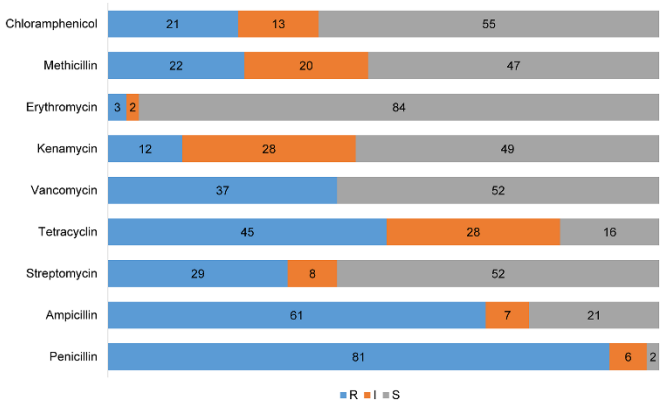
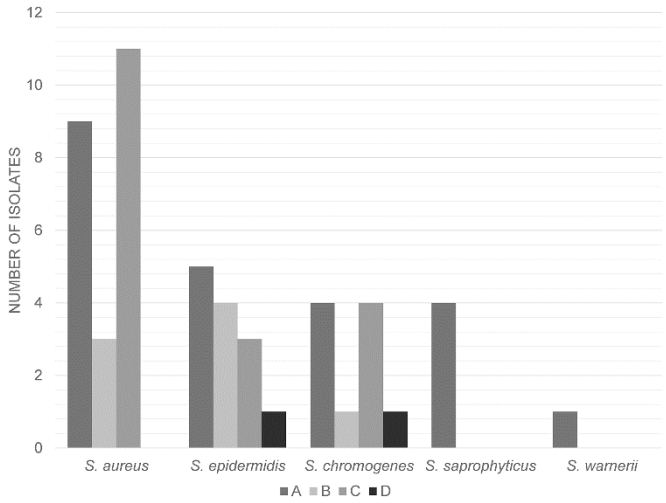


Table 2. Distribution of *Staphylococcus* spp. (CPS & CNS) isolates between the three different periods of lactation.

Species	CPS (+) or CNS (-)	Group I (n)	Group II (n)	Group III (n)	Total (n)
S. aureus	+	1	18	8	27
S. hyicus	+	NF	1	3	4
S. epidermidis	-	NF	12	13	25
S. saprophyticus	-	NF	7	NF	7
S. capitis	-	NF	4	3	7
S. chromogenes	-	NF	11	4	15
S. warnerii	-	NF	NF	2	2
S. cohnii	-	NF	NF	2	2
Total		1	53	35	89

CPS: coagulase-positive staphylococci; CNS: coagulase-negative staphylococci; NF: not found; Group I: early lactation; group II: mid lactation; group III: late lactation.

Figure 2. Distribution of different enterotoxins produced by staphylococci isolated from clinical mastitis.



Discussion

In many countries, mastitis is considered to be one of the most important infectious disease in dairy cattle farming [1,27]. Our investigation revealed a high diversity of different bacterial pathogens. From 79.6% of the milk samples, we detected only Gram-positive bacteria, while in the rest of the samples, the cultures grew either Gram-negative bacteria alone or both Gram-negative and -positive bacteria (Table 1). The diversity of mastitis pathogens has been reported for many years, and staphylococci are the most extensively studied cow mastitis pathogens [28]. Our results show that staphylococci were the most frequent Gram-positive species isolated (89/167; 53.3%), followed by enterococci and streptococci (33/167 and 14/167, respectively). This is in contrast to a previous report, which showed that the majority of pathogenic mastitis-causing bacteria were streptococci followed by staphylococci [29]. *S. aureus* is the most common pathogenic staphylococci associated with mastitis [7]. However, the importance of *S. aureus* in mastitis varies from country to country and between studies. In the study performed by Anderson *et al*., *S. aureus* was observed in 12.5% of cows with sub-clinical mastitis [30]. In another study from Turkey, *S. aureus* was found in 28.6% of mastitis cases [31]. In our study, *S. aureus* was detected in 58.6% of cases. In Australia, however, the presence of *S. aureus* is declining while other bacteria are emerging, and in recent years, *Streptococcus uberis* became their foremost mastitis pathogen [32].

