ANTIMICROBIAL RESISTANCE OF PATHOGENS FROM EWES SUBCLINICAL MASTITIS

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Abstract. Mastitis is a major health problem in dairy sheep flocks worldwide. It is associated with reduced productivity, low weaning weights, lamb mortality and the culling of affected ewes.

Subclinical mastitis is characterised by quantitative and qualitative changes to milk, mainly through increased numbers of somatic cells, and is frequently caused by the introduction and multiplication of pathogenic bacteria in the mammary glands. Causative bacteria include coagulase negative staphylococci, *Staphylococcus aureus*, *Streptococcus* spp., *Escherichia coli* and *Pseudomonas aeruginosa*. The aim of this study was to identify bacterial flora of subclinical mastitis and to determine antimicrobial resistance.

The study was carried out in 3 private Lithuanian ewes' farms. California mastitis test (CMT) was performed on 84 milk samples. For bacteriological investigation, ewes with CMT positive udder halves were sampled (n=23). Identification of common bacterial species isolated from all subclinical mastitis cases was done depending on morphological, cultural characterisation and biochemical tests. Antimicrobial susceptibility was determined by a disc diffusion method. Statistically significant associations were analysed by the Student test.

Subclinical mastitis and positive CMT results were detected in 47.6% of ewes and in 57.5% of udder halves. Bacteria were isolated from 91.3% of milk samples. The most prevalent bacterial species was *S. aureus* (38.1%) followed by *Bacillus* spp. (33.3%), *Escherichia coli* (9.5%), *Actinomyces* spp. (9.5%), *Serratia* spp. (4.8%) and *Pseudomonas aeruginosa* (4.8%). There was no statistically significant difference in the prevalence of bacteria isolates in milk samples between different productivity sheep breeds ($P > 0.05$). The results of antimicrobial susceptibility tests for β-lactamase positive *Staphylococcus aureus* isolates showed a high rate of resistance to ampicillin, cefalotin, cephalexin, gentamicin, streptomycin, erythromycin, oxytetracycline and sulphonamide with a percentage from 50.0 to 100.0. On the other hand, these isolates showed high sensitivity to amoxicillin/clavulanic acid (100.0%), methicillin (100.0%), cloxacillin (100.0%) and oxacillin (100.0%). The most effective antimicrobial agents against β-lactamase negative *S. aureus* isolates were methicillin (100.0%), cloxacillin (100.0%), cefalotin (100.0%), gentamicin (83.3%), erythromycin (83.3%), amoxicillin/clavulanic acid (66.6%) and streptomycin (66.6%). A high resistance rate to cephalexin, streptomycin, erythromycin, oxytetracycline and lincomycin was found in *E. coli* isolates.

Keywords: ewes, subclinical mastitis, microorganisms, antimicrobial susceptibility

Introduction

Mastitis is recognised as one of the most important diseases in ewes (Tormod et al., 2007). Although clinical cases of mastitis are a source of loss, more important economically is subclinical mastitis due its higher prevalence and associated decrease in milk production (Beheshti et al., 2010). Sheep's subclinical mastitis is characterised by quantitative and qualitative changes of milk, mainly through increased numbers of somatic cells. Especially high cell count is determined due to mammary infections caused predominantly by staphylococci (Bergonier, Berthelot 2003). The prevalence of subclinical sheep mastitis ranges between 7.05% and 92%, and it occurs worldwide (Ergun et al., 2009). The most frequently isolated microorganisms are staphylococci, streptococci and coliforms, but other microorganisms may infect the udder. Several reports indicate that coagulase-negative staphylococci (CNS) are the most common cause of subclinical mastitis in dairy ewes, while both CNS and *Staphylococcus aureus* (*S. aureus*) are frequent causes in meat sheep (Tormod et al., 2007). Infection of the mammary glands with *S. aureus* poses a number of different problems: first, the bacterium creates abscesses which provide the environment in which staphylococci survive well, and second, the bacterium has resistance mechanisms which make a number of routinely-used antibiotics ineffective (Abo-Shama, 2014).

