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Original article

PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITY OF BACTERIAL ISOLATES FROM SUBCLINICAL MASTITIS IN DAIRY FARMS IN KOSOVO

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Summary

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The aim of this study was to determine the prevalence of subclinical mastitis in dairy cows in Kosovo and the antimicrobial susceptibility of isolated bacteria. Six hundred and twenty four milk samples from individual quarters of 156 lactating cows were tested using California Mastitis Test (CMT) and the positive samples were further analysed using conventional methods for isolation of main mastitis causing agents and finally identified using biochemical tests. The overall prevalence of subclinical mastitis was 25.6%. Twenty one mastitis positive cows belonged to Holstein Friesian breed, 11 to Simmental and 8 were crossbred. Regarding age susceptibility, 13 cows resulting positive in the CMT test were 2–5 years of age and the other 27: 6–9 years of age. A total of 112 bacterial isolates were recovered. The most prevalent species were *Staphylococcus aureus* (28.6%) followed by coagulase negative staphylococci (21.4%), *E. coli* (13.4%), *Streptococcus agalactiae* (14.3%), *Streptococcus dysgalactiae* (3.6%), *Streptococcus uberis* (6.2%), *Corynebacterium* spp. (8%), and *Bacillus* spp. (4.5%). The isolates were subjected to antimicrobial susceptibility test using the disc diffusion method. *In vitro* tests of bacterial isolates revealed higher sensitivity to amoxycillin/clavulanic acid, oxytetracycline, trimethoprim and gentamicin. The lowest susceptibility was shown to penicillin and streptomycin except for *Corynebacterium* spp. and *Bacillus* spp.

Key words: bacterial pathogens, CMT, dairy cows, subclinical mastitis

INTRODUCTION

The dairy sector and especially raw milk quality in Kosovo is still not at the required level as set by the standards of current legislation. The new regulation on quality standards and grading of fresh milk came into force on January 1, 2007 (Table 1, MAFRD, 2006).

The majority of milk in the country is consumed on the farm, sold for subsistence, apart from semi-commercial farmer market it comes on the local Green Market without any prior quality control inspection. Large commercial farms which represent about 5% of the dairy market,

mainly sell to the processors who buy milk from milk collection centers. The level of colony forming unts (CFU) and somatic cell counts (SCC) are the parameters of the milk quality according to the national standards (Bytyqi *et al.*, 2014). Previous studies conducted on the quality of fresh milk in Kosovo have shown high CFU and SCC values, indicating high level of contamination during milking and storage of milk after milking (Bytyqi *et al.*, 2010). About 24% of milk collected was evaluated as being of poor hygiene, depicting values of more than 500,000 SCC cells/mL and around 50% of the samples had more than 3,000,000 CFU/mL of milk in a study carried out with dairy farms in Kosovo (Bytyqi *et al.*, 2011). The enforcement of the quality standard without intervention in milk preservation and handling infrastructure would take out of business about 90% of the dairy farmers who deliver the milk to the dairies.

The high SCC and CFU levels are associated with mastitis. It is caused by infectious agents, especially bacteria, but it can also be due to physical or chemical agents. It is a disease of dairy cattle that cause low milk production and high economic losses (Ericsson-Unnerstad *et al.*, 2009). SCC in milk are commonly used as indicators of mastitis, on the basis that an increase reflects an immune response to the presence of infection in the mammary gland (Green *et al.*, 2008). Although subclinical mastitis is the dominant form affecting cows, it frequently goes undetected or untreated for extended periods by most dairy producers (Hillerton & Berry, 2003). Clinical mastitis is characterised by visible changes in milk with appearance of flakes or blobs, oedema and pain in the udder. Subclinical mastitis has been defined as inflammation without clear signs. Subclinical mastitis is a widespread disease in dairy production where every clinical case of mastitis comes along with 15 to 40 subclinical cases (Kelly *et al.*, 2002). Several causative agents and predisposing factors have been implicated in dairy cows mastitis including bacterial, mycoplasmal and yeast pathogens. A variety of bacterial species are involved with the most common being the Gram positive staphylococci and streptococci and the Gram negative Enterobacteriaceae (Bannerman, 2009).