Staphylococci have become resistant to many antibiotics during the years and pose a serious problem to human health in all parts of the world [33,34]. In our study, more than 92.2% of the staphylococcal isolates were resistant to at least one antibiotic, with 91% of the isolates being resistant to penicillin G followed by ampicillin (69%,), tetracycline (51%), oxacillin (25%), and chloramphenicol (24%). Our data show that antibiotic resistance among staphylococcal isolates from clinical mastitis is significantly higher in Kosovo than what has been found in similar studies in most European countries and the United States [33]. This finding may be as the result of easy access to these antibiotics without prescription from veterinarians and the use of broad-spectrum antibiotics to treat infections without the benefit of culture and susceptibility testing. In many countries, a strict antimicrobial management policy exists in veterinary practice with respect to antibiotic use and, consequently, the prevalence of antibiotic resistant staphylococci is much lower [34].

It is interesting to observe that antimicrobial resistance against more than one antibiotic is quite widespread. We identified three *S. aureus* isolates that carried resistance to all antibiotics tested except erythromycin and vancomycin. In addition, there were six staphylococcal isolates with resistance to more than five antibiotics. These isolates were all from different lactation periods. Overall, 37 of 89 of the isolates were resistant to four or more antibiotics (Supplementary Table 1). One of most commonly used antimicrobial product in Kosovo is penicillin G [20]. In our study, high resistance was found to penicillin (91%) and ampicillin (68%), which is similar to previous findings [35]. It is of interest to note that, from eight different species of staphylococci found in the clinical mastitis samples in Kosovo, the level of resistance between isolates were not significantly different (Supplementary Table 2). The presence of resistant bacteria in milk might pose a health risk to humans. Out of 89 isolates, 40 produced at least one toxin, while 11 produced two enterotoxins. Studies in other countries have reported the production of a single enterotoxin in staphylococci from clinical mastitis, but the occurrence of staphylococci producing multiple enterotoxins has not been frequently observed [36-38]. Of 40 isolates, 27 (69%) that produced one or more toxins were also resistant to more than four antibiotics (Supplementary Table 2). The combination of enterotoxin production and multi-antibiotic resistance increases the risk of human infections and complicates medical treatment.

The occurrence of clinical mastitis was less frequent in early lactation (29/152 samples) than in either mid lactation (62/152) or late lactation (61/152). This may be because the early lactation period is shorter in duration and not due to factors that render cows

susceptible in early lactation. Interestingly, our results showed that the highest number (88 of 89 isolates) of staphylococci was found in mastitis samples obtained in the mid and late lactation stages, while in early lactation, only one sample was positive for staphylococci. In the early lactation stage, the most common mastitis-causing pathogen was enterococci (15/29) followed by Gram-negative bacteria, streptococci, and, in only one cow, staphylococci. This agrees with a previous study that showed that clinical mastitis was less frequent in the first stage of lactation than in later stages [39,40].

Diverse pathogens were isolated from clinical mastitis-infected cows in Kosovo. Staphylococci, especially *S. aureus*, were the most common isolate in clinical mastitis, followed by enterococci, Gram-negative bacteria, and streptococci. In many cases, staphylococcal isolates from bovine clinical mastitis were resistant to more than one antibiotic and were able to produce different enterotoxins.

Conclusions

Our results suggest that treatment of mastitis in Kosovo with antibiotics should be based on microbial isolation and antimicrobial susceptibility testing to ensure treatment with effective antibiotics. Ongoing surveillance will be important to detect changes in antimicrobial sensitivity in udder bacteria and adjust antibiotic treatment regimens accordingly.

Acknowledgements

This study was supported by a grant from Ministry for Foreign Affairs under the Program for Higher Education, Research and Development 2010-2014 Norway (HERD). We thank the local veterinarians for sample collection and the dairy farmers for collaboration.

Authors' contributions

IM: made the major contribution on this article in all aspects from the design of the study, analytical work, statistics and writing of the manuscript. BB: participated on design of the study, sample's collection, identification of cow's breeds, and helped in manuscript improvement with his comments and discussions. MM: participated on design of the experiment, sample's collection, laboratory work (part which has been done in Kosovo), and helped in manuscript improvement through his comments and discussions of manuscript. AA: participated on design of the study, interpretation and discussion of results, and was part of writing process for specific chapters of the manuscript. IFN & DBD: had a critical contribution on design of the study, supervision of analytical work, interpretation of results and helped in manuscript improvement with their scientific comments. All authors read and approved the final manuscript.

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Conflict of interests: No conflict of interests is declared.