Sheep with clinical mastitis are easily detected by inspection and palpation of teats and udder, and are, thus, treated. By contrast, animals with subclinical mastitis remain untreated because the disease may not be observed owing to the absence of macroscopic abnormalities in the udder and milk. Therefore, the use of laboratory assays is necessary to avoid persistent udder infection and the spread of the disease in ewes flocks (Miglio et al., 2013). Indirect tests, such as the California mastitis test (CMT), are among the methods most frequently used for diagnosing subclinical mastitis based on the increase in the number of somatic cells in milk from affected halves (Zafalon et al., 2016). Conventional methods for diagnosis of subclinical mastitis associate clinical evaluation of the udder with cyto-bacteriological examination of milk (Miglio et al., 2013).

Antimicrobial therapy is an important tool in the scheme of mastitis control, and the misuse or intensive use of antimicrobials can lead to the development of resistance among different bacterial strains and contamination of foodstuff with animal and human implications (Libera et al., 2010).

The aim of this study was to identify bacterial flora of subclinical mastitis and to determine antimicrobial resistance.

Material and methods

The study was carried out from December 2014 to August 2015 in 3 private Lithuanian ewes' farms. Ewes selected for this study were apparently healthy, and free of clinical mastitis and any other palpable udder lesion. All sheep were milked by hands. Twenty two Osfryz ewes (dairy breed), 10 meat productivity sheep breeds (Lithuanian black and Romanov) and 10 thin wool/meat productivity ewes breeds (Texel, Landrace) were included in this study. The milk samples were collected within 15–30 days after ewes lambing. California mastitis test (CMT) (Indirect diagnostic test, Krause, Denmark) was performed on all milk samples on the farms. Milk (3 mL) from each mammary half was mixed with a CMT reagent. After homogenisation, the samples were classified according to the degree of viscosity (Table 1).

Table 1. **Evaluation of subclinical mastitis by rapid CMT test**

Score	Milk viscosity	Somatic cell count (SCC) thousand/mL	SCC average thousand /mL
1	The consistency of the mixture is homogeneous, liquid, without visible change	<200	100
2	Forms slight flakes, which by turning the plate disappear	150−500	300
3 (+)	Clot is formed, the mixture viscosity is increased	400−1500	900
4 (++)	Viscous mixture, by turning the plate clot is visible, localised in one place	800−5000	2700
5 (+++)	Forms ropey, viscous mixture, significantly visible clot, pouring mixture falls of the plate	>5000	8100
1 – Negative reaction, 2 – trace, 3(+) – slightly positive, 4(++) – moderately positive, 5(+++) – strongly positive			

For bacteriological investigation, ewes were sampled before the morning milking and only CMT positive udder halves were sampled (n=23). Udder halves were cleaned and disinfected prior to sampling with 70% alcohol and dried with sterile cotton. The first 3 squirts of milk were discarded and approximately 5 mL of milk were taken in a sterile tube for bacteriological examinations.

From each milk sample, 100 µL was inoculated on 5% sheep blood agar (SBA), on MacConkey agar (Oxoid, England), on Pseudomonas cetrimide agar (Oxoid, England) and on Nutrition agar (Oxoid, England) plates. The Petri plates were incubated aerobically at +37°C and examined for growth between 24 and 48 hours. Bacterial strains were identified using routinely microbiological procedures such as colony morphology, microscopic characteristics and Gram staining (Diagnostica Merck, German), haemolysis pattern on 5% sheep blood agar, catalase and oxidase reactions. The isolates were identified to the species level by using API 20E system (BioMérieux, La Balme Les Grottes, France).