Approximately 70% of the antimicrobials used in dairy production are for treatment of clinical mastitis (Thomson *et al.*, 2008), but the cure rates are not always satisfactory. The efficacy of bovine mastitis treatment depends on the cause, clinical manifestation, antibiotic suscepti-

Table 1. Standards for CFU/mL and SCC/mL according to current legislation in Kosovo (MAFRD, 2006)

Year	Extra class	Class I	Class II	Class III
2007	<100,000 CFU/mL <400,000 SCC/mL	<200,000 CFU/mL <500,000 SCC/mL	<500,000 CFU/mL <600,000 SCC/mL	>500,000 CFU/mL >600,000 SCC/mL
2008	<80,000 CFU/mL <400,000 SCC/mL	<100,000 CFU/mL <400,000 SCC/mL	<200,000 CFU/mL <500,000 SCC/mL	<500,000 CFU/mL <600,000 SCC/mL
2009	<50,000 CFU/mL <300,000 SCC/mL	<80,000 CFU/mL <300,000 SCC/mL	<100,000 CFU/mL <400,000 SCC/mL	

bility of etiological agent and the efficiency of immune system. The abusive or incorrect use of antimicrobials has been outlined as the major selective pressure for the development of resistance (Levy, 2002).

There is no recent data about the prevalence of subclinical and clinical mastitis and its etiological agents in Kosovo. The overall annual financial losses calculated on 50 dairy farms in Kosovo reached € 42,263 per year, whilst the average annual financial loss per farm was € 845 due to poor quality of milk as around 66% of all studied commercial dairy farms produced raw milk of poor quality (Musliu *et al.*, 2009). The purpose of this survey was to investigate the prevalence and antimicrobial susceptibility of bacteria isolated from subclinical mastitis in dairy farms in some regions of Kosovo.

MATERIALS AND METHODS

This study was carried out in three regions of Kosovo (Prishtina, Prizren, Ferizaj) from January to April 2015. One hundred and fifty-six lactating cows from small holding dairy farms were screened for subclinical mastitis using CMT directly in farms with no prior mastitis control programmes in place. Regarding the breed and the age of animals, 47 cows were Friesian, 59 – crossbred and 50 – Simmental; 70 cows were 2–5 years of age and 86 cows: 6–9 years of age. Cows were selected randomly from these regions with farm populations ranging from 5 to 20 cattle. Most of the farms included in the study used open and bucket machine milking, representing the typical farm size and milking practices in Kosovo. Sampling for subclinical mastitis was performed by local veterinarians. Milk was collected from individual quarters into mastitis paddle wells, ensuring that the first strips were discarded. The procedures and the interpretation were according to Quinn *et al.* (1994) using CMT test. Six hundred and twenty four milk samples from individual quarters of 156 lactating cows were tested by the CMT and only positive milk samples were collected under aseptic conditions in sterile screw-capped bottles and transported to the laboratory for isolation and identification of main mastitis bacterial pathogens. The milk samples were inoculated on 5% sheep blood agar, MacConkey agar plates, Mannitol salt agar and Edward's agar. The plates were incubated aerobically at 37 ºC for 24–48 hours. Isolates that produced Gram positive cocci in clusters, catalase-positive, glucose-fermentative, not producing coagulase (negative coagulase reaction with rabbit plasma) were identified as coagulase negative staphylococci (CNS). *Staphylococcus aureus* isolates were differentiated from other coagulase-positive staphylococci (*S. intermedius* and some *S. hyicus* strains) on the basis of mannitol fermentation on Mannitol salt agar and by using biochemical test API Staph system (BioMèrieux SA). The enteric bacteria were identified on the basis of colony morphology, oxidase test, lactose fermentation on MacConkey agar, indole production test, citrate utilisation, and other standard biochemical tests as described by Quinn *et al.* (1994). *Str. agalactiae* (aesculin-negative, blue colonies) isolates were differentiated from group D streptococci (aesculin-positive, black colonies) using Edward medium while remaining streptococci were identified using the API 20 Strep strips (BioMèrieux).