Annex 1 – Supplementary Items

Supplementary Table 1. Distribution of bacterial pathogens isolated from 152 milk cows with clinical mastitis.

Breed	Lactation time	Gram-positive (+) Gram-negative (-)	ID of bacteria and cell counts (CFU/mL)
Busha	I[a]	+	C; 3×10^4
Busha	I[a]	+	C; 12×10^4
Busha	I[a]	+	C; 2×10^4
Busha	I[a]	+	C; 3×10^3
Busha	I[a]	+	C; 3×10^3
Tyrolean Grey	I[a]	+	C; 14×10^4
Tyrolean Grey	I[a]	+	C; 43×10^4
Tyrolean Grey	I[a]	+	C; 52×10^4
Simmental	I[b]	+ & -	C; 82×10^3 & D; 45×10^3
Simmental	I[b]	-	D; 57×10^3
Simmental	I[b]	-	D; 89×10^3
Simmental	I[b]	+	C; 12×10^4
Simmental	I[b]	+	C; 23×10^4
Simmental	I[b]	+	C; 34×10^4
Cross-breeds	I[b]	+	C; 46×10^4
Cross-breeds	I[b]	-	D; 65×10^4
Cross-breeds	I[b]	+ & -	C; 33×10^3 & D; 87×10^3
Cross-breeds	I[b]	+ & -	C; 14×10^4 & D; 71×10^3
Montafon	I[b]	+	C; 3×10^4
Montafon	I[b]	+	A; 56×10^3 & C; 57×10^4
Holstein Friesian	I[c]	+ & -	C; 62×10^4 D; 56×10^4
Holstein Friesian	I[c]	-	D; 67×10^5
Holstein Friesian	I[c]	+	C; 52×10^4
Holstein Friesian	I[c]	+	C; 37×10^4
Holstein Friesian	I[c]	+	C; 23×10^4
Holstein Friesian	I[c]	+	B; 28×10^4
Jersey	I[c]	-	C; 31×10^4
Jersey	I[c]	+	B; 5×10^5
Holstein Friesian	I[c]	+	C; 48×10^4
Busha	II[a]	+	B; 26×10^4
Busha	II[a]	+	C; 51×10^4
Busha	II[a]	+	C; 17×10^4
Busha	II[a]	+	A; 71×10^4
Busha	II[a]	+	A; 71×10^5
Busha	II[a]	+	A; 21×10^4
Busha	II[a]	+	B; 41×10^4
Norwegian Red	II[a]	-	D; 22×10^2
Norwegian Red	II[a]	+	A; 31×10^4
Tyrolean Grey	II[a]	+	A; 41×10^4
Tyrolean Grey	II[a]	+	A; 34×10^5
Tyrolean Grey	II[a]	+	A; 52×10^4
Tyrolean Grey	II[a]	+	A; 31×10^4
Tyrolean Grey	II[a]	+	A; 34×10^4
Simmental	II[b]	+	A; 21×10^5
Simmental	II[b]	-	D; 64×10^4
Simmental	II[b]	-	D; 91×10^4
Simmental	II[b]	+	A; 21×10^5
Simmental	II[b]	-	D; 23×10^5
Simmental	II[b]	+	A; 17×10^4
Simmental	II[b]	+	A; 15×10^4
Simmental	II[b]	+	A; 3×10^4 & B; 16×10^4
Simmental	II[b]	+	B; 4×10^3
Simmental	II[b]	+	B; 34×10^3
Simmental	II[b]	+	B; 67×10^3
Simmental	II[b]	+	B; 42×10^4
Simmental	II[b]	+	B; 31×10^4

