Antibiotic sensitivity of the causative microorganisms of subclinical mastitis in lactating sheep

Aleksandar Stoimenov*, Teodora Popova, Georgi Stoimenov, Kalin Hristov
University of Forestry, Faculty of Veterinary medicine, Sofia, Bulgaria
*E-mail: a.stoimeniv@ltu.bg

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Abstract

The aim of the presented research is to determine the antibiotic sensitivity and resistance of the microorganisms causing subclinical mastitis in dairy sheep. To achieve this, 120 milk samples from 4 farms located in 3 regions of the country were obtained and examined. The results showed that the isolates from the different farms were sensitive to Ciprofloxacin and Enrofloxacin, as well as to the combination Sulfamethoxazole+Trimethoprim. Resistance was found most often to Kanamycin, Colistin and antibiotics from the penicillin group.

Key words: antibiotic, sensitivity, sheep, subclinical, mastitis

Антибиотична чувствителност на микроорганизмите, причинители на субклинични мастити при лактиращи овце

Александър Стоименов*, Теодора Попова, Георги Стоименов, Калин Христов
Лесотехнически Университет, Факултет ветеринарна медицина, София
*E-mail: a.stoimeniv@ltu.bg

Абстракт

Целта на проведеното изследване бе установяване на чувствителността и резистентността на микроорганизмите, причинители на субклинични мастити при млечни породи овце, към антибиотици от различни групи. За осъществяването ѝ бяха получени и изследвани 120 млечни проби от 4 ферми, разположени в 3 региона на страната. Резултатите показаха, че изолатите от различните ферми са чувствителни към Ciprofloxacin и Enrofloxacin, както и към комбинацията Sulfamethoxazole+Trimethoprim. Резистентност се установи най-често към Kanamycin, Colistin и антибиотици от групата на пеницилините.

Ключови думи: антибиотична, чувствителност, овце, субклиничен, мастит

Introduction

Because of its high prevalence, subclinical mastitis causes significant losses to dairy sheep farming. Worldwide, the authors estimate the prevalence of this disease between 9% and 66% (Leitner et al., 2007; Vasileiou et al., 2018; Knuth et al., 2022). The widespread and in some cases

incorrect use of antibiotics to treat mastitis and various other diseases in sheep leads to the development of resistance of microorganisms to antibacterial drugs. In recent years, strains of antibiotic-resistant microorganisms have been recognized as an emerging threat to public health (Ventola, 2015). This resistance is a major reason for the unsatisfactory results observed in the treatment of subclinical mastitis in this animal species.

Studies have shown that most often the bacteria causing subclinical mastitis show sensitivity to Doxycycline, Levofloxacin, Ciprofloxacin, Vancomycin and Sulfamethoxazole-Trimethoprim (Abed et al., 2022). From the research carried out, it is known that the most frequently isolated causative agents of this disease are resistant to penicillin antibiotics (Ebrahimi et al., 2007; Azzi et al., 2020; Vezina et al., 2022). In recent years, there has also been an increasing resistance of pathogens to second-generation cephalosporins (Abed et al., 2022; Vezina et al., 2022). A detailed study of the antibiotic sensitivity of the microorganisms causing this disease would help to reduce the losses and its successful treatment.

Material and methods

Studied animals

In order to determine the antibiotic sensitivity of pathogenic microorganisms, we collected and processed milk samples from four farms – farm A, B, C and D. Before sampling, all animals underwent a clinical examination, after which 120 milk samples were obtained from ewes without clinical signs of mastitis. All immunoprophylactic and antiparasitic measures were carried out in the farms according to the normative veterinary medical requirements and the specific health status of the animals. The farms were of different sizes and farming methods. In Farm A, sheep of the Tsigai breed were bred. The farming system was semi-intensive. On farm B, the animals bred were of the Asaf breed. The farm system was intensive. In the third studied farm (C), the sheep bred were of the Bulgarian dairy synthetic population, raised semi-intensively. The animals from farm D were of the Lacon breed, the breeding was intensive. The age of the examined animals from all farms was between 3 and 5 years, and the lactation period was between 8 and 10 weeks.