All isolates were tested for antimicrobial susceptibility on Mueller Hinton agar (HIMEDIA) by disk diffusion method (CLSI, 2012). For this purpose the follow-

Table 2. Relationship between the incidence of subclinical mastitis and breed and age of cows

	Number of tested cows	Number (%) of infected cows	Number (%) of tested/infected quarters
Breeds of cows			
Friesian	47	21 (44.7)	188/44 (23.4)
Crossbred	59	8 (13.5)	236/12 (5.1)
Simmental	50	11 (22.0)	200/20 (10.0)
Total	156	40 (25.6)	624/76 (12.2)
Age of cows			
2–5 years	70	13 (18.6)	280/19 (6.8)
6–9 years	86	27 (31.4)	344/57 (16.6)
Total	156	40 (25.6)	624/76 (12.2)

Table 3. Prevalence of subclinical mastitis in cows detected by CMT from the three studied regions

Region	Total number of animals	Positive animals for mastitis	Prevalence
Prishtina	48	8	16.7 %
Prizren	53	15	28.3 %
Ferizaj	55	17	30.9%
Total	156	40	25.6 %

ing antibiotic disks were used: penicillin P (10 IU, Oxoid), amoxicillin/clavulanic acid AMC (30 µg, Oxoid), oxytetracycline T (30 µg, BD BBL^{TM}), streptomycin S (10 µg, Liofilchem), cloxacillin CX (5 µg, Liofilchem), gentamicin CN (10 µg, Liofilchem), trimethoprim TM (2.5 µg, Liofilchem).

The plates were incubated at 35 ºC for 24 hours. The zone of inhibition around each disc was measured and the interpretation was made as per the zone size interpretation chart provided by the disc manufacturer.

RESULTS

In the CMT, 156 lactating cows from 32 farms (624 foremilk quarter samples) were tested for subclinical mastitis. In total, 40 (25.6%) cows and 76 (12.2%) quarters were diagnosed with subclinical mastitis. Regarding breed susceptibility, out of the 40 cows positive for subclinical mastitis by CMT, 21 were Holstein Friesian (44.7%), 11 were Simmental (22%) and 8 were crossbred (13.5%) (Table 2). As shown in Table 2, the prevalence of subliclinical mastitis was higher in cows aged 6–9 years (31.4%) than in those aged 2–5 years (18.6%).

The prevalence of mastitis in three regions is given in Table 3. Out of 76 positive milk samples, a total of 112 different bacterial isolates were isolated and the biochemical tests identified 8 species as shown in Table 4. The results of this study showed that the most predominant species were *S. aureus* (n=32; 28.6%), followed by CNS (n=24; 21.4%), *E. coli* (n=15 or

Table 4. Bacterial isolates from milk samples of the cows with subclinical mastitis in the three studied regions in Kosovo

Bacterial isolates	Number (%)
Staphylococcus aureus	32 (28.6)
Coagulase negative staphylococci (CNS)	24 (21.4)
E. coli	15 (13.4)
Streptococcus agalactiae	16 (14.3)
Streptococcus dysgalactiae	4 (3.4)
Streptococcus uberis	7 (6.2)
Corynebacterium spp.	9 (8)
Bacillus spp.	5 (4.5)
Total	112 (100)

13.4%), *Str. agalactiae* (n=16; 14.3%) *Corynebacterium spp.* (n=9; 8%), and *Bacillus spp*. (n=5; 4.5%).

