Occurrence and Antibiotic Susceptibility Profile of *Streptococcus* spp. Isolated from Ewe's Milk

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Abstract: The purpose of this study was to determine the occurrence and antimicrobial resistance of *Streptococcus* spp. isolated from fresh ewe's milk and consequently to draw conclusions concerning the health condition of the animal flocks and the current trends of antibiotic use in small ruminants. A total of 77 mountainous sheepfolds were investigated representing 10% of all transhumant sheep and goats flocks in the area. Duplicate milk samples were collected from January to February transferred to the laboratory and analyzed for the presence of *Streptococcus* spp. by using the serial dilution method and plating on the surface of Slanetz and Bartley agar. The isolates were tested against nine antimicrobial agents with the Kirby-Bauer disk diffusion method. The results showed that *Streptococcus* spp. occurred in 84.4% of the samples and in counts ranged between 1.8 log CFU/mL and 4.6 log CFU/mL. Concerning susceptibility, only 1.3% of the isolates were resistant to all antimicrobials while 27.3% were fully susceptible. Resistance to cefepime was the most common (93.1%) observation followed by resistance to ampicillin (82.1%), ciprofloxacin (58.3%) and meropenem (51.9%). The isolates were vancomycin susceptible at a rate of 83.6%. Multidrug resistance to at least three antibiotics was observed to be 22.1% of *Streptococcus* spp.. Given the prevalence of *Streptococcus* spp. and the elevated resistance of isolates to various antibiotics it is concluded that further examinations and even interventions are required in order to pertain the appropriate animal health and milk quality.

Key words: Antibiotic, ewe's milk, *Streptococcus*.

1. Introduction

Ewe farming is an important branch of the national economy for many Mediterranean countries supporting the meat and dairy industry [1]. In Greece, ewe's and goat's milk is the key material for production of Greek yogurt, feta and other protected designation of origin (PDO) cheeses. Milk production in Greece has been estimated to be about 680,000 t/year, which is the second larger production among the European

countries [2].

Among the various factors which affect the milk quality [3], mastitis caused by bacteria resistant to various antimicrobial agents is undoubtedly the major one [4]. Mastitis in ewes, goat or bovines can be caused by many different bacterial species, the most common of which are *Staphylococcus* and *Streptococcus* genera [5]. Due to the variety of antimicrobials in use used for the prevention and treatment, an inevitable resistance to antibiotics is expected [6, 7]. As a result, various effects anticipated for the livestock health and production [8, 9] or for the public [10, 11]

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accompanied by an increased economic cost [12].

Streptococcal species are major mastitis pathogens along with *Staphylococcus aureus* and coliforms. Some of the *Streptococcus* spp., as for example *Streptococcus agalactiae* in cows are animal associated and well adapted to their mammary glands whereas others (*S. disgalactiae*, *S. uberis*, *S. bovis*, *S. oralis*, etc.) are environmental strains acting as opportunistic pathogens [13]. However, various studies have shown that such environmental streptococci are becoming increasingly resistant to many antimicrobial agents and are known to be reservoirs of resistant genes, transferring different resistant traits to more pathogenic organisms [14, 15].

Although several studies have been conducted on the antimicrobial resistance of streptococci [16-18], only limited data are available on multidrug resistance of various *Streptococcus* species from ewe's milk. The aim of this study was to study the occurrence of gram-positive bacteria belonging to the *Streptococcus* genus and to determine their resistance to various antibiotics.

2. Materials and Methods

2.1 Microbiological Determinations

A total of 10% of transhumant sheep and goats flocks, representing approximately 20,000 milked ewes, were investigated. Milk samples were collected from refrigerated tanks during the period of January and February 2012 just before the milk carried to the milk plant. Samples were collected from farms located in mountainous areas in Central Greece. Milking of the flock was supervised by team members. After a visual inspection of each animal, the udder exterior was washed and carefully dried with a clean towel and the teats swabbed with 70% alcohol. Duplicate milk samples were placed in an ice container and transferred to the laboratory for bacteriological examination.

2.2 Bacteriological Examination

The collected milk samples were placed in a water

bath at 36 °C for 30 min and afterwards they serially diluted in tubes containing sterile saline (0.9% NaCl). Aliquots of 100 µL were plated onto Petri dishes with Slanetz & Bartley agar and incubated initially at 36 °C. Four hours later the incubation temperature was raised to 44 °C and remained there for 48 h as an additional step towards selection of streptococci. The isolates were identified by their characteristic morphology as showed in Gram stain, catalase-negative reaction and esculin hydrolysis. Plates with isolates in a range of 25-250 were used for enumeration (CFU/mL of milk).

2.3 Antibiotic Sensitivity Testing

Antimicrobial resistance of *Streptococcus* spp. was tested using the disk diffusion method. Three to five isolates from each plate were harvested and diluted in sterile saline at a concentration of 0.5 turbidity units of the McFarland scale to obtain an inoculum with approximately 1.5×10^8 CFU/mL. The surface of agar plates contained cation-adjusted Muller-Hinton was inoculated with a sterile swap and the antibiotic disks were placed after 15 min. Subsequently, the plates were incubated aerobically for 16 h to 20 h at 35 °C and then the diameter of the inhibition zone was measured. The following disks with antibiotics were used: amoxicillin (25 µg), ampicillin (10 µg), cefepime (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), meropenem (10 µg), piperacillin (100 µg), piperacillin/tazobactam (100/10 µg) and vancomycin (10 µg).

The breakpoints used were those published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2012) for *Streptococcus* and/or *Enterococcus* spp.. Multidrug resistance was defined as resistance to three or more agents [19].