Sample collection

The milk samples were obtained aseptically from all 120 udder halves. Before the sampling, the mammary gland and papillae were cleaned of contaminants, followed by dipping of the papillae with 70° alcohol. From each half, after removal of the first jets of milk, we took double samples in sterile 10 ml test tubes for microbiological examination. The milk samples were stored and transported to the laboratories in a cooler at a temperature of 4 °C, and the examination of the same was carried out up to 4 hours after their collection.

Microbiological analysis of the samples

To isolate and identify the microorganisms causing subclinical mastitis, cultures were made from the milk samples on selective and elective nutrient media - Colorex Chromogenic Orientation Candida agar (HiMeida Laboratories Pvt. Ltd. Mumbai India), Columbia blood agar, also agars of Chapman, Endo, Eosin methylene blue and Mueller - Hinton. The results were reported after incubation under aerobic conditions at 37 °C for 48–72 hours.

In order to perform the taxonomic identification of the isolated microorganisms, we used a microscopic examination by staining according to the classical methods of Gram, Pfeiffer, Klet and Moeller. Taxonomic identification of all isolates was performed by conventional methods according to the Bergey's Manual of Determinative Bacteriology (Guerrero, 2001). The determination of the cultural and hemolytic properties was perform on solid and liquid media. The biochemical characterisation of the pathogens was made by using Polymicrotest (National Centre for Infectious and Parasitic Diseases, Sofia, Bulgaria) and STAPHYtest 24 (Erba Mannheim).

Antimicrobial agents and determination of isolates sensitivity

The determination of the antibiotic sensitivity of the isolated microorganisms was performed according to the classical agar-gel diffusion method of Bauer et al. (1966). Standard disks for antibioticograms (Bul-Bio - Sofia) were used, well as prepared by us, after inoculation of bacterial suspensions in exponential growth phase with a concentration of 2.106 cells/ml, determined by the Mac Farland optical standard, on blood agar (Bul-Bio - Sofia) or Mueller - Hinton agar (Antisel - Sharlau Chemie S. A., Spain). Cultivation was performed at 37 °C for 24 hours. The results were interpreted according to the three-step system of Bauer et al. (1966) after measuring the diameters of the inhibitor zones in millimeters.

Results and discussion

The sensitivity of the microorganisms isolated from farm A to antimicrobial agents in vitro is presented in Table 1. In this farm *S. aureus ssp. aureus*, *S. epidermidis* and *Enterococcus sp.* were identify as the causative agent of subclinical mastitis. The microorganisms showed significant sensitivity to Chloramphenicol, Tetracycline, Enrofloxacin, Ciprofloxacin and Sulfamethoxazole+Trimethoprim. Resistance was found to Kanamycin, Oxacillin, Ampicillin, Gentamicin and some penicillin antibiotics. This resistance is most likely due to the widespread use of these antibiotics in veterinary practice. Our results support the study of Ebrahimi et al. (2007) who found similar resistance in *S. aureus ssp. aureus* to antibiotics. Lollai et al. (2008) also found that Ampicillin was ineffective in 2–12% of bacterial isolates from sheep with subclinical mastitis.

Results showing the sensitivity of the bacteria isolated from the milk samples obtained from farm B to antimicrobial agents in vitro are presented in Table 2. The sensi-

Table 1. Sensitivity of the isolated bacteria from farm A to antimicrobial agents *in vitro*
Таблица 1. Чувствителност на изолираните бактерии от ферма А към антимикробни средства *in vitro*