The antimicrobial susceptibility and resistance profiles of bacterial isolates from subclinical cases of mastitis to antibiotics are shown in Table 5. In this study, *S. aureus* isolates were found to be highly susceptible to amoxicillin/clavulanic acid (90.6%), followed by oxytetracycline (78.1%), trimethoprim (75%) and gentamicin (71.9%). However these isolates were highly resistant to penicillin (71.9%), streptomycin (75%) and cloxacillin (53.1%). Coagulase negative staphylococci were also more sensitive to amoxycillin/clavulanic acid (83.3%), trimethoprim (79.2%), oxytetracycline (75%), gentamicin (62.5%), cloxacillin (54.2%). *E. coli* (13.4% of all isolates) were highly susceptible to oxytetracycline (86.7%), trimethoprim (80%), gentamicin (66.7%), cloxacillin (53.3%) but not to amoxicillin/clavulanic acid (20%). None of the

Table 5. Antimicrobial susceptibility of bacterial isolates from cows with subclinical mastitis to antibiotics – percentage of sensitive (S) and resistant (R) isolates

Bacterial isolates		Tested antimicrobial drugs						
		AM/CLA	OXY	CLO	GEN	TRI	PEN G	STR
S. aureus	S	90.6	78.1	46.9	71.9	75	28.1	25
(n=32)	R	9.4	21.9	53.1	28.1	25	71.9	75
CNS	S	83.3	75	54.2	62.5	79.2	25	41.7
(n=24)	R	16.7	25	45.8	37.5	20.8	75	58.3
E. coli	S	80	86.7	53.3	66.7	80	20	26.7
(n=15)	R	20	13.3	46.7	33.3	20	80	73.3
S. agalactiae	S	93.75	75	81.25	68.75	87.5	56.25	62.5
(n=16)	R	6.25	25	18.75	31.25	12.5	43.75	37.5
S. dysgalactiae	S	100	75	50	75	100	25	25
(n=4)	R	–	25	50	25	–	75	75
S. uberis	S	85.7	57.1	85.7	71.4	57.1	28.6	28.6
(n=7)	R	14.3	42.9	14.3	28.6	42.9	71.4	71.4
Corynebacterium	S	77.8	88.9	66.7	77.8	88.9	66.7	66.7
spp. *(n=9)*	R	22.2	11.1	33.3	22.2	11.1	33.3	33.3
Bacillus spp.	S	80	60	80	80	80	80	60
(n=5)	R	20	40	20	20	20	20	40

Legend: AM/CLA: amoxillin/clavulanic acid; OXY: oxytetracycline; CLO: cloxacillin; GEN: gentamicin; TRI trimethoprim; PEN G: penicillin G; STR: streptomycin.

four *Str. dysgalactiae* isolates was resistant to amoxicillin/clavulanic acid and trimethoprim.

DISCUSSION

In the present study, the prevalence of subclinical mastitis was 25.6% as measured on a cow basis, and 12.2% as measured on a quarter basis. This prevalence rate is relatively high, although substantially higher values have been also reported, for instance the incidence of subclinical mastitis in Uruguay was 52.4% as measured on a cow basis and 26.7% on a quarter basis (Gianneechini *et al.*, 2002). The CMT results in the present study showed that the prevalence of subclinical mastitis at cow level was higher in Holstein Friesian (44.7%) than in the Simmental (22%) and crossbred cows (13.5%). This might be primarily in association with genetic resistance and also with poor adaptation of these cows to local environment and climate (Girma, 2001; Dego & Tarek, 2003), but future studies are needed to explain different udder infection rates in cow breeds. Regarding age susceptibility, higher percentage of subclinical mastitis was established in cows aged 6–9 years (31.4%) than in those aged 2–5 years (18.6%). This age-related variation in the prevalence of subclinical mastitis is attributed to the fact that cows with multiple parturitions are more prone to mastitis than younger cows due to physical alterations of udder, and is in line with findings reported by other authors (Carlen *et al.*, 2004; Zwald *et al.*, 2004; Abdel-Rady *et al.*, 2009). This occurrence could be in association with differences in management systems between farms, stage of lactation, parity, breed (Almaw *et al.*, 2008) and milking hygiene (Haltia *et al.*, 2006).