Pathogenic *Staphylococcus* strains were analysed for haemolysis pattern on 5% SBA, lecithinase activity on Baird Parker medium (Oxoid, UK) supplemented with 5% egg yolk tellurite emulsion (Oxoid, UK), DNase on toluidine blue-DNA agar (DNase, Italy), free coagulase and bound coagulase production (Staphylase test, France) by coagulase plasma-EDTA (Coagulase plasma-EDTA, France) and staphylase (Staphylase test, France) systems, following the manufacturer's instructions. Latex slide agglutination test (Staphytect plus kit, England) was conducted for the determination of the clumping factor, protein A and certain polysaccharides were exclusively found in *S. aureus* strains. Beta lactamase test (Liofilchem, Italy*)* was used to identify β-lactamase producing *S. aureus* isolates.

Antimicrobial susceptibility was determined by a disc diffusion method. Susceptibility testing was performed according to the recommendations of National Committee for Clinical Laboratory Standards (NCCLs, 2010). Five colonies of each bacteria isolates from Nutrition agar (Oxoid, England) were suspended in 9 mL sterile saline to a density approximately equal to 0.5 MacFarland opacity densities. The bacterial suspension was inoculated onto Mueller-Hilton agar (Oxoid, England). Then, the discs containing ampicillin (10 µg), amoxicillin/clavulanic acid (20 µg + 10 µg), cloxacillin (30 µg), methicillin (10 µg), gentamicin (10 µg), oxytetracycline (30 µg), sulfonamide (30 µg), streptomycin (10 µg), cefalotin (30 µg), cephalexin (30 µg), oxacillin (1 µg), lincomycin (30 µg), and erythromycin (15 µg) (Liofilchem, Italy) were applied. The Petri plate was assessed after 24 hours of incubation at +37ºC, and the diameter of the inhibition zone for each antimicrobial disc was committed in millimetres. The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2010), and intermediate results were classified as resistant. In addition, oxacillin was used to determine methicillin resistance of staphylococci.

Statistically significant associations were determined by the Student test (Microsoft Office Excel 2010 app). Results were considered statistically significant if $P \leq 0.05$.

Results

Subclinical mastitis and positive CMT results were detected in 20 ewes (47.6%) and in 23 udder halves (57.5%). The average prevalence of SCCs in milk samples was from 400,000 to 1,500,000 cells/mL. The prevalence of subclinical mastitis in ewes was higher (86.96%) in one half of the udder than in both sides of the udder (13.0%).

For bacteria isolation, 23 milk samples were tested bacteriologically. Bacteria were cultured from 21 samples (91.3%), and 2 samples (8.7%) yielded no bacterial growth. The microorganism isolation rate from ewes milk samples is shown in Figure 1. The most prevalent bacterial species from the mammary gland was *S. aureus* (38.1%, n=8). *S. aureus* as a primary pathogen was isolated from 28.6% (6) and in association with *Bacillus* spp. from 9.5% (2) of the milk samples. *Bacillus* spp. was the second most prevalent bacterial group isolated from the samples. Other bacteria isolated at a low frequency were *Escherichia coli, Actinomyces* spp, *Serratia* spp. and *Pseudomonas aeruginosa* representing 2, 2, 1 and 1 of isolated strains, respectively. There was no statistically significant difference of occurrence of isolated bacteria in milk samples (P > 0.05).