Antimicrobial agent	Disc concentration (μg/ disc)	Inhibitory zones in mm and strain sensitivity		
		S. aure us ssp. aureus	*S. epidermidis*	*Enterococcus sp*
Chloramphemicol	30 μg	22.2 ± 1.33 (S)	28.5 ± 7.5 (S)	30 ± 0.5 (S)
Tetracycline	30 μg	26.8 ± 2.78 (S)	32.5 ± 9.5 (S)	40 ± 1.5 (S)
Clindamycin	10 μg	21.6 ± 1.85 (S)	30 ± 13 (S)	29 ± 2.5 (S)
Penicillin	10 μg	23.4 ± 11.7 (R)	25 ± 17 (R)	40 ± 2.0 (S)
Oxacillin	1 μg	12.2 ± 3.18 (R)	24.5 ± 16.5 (S)	18 ± 2.5 (S)
Ampicillin	30 μg	22.2 ± 12.4 (R)	26 ± 15 (I)	40 ± 3.0 (S)
Amoxycillin	10 μg	22.6 ± 13.7 (S)	14 ± 8 (R)	42 ± 4 (R)
Cefuroxim	30 μg	16.2 ± 8.1 (I)	26 ± 17 (S)	41 ± 3.5 (S)
Ceftriaxone	30 μg	17.2 ± 3.9 (I)	28.5 ± 13.5 (S)	41 ± 3.0 (S)
Novobiocin	30 μg	23.6 ± 2.87 (S)	31.5 ± 13.5 (S)	8 ± 1.5 (S)
Gentamicin	10 μg	14.4 ± 3.8 (S)	24 ± 14 (S)	31 ± 4 (R)
Kanamycin	5 μg	9.4 ± 1.6 (R)	17 ± 7 (S)	19 ± 2.0 (S)
Enrofloxacin	5 μg	31 ± 1.8 (S)	37.5 ± 7.5 (S)	38 ± 3 (S)
Ciprofloxacin	5 μg	28.8 ± 1.3 (S)	35 ± 7 (S)	40 ± 5.0 (S)
Sulfamethoxazole+ Trimethoprim	23.75/1.25 μg	25.6 ± 7.2 (S)	32 ± 10 (S)	42 ± 7.0 (S)

S (sensitive); I (intermediate); R (resistant)

tivity of the isolated microorganisms from this study was most significant to Gentamicin, Enrofloxacin, Ciprofloxacin and Sulfamethoxazole+Trimethoprim. Resistance of the microorganisms from the second farm (farm B) was established to Doxycycline, Kanamycin and Colistin, as well as to some of the penicillin and cephalosporin antibiotics. In the milk samples from this farm, we also isolated pathogens intermediate sensitive to Ceftriaxone, a representative of the third generation cephalosporins. Most published works report resistance to second-generation representatives (Azzi et al., 2020; Katsarou et al., 2021; Abed et al., 2022). This result is unfavorable regarding the future use and efficacy of antibiotics of this group. The results show that *S. epidermidis* shows the lowest sensitivity to the tested antibiotics (to 6/15), while *Str. sanguinis* shows the highest one (at 10/15).

The sensitivity of the isolated bacteria of all established species to antimicrobial agents from the third investigated farm (C) in vitro is presented in Table 3. All the microorganisms were sensitive to Tetracycline, Clindamycine, Ampiciline, Enrofloxacin, Ciprofloxacin and Sulfamethoxazole+Trimethoprim. The isolates showed the most significant resistance to Novobiocin and Kanamycin. Most of the pathogens are also resistant to Penicillin and Amoxycillin. Intermediate sensitivity was found to Chloramphemicol, Cefuroxim and Ceftriaxone, which is in line with the results of previous farms. From the studies carried out on the resistance of the pathogens isolated from this farm, we came to the conclusion that the most serious resistance to antibiotic preparations was shown by *S. xylosus* and *Staphylococcus hyicus*, followed by *S. epidermidis* and *S. chromogenes.*

Table 2. Sensitivity of the isolated bacteria from farm B to antimicrobial agents *in vitro*
Таблица 2. Чувствителност на изолираните бактерии от ферма В към антимикробни средства *in vitro*