2.4 Statistical Analysis

Distribution of *Streptococcus* spp. isolates and antibiotic resistance are presented by frequency and percentages. Computational values are based on logarithmically transformed counts. Comparison of occurrence and susceptibility rates was performed by

using Fisher's exact chi-squared test. Significant levels were defined at $P < 0.05$.

3. Results and Discussion

During this study, 77 ewe's milk samples were examined for the presence of various *Streptococcus* spp.. Of all samples, 12 (15.6%) were negative regarding this genus and 65 (84.4%) were positive (Table 1). Counts in positive samples were ranged between 70 CFU/mL (1.8 log CFU/mL) and 40,000 CFU/mL (4.6 log CFU/mL). In general an average of 2.67 ± 1.3 log CFU/mL was recorded. These counts are consistent with those published by Kondyli et al. [3] who reported that the mean values of *Enterococcus* and *Streptococcus* combined in ewe's milk was between 3.13 log CFU/mL and 3.80 log CFU/mL. On the contrary, other investigators from European countries and elsewhere reported lower incidence patterns. El-Jakee et al. [20] reported a 50.4% of positive samples in Egypt among 270 tested ewe farms. Marogna et al. [4] reported that streptococci were the second most common finding in milk samples from Sardinia with *Streptococcus* spp. and *Enterococcus* spp. in combination isolated from 16.6% of the samples. In a similar study from Spain, both groups represented the 6% of all isolates [21] while the corresponding number from Scotland was 2.8% [22]. *Streptococcus bovis*, *S. suis* and *S. uberis* were isolated from six out of 354 ewe's milk samples examined from Arsenault et al. [23] in Quebec, Canada. The distribution of streptococci in another study from Italy ranged from 9.6% to 10.9% [24]. These findings suggest that a relatively low microbiological quality of raw ewe's milk is recorded in the study. Based on the literature this could be related to factors such as area, animal breed, stage of lactation, hygienic conditions of milk production and handling [24-27].

In this study, streptococci were tested for resistance to nine antibiotics belonging to six different classes. Table 2 presents the percentages of antibiotic resistant patterns for the isolates during this study. The most

effective antimicrobial agents were vancomycin and levofloxacin with a resistance of 9.1% and 1.2%, respectively. Amoxicillin and piperacillin from penicillins were effective enough (resistant at 19.2% and 14.8%) while ampicillin, cefepime, meropenem and ciprofloxacin were the least effective with a resistance rate of 82.1%, 93.1% and 58.3%, respectively. The data for streptococci resistance to antimicrobials in sheep's milk are not as extensive as for other pathogens, and for comparison data from cow's milk have often been used. Ebrahimi et al. [28] identified that streptococci isolated from cows showed high resistance to amoxicillin (76.9%) and ampicillin (61.5%-88.8%). The strains in the study were susceptible to piperacillin (48.1%) and amoxicillin (63.5%) but resistant to ampicillin (82.1%). In a retrospective study of Lollai et al. [29], *Streptococcus* isolates showed rates of resistance to ampicillin (up to 12.2%) comparable to those reported for the genus of cattle farms. El-Jakee et al. [20] reported a 20% and 60%

Table 1 Occurrence of *Streptococcus* spp. isolated from ewe's milk ($n = 77$).

Counts (CFU/mL)	Counts (log ₁₀ CFU/mL)	Number of samples	Percentage of samples
Non detected		12	15.6
1-100	0-2	6	7.8
101-500	2-2.7	13	16.9
501-1,000	2.7-3	7	9.0
1,001-5,000	3-3.7	23	29.9
5,001-10,000	3.7-4	12	15.6
> 10,000	> 4	4	5.2
Total		77	100

Table 2 Percentages of antibiotic resistance patterns of *Streptococcus* spp. isolated from ewe's milk.

Class	Antibiotic	R%	I%	S%
Penicillins	Amoxicillin	19.2	17.3	63.5
	Ampicillin	82.1	10.7	7.1
	Piperacillin	14.8	37.0	48.1
β-lactam/β-lactamase inhibitors	Piperacillin/tazobactam	35.2	42.6	22.2
Cephalosporin IV	Cefepime	93.1	0.0	6.9
Carbapenem	Meropenem	51.9	0.0	48.1
Fluoroquinolones	Ciprofloxacin	58.3	20.8	20.8
	Levofloxacin	1.8	0.0	98.2
Glycopeptides	Vancomycin	9.1	7.3	83.6

Table 3 Frequency of multidrug resistance of *Streptococcus* spp. isolated from ewe's milk.

Number of antibiotics	Frequency	%	Cumulative %
0	21	27.3	
1	12	15.6	15.6
2	18	23.4	38.9
3	9	11.7	50.6
4	10	13.0	63.6
5	5	6.5	70.1
6	1	1.3	71.4
7	1	1.3	72.7
Total	77	100.0	

of resistance to ampicillin and vancomycin, respectively, from Egypt. Moatamedi et al. [30] revealed susceptibility of streptococci to amoxicillin and ciprofloxacin. The increase of resistance to vancomycin observed in some of the studies already mentioned and also in a few European countries [31] is a matter of concern, but similar conclusions could not be drawn from the present study as most of the strains (83.6%) were proven to be susceptible to this agent.

In overall, only 27.3% (21/77) of the isolates of this study were susceptible to all antibiotic agents (Table 3). About 23.4% (18/77) were resistant to two antibiotics while multidrug resistance (to at least three agents) was observed for the 22.1% (17/77). Finally, isolates from only one milk sample showed to be resistant to all antibiotics.

4. Conclusions

Considering the elevated prevalence and overall resistance to antibiotics of *Streptococcus* spp. isolated from milk samples, further examinations and even interventions are required in the area in order to ensure both the animal health and the quality of the milk.

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