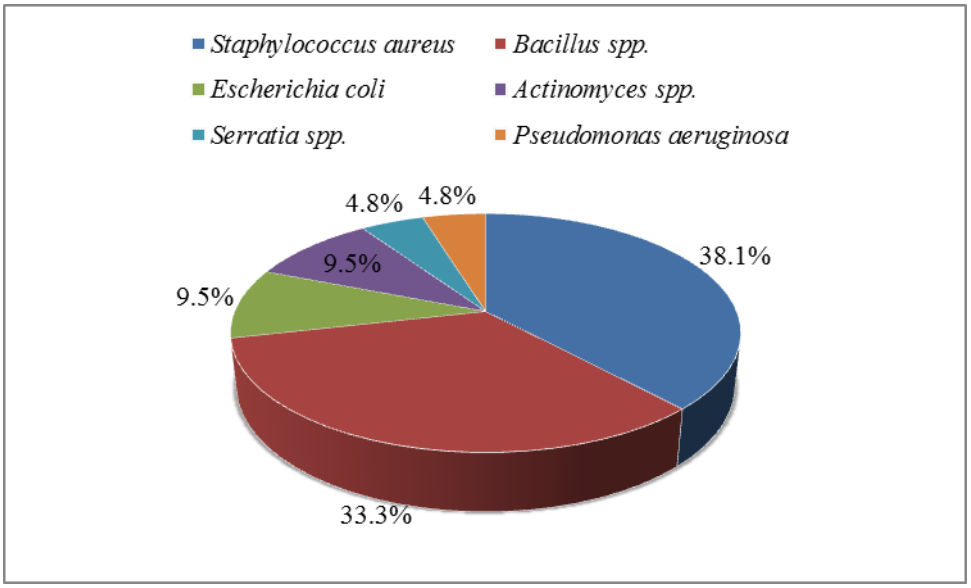

Figure 1. Bacteria strains isolated from ewes with subclinical mastitis

Five different bacterial genera were found in the milk samples of dairy ewes breeds: *Bacillus* spp. (42.9%, n=6), *S. aureus* (28.6%, n=4), *Escherichia coli* (14.3%, n=2), *Serratia* spp. (7.1%, n=1) and *Actinomyces* spp. (7.1%, n=1). Two bacterial genera were identified in meat sheep breeds: *S. aureus* (50.0%, n=1) and *Pseudomonas aeruginosa* (50.0%, n=1). *S. aureus* (60.0%, n=3), *Actinomyces* spp. (20.0%, n=1) and *Bacillus* spp. (20.0%, n=1) were isolated from wool-meat productivity direction ewes breeds. This difference was not statistically significant (P > 0.05).

Antimicrobial susceptibility of 8 *Staphylococcus aureus* isolates was evaluated using 13 antimicrobial agents. Two (25.0%) *S. aureus* isolates were positive for β-lactamase production, and 6 (75.0%) isolates were β-lactamase negative. Susceptibility studies disclose that all β-lactamase positive *S. aureus* isolates were most susceptible to amoxicillin/clavulanic acid (100.0%), methicillin (100.0%), cloxacillin (100.0%) and oxacillin (100.0%). Half (50.0%) of these isolates were found to be resistant to ampicillin, gentamicin, erythromycin and oxytetracycline (more than 3 antimicrobial classes). All (100.0%) β-lactamase producing isolates were resistant to cefalotin, cephalexin, streptomycin, lincomycin and sulphonamide.

The most effective antimicrobial agents against *S. aureus* strains that were negative for β-lactamase were methicillin (100.0%), cloxacillin (100.0%) and cefalotin (100.0%) followed by gentamicin (83.3%), erythromycin (83.3%), amoxicillin/clavulanic acid (66.6%), streptomycin (66.6%) and oxacillin (66.6%). Two (33.3%) of these strains were resistant to oxytetracycline and sulphonamide. Antimicrobial resistance to ampicillin and cephalexin was found in 100.0% of β-lactamase negative *S. aureus* strains.

E. coli isolates were most susceptible to gentamicin, cephalexin and sulphonamide in the ratio of 100.0%. Two (100.0%) *E. coli* isolates were resistant to cephalexin, streptomycin, erythromycin, oxytetracycline and lincomycin. *Pseudomonas aeruginosa* isolates were susceptible to gentamicin (100.0%) and resistant to the rest tested antimicrobial agents (100.0%). *Bacillus* spp. was susceptible to amoxicillin/clavulanic acid (100.0%), gentamicin (100.0%) and methicillin (100.0%). *Actinomyces* spp. was susceptible to amoxicillin/clavulanic acid (100.0%), streptomycin (100.0%), ampicillin (100.0%), cefalotin (100.0%), oxytetracycline (50.0%) and sulphonamide (50.0%). All (100.0%) isolates were resistant to methicillin, gentamicin, erythromycin, lincomycin and cephalexin.