Breed	Lactation time	Gram-positive (+) Gram-negative (-)	ID of bacteria and cell counts (CFU/mL)
Simmental	II[b]	-	D; 41×10^5
Simmental	II[b]	+	A; 15×10^4
Simmental	II[b]	+	A; 15×10^4
Simmental	II[b]	+	A; 16×10^4
Simmental	II[b]	+	A; 21×10^4
Simmental	II[b]	+	A; 15×10^4
Simmental	II[b]	+	A; 2×10^4
Simmental	II[b]	+	A; 4×10^4
Simmental	II[b]	+	A; 12×10^4
Simmental	II[b]	+	A; 23×10^4
Simmental	II[b]	+	A; 18×10^4
Cross-breeds	II[b]	-	D; 61×10^5
Cross-breeds	II[b]	+	B; 14×10^4
Cross-breeds	II[b]	-	D; 51×10^5
Cross-breeds	II[b]	-	D; 32×10^4
Cross-breeds	II[b]	+ & -	C; 57×10^3 & D; 12×10^4
Cross-breeds	II[b]	-	D; 52×10^5
Cross-breeds	II[b]	+	A; 52×10^4
Cross-breeds	II[b]	+	A; 6×10^5
Cross-breeds	II[b]	+	C; 63×10^3
Cross-breeds	II[b]	+	C; 75×10^3
Cross-breeds	II[b]	-	D; 58×10^3
Montafon	II[b]	+	A; 41×10^4
Montafon	II[b]	+	A; 18×10^4
Montafon	II[b]	+	A; 47×10^4
Montafon	II[b]	-	D; 53×10^3
Montafon	II[b]	+	B; 14×10^4
Montafon	II[b]	+	A; 42×10^4
Montafon	II[b]	+	A; 45×10^4
Montafon	II[b]	+	A; 26×10^4
Montafon	II[b]	+	A; 4×10^5
Montafon	II[b]	+	A; 41×10^4
Montafon	II[b]	+	A; 24×10^4
Montafon	II[b]	+	A; 2×10^5
Montafon	II[b]	+	A; 23×10^4
Holstein Friesian	III[c]	-	D; 61×10^4
Holstein Friesian	III[c]	-	D; 13×10^4
Holstein Friesian	III[c]	-	D; 28×10^4
Holstein Friesian	III[c]	+	A; 14×10^4
Holstein Friesian	III[c]	+	A; 5×10^5
Brown Swiss	III[c]	+	A; 25×10^4
Hollshtajn Frizis	III[c]	-	D; 56×10^4
Brown Swiss	III[c]	+	A; 2×10^4
Holstein Friesian	III[c]	+	A; 47×10^4
Holstein Friesian	III[c]	+	A; 19×10^4
Holstein Friesian	III[c]	+	A; 1×10^5
Holstein Friesian	III[c]	+	A; 24×10^4
Holstein Friesian	III[c]	+	A; 26×10^4
Holstein Friesian	III[c]	+	A; 25×10^4
Brown Swiss	III[c]	+	A; 34×10^4
Jersey	III[c]	+	A; 4×10^5
Holstein Friesian	III[c]	+	A; 56×10^3
Holstein Friesian	III[c]	+	A; 65×10^3
Holstein Friesian	III[c]	+	A; 72×10^3
Jersey	III[c]	+	A; 4×10^5
Busha	III[a]	+	A; 8×10^4
Busha	III[a]	+	A; 25×10^4
Busha	III[a]	+	A; 22×10^4
Tyrolean Grey	III[a]	-	D; 33×10^4
Tyrolean Grey	III[a]	+	A; 19×10^4
Simmental	III[b]	+	A; 17×10^4

Breed	Lactation time	Gram-positive (+) Gram-negative (-)	ID of bacteria and cell counts (CFU/mL)
Simmental	III[b]	+	A; 14×10^4
Simmental	III[b]	-	D; 26×10^4
Simmental	III[b]	+	A; 19×10^4
Simmental	III[b]	+	A; 25×10^4
Simmental	III[b]	+	A; 48×10^4
Simmental	III[b]	+	A; 2×10^5
Cross-breeds	III[b]	-	D; 43×10^4
Cross-breeds	III[b]	+	A; 4×10^5
Cross-breeds	III[b]	-	D; 56×10^4
Cross-breads	III[b]	+	A; 54×10^4
Cross-breads	III[b]	+	A; 41×10^4
Cross-breeds	III[b]	+	A; 2×10^5
Cross-breeds	III[b]	+	A; 46×10^4
Cross-breeds	III[b]	+	A; 37×10^4
Cross-breeds	III[b]	+	A; 4×10^4
Cross-breeds	III[b]	-	D; 87×10^4
Cross-breeds	III[b]	+	A; 12×10^4
Montafon	III[b]	+	A; 23×10^4
Montafon	III[b]	+	A; 26×10^4
Montafon	III[b]	+	A; 38×10^4
Montafon	III[b]	+	A; 46×10^5
Montafon	III[b]	+	A; 41×10^4
Montafon	III[b]	+	A; 38×10^5
Montafon	III[b]	+	A; 51×10^4
Montafon	III[b]	+	A; 38×10^5
Montafon	III[b]	+	A; 67×10^5
Holstein Friesian	III[c]	+	A; 41×10^4 & C; 78×10^3
Holstein Friesian	III[c]	+	B; 24×10^4
Holstein Friesian	III[c]	+	A; 55×10^4 & B; 38×10^3
Holstein Friesian	III[c]	+	B; 41×10^4 & C; 41×10^3
Brown Swiss	III[c]	+ & -	A; 71×10^4, C; 61×10^3 & D; 51×10^3
Brown Swiss	III[c]	+	A; 34×10^4 & C; 3×10^4
Brown Swiss	III[c]	+ & -	A; 71×10^4, C; 78×10^3 & D; 63×10^3
Jersey	III[c]	+	A; 43×10^4 & C; 86×10^3
Jersey	III[c]	+	A; 47×10^5