The most commonly isolated pathogens in our study were *S. aureus* with a frequency of 28.6%, followed by CNS: 21.4%, *S. agalactiae:* 14.3%, and *E. coli:* 13.4%. In previous studies, *S. aureus* was also reported as major pathogen (Rabello *et al.*, 2005; Botrel *et al.*, 2009). The present study showed that staphylococci and streptococci represented more than half (73.9%) of bacterial isolates involved in subclinical mastitis, followed by other bacteria. Similar results were also reported in Italy (Moroni *et al.*, 2006). Bacteria that most frequently cause mastitis can be classified into two main categories major and minor pathogens (Sargeant *et al.*, 2001). *S. aureus, Str. agalactiae* and *E. coli* are the most common etiological agents involved in subclinical and clinical cases of mastitis in dairy cows (Gonzalo *et al.*, 2002). The high rate of isolation of staphylococci may be attributed to the fact that the principal reservoirs of these organisms are the udder skin and milk of the infected gland. The high frequency of staphylococcal mastitis is considered to be due to the existence of inadequate hygiene in the dairy industry, poor animal health services, and lack of proper attention to the health of the mammary gland in general.

The antimicrobial susceptibility tests carried out in this study show in general a high susceptibility for *S. aureus* to amoxicillin/clavulanic acid (90.6%), oxytetracycline (78.1%), trimethoprim (75%) and gentamicin (71.9%) and resistance to streptomycin (75%), penicillin G (71.9%) and cloxacillin (53.1%). The majority of authors have noted an increase in the resistance to antibiotics of bacteria, mostly staphylococci, isolated from mastitis (Pitkala *et al.*, 2004; Turutoglu *et al.*, 2006; Pyorala & Taponen, 2009). During this study the data were collected from local

veterinarians on antibiotics most frequently used for the treatment of clinical mastitis in cows. Antimicrobials such as penicillin+streptomycin (PENSTREP 20/20 administered by parenteral routes), oxytetracycline (ALAMYCIN 100 mg/mL, Limoxin 100, TOPOXY 10, administered by parenteral routes), procaine benzylpenicillin+streptomycin sulphate+neomycin sulphate (Mastiquick 5 g intramammary injector), colistin sulphate+ metampicillin sodium+cloxacillin sodium (Mastidian forte 10 mL intramammary injector) were used most often in cases of clinical mastitis in lactating cows in these regions and very rarely, sulfadiazine+trimethoprim (Norodine 24 administered by parenteral routes).

The present study revealed higher sensitivity for *S. agalactiae*, *S. dysgalactiae*, *S. uberis, Corynebacterium* spp. and *Bacillus* spp. to the same antibiotics as amoxicillin/clavulanic acid, oxytetracycline, trimethoprim, gentamicin and cloxacillin, except for *Str. dysgalactiae* and *Str. uberis* isolates that were more resistant to penicillin and streptomycin. None of *Str. dysgalactiae* isolates was resistant to amoxicillin/clavulanic acid and trimethoprim which shows the efficacy of these antibiotics in treatment of mastitis. Idriss *et al.* (2014) has found that *Str. agalactiae, Str. uberis, S. aureus,* CNS and *E. coli* were sensitive to amoxicillin/clavulanic acid, which support our findings but the other antibiotics in our case showed different results which might have different association.

In conclusion, this study provides the proof about a very high prevalence of subclinical mastitis on dairy farms in three Kosovo regions (25.6%). The most commonly isolated pathogens were *S. aureus*, followed by coagulase negative staphylococci (CNS), *Str. agalactiae,* and *E. coli*. The antimicrobial susceptibility tests shows higher susceptibility of *S. aureus, CNS, E. coli*, *Str. agalactiae*, *Str. dysgalactiae*, *Str. uberis, Corynebacterium* spp*.,* and *Bacillus* spp*.,* to amoxicillin/clavulanic, oxytetracycline and trimethoprim. Good management practices and routine tests with CMT can help for early detection of infection and reduction of subclinical mastitis prevalence. Identification of mastitis pathogens and antimicrobial susceptibility testing are essential to control the disease, achieve effective therapy and consequently improve the farm profitability.

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