Discussion

This study was accomplished to determine the prevalence of subclinical mastitis in ewes. CMT and microbiological examination were used to assess subclinical mastitis at the udder half level. In the present study, the prevalence of subclinical mastitis was reported as 47.6% for ewes and 57.5% for udders halves. These infection rates are comparable with those reported in Israel (55.0%) and the United States (51.1%) (Bor et al., 1989; Moroni et al., 2007). Lower prevalence rates of subclinical mastitis have been reported in Iran (20.3%), Italy (17.5%), England (12.0%) and Spain (34.6%) (Watkins et al., 1991; Albenzio et al., 2002; Batavani et al., 2003; Davasztabrizi et al., 2013). The highest subclinical mastitis prevalence rates have been reported in Portugal (92%) (Quiroga et al., 1997). In Lithuania, there are rather small ewes herds, which could explain the smaller number of subclinical mastitis in ewes. High stocking density, particularly in intensively managed herds, may be associated with large concentrations of microorganisms in environment (Persson, Olofsson 2011).

In the present study, 91.3% of the milk samples were simultaneously positive for a bacterial pathogen and somatic cells. Our data indicated that for the determination of the infection rate in a herd both microbiological status and SCC in milk should be taken into account, especially when prevalence within a herd is not known. CMT should still be considered an effective screening test for subclinical mastitis in ewes. Nine samples yielded no bacterial growth but had a high SCC value. The absence of bacterial growth on blood agar may be due to bacteria, such as mycoplasma, or due to non-bacterial causes, including physiological factors (Hariharan et al., 2004).

Although a wide range of microorganism species may cause sheep subclinical mastitis, most cases are reported to be due to staphylococci (Mørk et al., 2007). In the present study, subclinical mastitis was most frequently caused by *Staphylococcus aureus* (38.1%). *S. aureus* were the most common isolates from subclinical mastitis in ewes in Jordan and Iran (Lafi, Hailat, 1998; Narenji et al., 2015). Lafi and Hailat (1998) reported that *S. aureus* were the predominant bacteria strain (50.0%) in milk from ewes, followed by *E. coli* (27.0%) and *Pseudomonas aeruginosa* (7.0%). Narenji et al. (2015) reported that *Staphylococcus aureus* (72.2 %) and coagulase-negative staphylococci (66.6%) were the most common isolates from dairy ewes' subclinical mastitis. The main *S. aureus* reservoirs in sheep are suggested to be infected mammary glands and teat lesions. However, *S. aureus* can also be cultured from intact teat skin and other body sites (Scott, Jones 1998; Vautor et al., 2005). The main mechanism for spreading *S. aureus* in dairy ewes flocks was milking procedures. Lambs can promote the spread of *S. aureus* in flocks by their sucking other ewes than their dams (Bergonier et al., 2003; Tormod et al., 2007). The contamination of milkers' hands, washcloths, milking machine cups and bedding grounds may increase the incidence of *Staphylococcus aureus* mastitis (Lafi, Hailat 1998*).* In some countries, to reduce the reservoir of *S. aureus*, routine examination of teats and udders is performed after weaning, and ewes with palpable abnormalities or which have experienced clinical mastitis are usually slaughtered before the breeding season (Tormod et al., 2007).

Bacillus spp. (33.3%) was the second most prevalent bacterium isolated from the milk samples. The occurrence of *Bacillus* spp. in milk samples from ewes can be high, and the environment may play the main role. Some studies demonstrated that *Bacillus* spp. was predominant in milk samples from ewes on pasture (Hariharan et al., 2004). The prevalence of *Escherichia coli, Actinomyces* spp. *Serratia* spp. and *Pseudomonas aeruginosa* in the present study was low and lower than that reported by Zafalon et al. (2016) and Lafi and Hailat (1998).