A: staphylococci; B: streptococci; C: enterococci; D: Gram-negative bacteria; I: lactation period between weeks 0 and 8; II: lactation period between weeks 9 and 22; III: lactation period between week 23 and end of lactation.

Supplementary Table 2. Distribution of staphylococci, their antibiotic susceptibility and toxin production.

Races	Lactation[a]	ID	Isolates	ANTIBIOTIC									TOXINS			
				PEN	AMP	STR	TET	VAN	KEN	ERY	OXA	CHL	A	B	C	D
Montafon	5	RKS10038	*S. chromogenes*	R	R	R	I	I	S	S	R	S	+	-	-	-
Tyrolean Grey	7	RKS10004	*S. aureus*	S	S	S	S	S	S	S	S	S	-	-	-	-
Tyrolean Grey	7	RKS10006	*S. aureus*	I	S	S	S	S	S	S	S	I	-	-	-	-
Cross-breeds	7	RKS10022	*S. chromogenes*	R	R	R	R	I	I	S	R	S	-	-	+	-
Busha	8	RKS10003	*S. aureus*	S	S	S	S	S	S	S	S	S	-	-	-	-
Montafon	8	RKS10021	*S. chromogenes*	R	R	R	R	I	R	S	R	S	-	-	+	-
Brown Swiss	8	RKS6532	*S. epidermidis*	R	S	S	R	S	S	S	S	R	-	-	+	-
Norwegian Red	8	RKS6578	*S. epidermidis*	R	R	R	S	S	S	S	S	S	-	-	-	-
Simmental	9	RKS10007	*S. aureus*	R	R	R	R	I	R	S	R	I	+	-	+	-
Simmental	9	RKS10009	*S. aureus*	R	R	R	R	I	R	S	R	R	+	-	+	-
Simmental	9	RKS10011	*S. aureus*	R	R	R	R	I	R	S	R	R	+	-	+	-
Holstein Friesian	9	RKS10024	*S. chromogenes*	R	R	R	R	I	I	S	I	S	+	-	-	-

Races	Lactation[a]	ID	Isolates	ANTIBIOTIC									TOXINS			
				PEN	AMP	STR	TET	VAN	KEN	ERY	OXA	CHL	A	B	C	D
Tyrolean Grey	9	RKS6523	*S. capitis*	R	S	S	R	S	S	S	S	S	-	-	-	-
Simmental	10	RKS10010	*S. aureus*	R	R	R	R	I	R	S	R	R	+	-	+	-
Simmental	10	RKS10020	*S. chromogenes*	R	R	R	S	I	R	S	S	S	-	-	+	-
Cross-breeds	10	RKS10034	*S. aureus*	R	R	R	I	I	S	S	R	S	-	-	-	-
Simmental	11	RKS10008	*S. aureus*	R	R	R	R	I	R	S	S	I	+	-	+	-
Montafon	11	RKS6520	*S. saprophyticus*	R	S	S	R	S	S	S	S	S	-	-	-	-
Busha	12	RKS10002	*S. aureus*	R	I	S	S	I	S	S	S	S	-	-	-	-
Tyrolean Grey	12	RKS10005	*S. aureus*	I	S	I	S	S	S	S	S	I	-	-	-	-
Simmental	12	RKS10017	*S. saprophyticus*	R	R	S	R	I	S	S	S	R	+	-	-	-
Cross-breeds	12	RKS6568	*S. epidermidis*	R	R	S	R	I	S	S	S	R	-	-	-	-
Simmental	13	RKS10014	*S. saprophyticus*	R	R	S	S	I	I	S	S	I	+	-	-	-
Simmental	13	RKS10018	*S. chromogenes*	R	R	R	S	I	R	S	I	S	+	-	-	-
Cross-breeds	13	RKS10023	*S. chromogenes*	R	R	R	R	I	I	S	I	S	-	+	+	-
Montafon	13	RKS6545	*S. epidermidis*	R	R	S	R	S	S	S	S	S	-	-	-	-
Holstein Friesian	14	RKS10026	*S. chromogenes*	R	S	I	I	S	S	I	I	S	-	-	-	-
Montafon	14	RKS6552	*S. epidermidis*	R	R	S	R	S	S	S	I	I	-	-	-	-
Busha	14	RKS6570	*S. epidermidis*	R	R	S	R	S	S	S	S	R	-	-	-	-
Simmental	15	RKS10012	*S. aureus*	R	R	S	S	I	I	S	I	I	+	-	+	-
Simmental	15	RKS10015	*S. saprophyticus*	R	R	S	R	I	S	S	I	R	+	-	-	-
Simmental	15	RKS10019	*S. chromogenes*	R	R	R	S	I	R	S	I	S	+	-	-	-
Holstein Friesian	15	RKS10028	*S. aureus*	R	S	I	I	S	S	S	S	S	-	-	+	-
Simmental	15	RKS10032	*S. chromogenes*	I	I	R	I	S	S	S	I	S	-	-	-	-
Simmental	16	RKS10013	*S. aureus*	R	R	S	S	I	I	S	S	I	-	-	-	-
Simmental	16	RKS10016	*S. saprophyticus*	R	R	S	R	I	S	S	R	R	+	-	-	-
Holstein Friesian	16	RKS10027	*S. aureus*	R	S	I	I	S	S	S	S	S	-	+	+	-
Brown Swiss	16	RKS6528	*S. epidermidis*	R	R	S	R	S	S	S	I	S	-	-	-	-
Holstein Friesian	16	RKS6538	*S. epidermidis*	R	R	S	I	S	S	S	I	R	-	+	+	-
Montafon	16	RKS6544	*S. saprophyticus*	R	S	S	R	S	S	S	S	S	-	-	-	-
Holstein Friesian	17	RKS10025	*S. chromogenes*	R	S	I	I	S	S	I	S	S	-	-	-	+
Montafon	17	RKS6535	*S. chromogenes*	R	S	S	R	S	S	S	I	R	-	-	-	-
Montafon	17	RKS6537	*S. hyicus*	R	R	S	I	I	S	S	I	S	-	-	-	-
Montafon	18	RKS10046	*S. hyicus*	R	R	S	I	I	S	S	I	S	-	-	-	-
Holstein Friesian	18	RKS6525	*S. chromogenes*	R	R	S	R	S	S	S	S	S	-	-	-	-
Montafon	18	RKS6536	*S. epidermidis*	R	R	S	I	S	S	S	S	R	+	-	-	-
Holstein Friesian	18	RKS6547	*S. epidermidis*	R	R	S	R	S	S	S	S	I	+	-	-	+
Cross-breeds	18	RKS6572	*S. capitis*	R	R	R	R	S	S	S	S	S	-	-	-	-
Tyrolean Grey	18	RKS6573	*S. epidermidis*	R	R	S	R	S	S	S	S	R	-	-	-	-
Jersey	19	RKS10029	*S. aureus*	R	S	I	I	S	S	S	I	S	+	-	-	-
Montafon	19	RKS6522	*S. capitis*	R	S	S	R	S	S	S	S	S	-	-	-	-
Jersey	19	RKS6527	*S. chromogenes*	R	R	S	R	S	S	S	I	S	-	-	-	-
Holstein Friesian	20	RKS6529	*S. capitis*	R	R	S	R	S	S	S	S	S	-	-	-	-