The present study showed that the prevalence of resistance among *Staphylococcus* strains was generally high (33.3–100.0%), in comparison with studies reported in Turkey (Ergun et al., 2009) or Brazil (Libera et al., 2010). B-lactamase positive *S. aureus* isolates were most resistant to ampicillin, cefalotin, cephalexin, gentamicin, streptomycin, erythromycin, oxytetracycline, lincomycin and sulphonamide. B-lactamase negative *S. aureus* strains were commonly resistant to ampicillin, cephalexin, oxytetracycline and sulphonamide. These results were in agreement with the study of Ergun et al. (2009) who recorded that staphylococcus isolated from subclinical mastitis of sheep showed the highest resistance to penicillin (56.4%) and ampicillin (42.3%) followed by the resistance of isolates to tetracycline (24.4%), erythromycin (15.4%) and gentamycin (7.7%). Our findings were in disagreement with the studies of Abed and Hamim (2015) and Abo-Shama (2014). Abed and Hamim (2015) recorded that *Staphylococcus aureus* and isolates from clinical ewes mastitis showed the highest resistance to penicillin, oxacillin, ciprofloxacin, and amoxillin/clavulanic acid with a percentage of 100.0%, 82.0%, 75.5%, and 74.5%, respectively. Abo-Shama (2014) reported to have found the resistance of *Stapylococcus* spp. to amoxicillin/clavulanic acid, ampicillin, oxacillin and penicillin. The resistance of staphylococcus isolates may be due to the structural modification of enzymatic action (β-lactame action) or the prevention of access to target by altering the outer membrane permeability and may be due to the alternation of the antibiotic target site, and sometimes the resistance is due to the efflux pump which pumps out the antibiotic (Abed and Hamim 2015). In our study, 25.0% of *S. aureus* isolates were positive for β-lactamase production and half of them were resistant to more than 3 antimicrobial classes. Multi-drug resistance to antimicrobial drugs among *S. aureus* isolates complicates therapeutic management of subclinical mastitis infection. Our susceptibility studies disclose that subclinical mastitis caused by *S. aureus* isolates may be treated with amoxicillin/clavulanic acid, methicillin, cloxacillin, gentamicin and erythromycin.

Antimicrobial resistance represents a serious problem in the treatment of mastitis caused by *E. coli* (Hawari et al., 2014). In the present study, among the 2 *E. coli* included for antimicrobial resistance patterns, all were resistant to cephalexin, streptomycin, erythromycin, oxytetracycline and lincomycin. A previous study showed that *E. coli* isolated

from domestic animals (ewes were also included) were resistant to ampicillin (70.0%), tetracycline (75.0%), neomycin (75.0%), sulfamethaxazole (85.0%) and gentamicin (5.0%) (Hawari et al., 2014). Surveillance of drug resistance shows growing population of resistant bacteria to commonly used drugs in ewes' subclinical mastitis.

Conclusion

Subclinical mastitis and positive CMT results were detected in 20 ewes (47.6%) and in 23 udders halves (57.5%). The most common isolates from subclinical mastitis were *Staphylococcus aureus* (38.1%) followed by *Bacillus* spp. (33.3%), *Escherichia coli* (9.5%), *Actinomyces* spp. (9.5%), *Serratia* spp. (4.8%*)* and *Pseudomonas aeruginosa* (4.8%). B-lactamase positive *Staphylococcus aureus* isolates showed a high rate of resistance to ampicillin, cefalotin, cephalexin, gentamicin, streptomycin, erythromycin, oxytetracycline and sulphonamide with a percentage from 50.0 to 100.0. The highest rate of sensitivity was found to amoxicillin/clavulanic acid (100.0%), methicillin (100.0%), cloxacillin (100.0%) and oxacillin (100.0%). *Escherichia coli* were resistant to cephalexin, streptomycin, erythromycin, oxytetracycline and lincomycin.

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