Races	Lactation[a]	ID	Isolates	ANTIBIOTIC									TOXINS			
				PEN	AMP	STR	TET	VAN	KEN	ERY	OXA	CHL	A	B	C	D
Montafon	24	RKS6534	*S. epidermidis*	R	R	S	I	S	S	S	S	R	-	-	-	-
Holstein Friesian	25	RKS6548	*S. capitis*	R	R	S	R	S	S	S	S	R	-	-	-	-
Montafon	25	RKS10039	*S. aureus*	R	S	R	I	S	S	S	R	S	-	-	-	-
Montafon	25	RKS6546	*S. epidermidis*	R	R	S	R	S	S	S	R	S	-	+	-	-
Montafon	26	RKS6553	*S. epidermidis*	R	R	S	R	S	S	S	R	I	+	-	-	-
Montafon	26	RKS6558	*S. epidermidis*	R	R	S	R	S	R	R	R	I	+	-	-	-
Simmental	26	RKS10037	*S. capitis*	R	R	S	R	S	S	S	S	S	-	-	-	-
Brown Swiss	26	RKS10041	*S. aureus*	R	R	R	I	I	S	S	R	S	+	+	-	-
Holstein Friesian	26	RKS10048	*S. hyicus*	R	R	S	I	I	S	S	S	S	-	-	-	-
Holstein Friesian	26	RKS6540	*S. epidermidis*	R	R	S	I	S	S	S	S	R	-	-	-	-
Busha	27	RKS10031	*S. aureus*	I	I	I	I	S	S	S	S	S	-	-	+	-
Busha	27	RKS6530	*S. epidermidis*	I	S	S	S	S	S	S	S	S	-	-	-	-
Cross-breeds	27	RKS6574	*S. epidermidis*	R	R	S	R	S	S	S	S	R	-	-	-	-
Brown Swiss	27	RKS10042	*S. aureus*	R	R	R	I	I	S	S	R	S	-	+	-	-
Brown Swiss	27	RKS6533	*S. saprophyticus*	R	S	S	R	S	S	S	S	S	-	-	-	-
Montafon	27	RKS6556	*S. capitis*	R	R	S	R	S	S	S	I	S	-	-	-	-
Cross-breeds	27	RKS6577	*S. epidermidis*	R	R	R	S	S	S	S	I	S	+	-	-	-
Cross-breeds	28	RKS10000	*S. aureus*	R	I	S	S	I	S	S	R	S	-	-	+	-
Jersey	28	RKS10044	*S. aureus*	R	R	R	I	I	S	S	R	S	-	-	-	-
Simmental	28	RKS10045	*S. epidermidis*	R	R	S	R	S	S	S	S	R	-	+	-	-
Simmental	28	RKS6555	*S. cohnii*	R	R	S	R	S	S	S	S	S	-	-	-	-
Tyrolean Grey	28	RKS6561	*S. warneri*	R	R	S	I	S	S	S	S	S	-	-	-	-
Jersey	28	RKS6579	*S. epidermidis*	R	R	S	R	I	S	S	S	R	-	-	-	-
Montafon	29	RKS10047	*S. hyicus*	R	R	S	I	I	S	S	S	S	-	-	-	-
Montafon	29	RKS6559	*S. epidermidis*	R	R	S	R	S	R	R	I	I	-	-	-	-
Brown Swiss	30	RKS10043	*S. aureus*	R	R	R	I	I	S	S	R	S	+	-	-	-
Simmental	30	RKS6554	*S. cohnii*	R	R	S	R	S	S	S	S	S	-	-	-	-
Holstein Friesian	30	RKS6560	*S. epidermidis*	R	R	S	R	S	R	R	R	I	-	+	-	-
Busha	31	RKS10030	*S. warneri*	I	S	I	I	S	S	S	S	S	+	-	-	-
Montafon	31	RKS10036	*S. chromogenes*	R	I	R	I	I	S	S	S	S	-	-	-	-
Montafon	32	RKS10001	*S. aureus*	R	I	S	S	I	S	S	I	S	-	-	+	-
Simmental	33	RKS10033	*S. aureus*	R	R	R	I	I	S	S	R	S	-	-	-	-
Cross-breeds	33	RKS6576	*S. epidermidis*	R	S	R	R	S	S	S	S	R	-	-	-	-
Cross-breeds	35	RKS6531	*S. epidermidis*	R	R	R	R	S	S	S	S	R	-	-	+	-
Montafon	48	RKS10035	*S. aureus*	R	I	R	I	I	S	S	R	S	-	-	-	-
Holstein Friesian	56	RKS10040	*S. aureus*	R	S	R	I	I	S	S	R	S	-	-	-	-

[a] Week of lactation period; PEN: penicillin G; AMP: ampicillin; STR: streptomycin; TET: tetracycline; KEN: kanamycin; VAN: vancomycin; ERY: erythromycin; OXA: oxacillin; CHL: chloramphenicol; Cross-breeds are dairy cattle that have been cross-bred with beef cattle.