Antimicrobial agent	Disc concentration (μg/disc)	Inhibitory zones in mm and strain sensitivity				
		Bacillus spp.	*Dermatococcus nishinomiyaensis*	*S. chromogenes*	*S. epidermidis*	*Str. sanguinis*
Doxycycline	30 μg	10.3 ± 3.2 (R)	31 ± 3.5 (S)	10.5 ± 2.8 (R)	33.4 ± 4.2 (S)	11.5 ± 1.4 (R)
Penicillin	10 u	13 ± 4.7 (R)	38.4 ± 7.2 (S)	27 ± 3.1 (R)	9 ± 3.7 (R)	38 ± 3.2 (S)
Oxacillin	1 μg	9.6 ± 1.7 (R)	12.6 ± 3.8 (I)	12.4 ± 2.7 (I)	7.4 ± 2.4 (R)	15 ± 1.5 (S)
Ampicillin	30 μg	12 ± 1.7 (I)	36.1 ± 5.2 (S)	30.1 ± 4.6 (S)	7.1 ± 1.4 (R)	35.5 ± 2.5 (S)
Amoxycillin	10 μg	27.4 ± 5.8 (I)	34.9 ± 6.5 (S)	14.7 ± 5.7 (R)	15.1 ± 2.9 (R)	32.4 ± 2.8 (S)
Amoxycillin/Clav	10 μg	28 ± 4.7 (S)	28 ± 3.9 (S)	20 ± 7.3 (I)	13.3 ± 4.4 (R)	32 ± 3.5 (S)
Cefotaxime	30 μg	25.8 ± 4.3 (S)	7.2 ± 1.5 (R)	15.8 ± 3.1 (S)	10.2 ± 2.1 (R)	11.5 ± 2.4 (R)
Ceftriaxone	30 μg	28.3 ± 7.2 (S)	20 ± 1.9 (I)	27.2 ± 2.7 (S)	30.1 ± 3.5 (S)	20.8 ± 2.8 (I)
Novobiocin	30 μg	30.5 ± 2.5 (S)	17.6 ± 2.9 (R)	31.2 ± 3.1 (S)	20.4 ± 1.9 (I)	34.3 ± 1.6 (S)
Gentamicin	10 μg	20 ± 3.1 (S)	20.9 ± 1.7 (S)	20 ± 2.3 (S)	24.4 ± 1.5 (S)	24.3 ± 2.1 (S)
Kanamycin	5 μg	8.7 ± 1.4 (R)	13 ± 2.4 (R)	13.1 ± 1.9 (I)	10.1 ± 0.9 (R)	16 ± 1 (I)
Colistin	10 μg	14.2 ± 0.7 (I)	6 ± 0.5 (R)	8.1 ± 1.3 (R)	14.3 ± 3.2 (I)	8 ± 1.5 (R)
Enrofloxacin	5 μg	27.9 ± 3.1 (S)	38.1 ± 3.9 (S)	28.4 ± 3.7 (S)	32 ± 2.7 (S)	31.4 ± 3.3 (S)
Ciprofloxacin	5 μg	29.2 ± 2.8 (S)	25.8 ± 3.7 (S)	24.9 ± 2.9 (S)	33.1 ± 1.6 (S)	29.4 ± 2.7 (S)
Sulfamethoxazole+ Trimethoprim	23.75/1.25 μg	21.3 ± 2.2 (S)	32.4 ± 3.8 (S)	27.4 ± 3.1 (S)	30.5 ± 3.5 (S)	28.5 ± 2.5 (S)

S (sensitive); I (intermediate); R (resistant)

Table 3. Sensitivity of the isolated bacteria from farm C to antimicrobial agents *in vitro*
Таблица 3. Чувствителност на изолираните бактерии от ферма С към антимикробни средства *in vitro*

Antimicrobial agent	Disc concentration (μg/disc)	Inhibitory zones in mm and strain sensitivity			
		S. xylosus	*S. epidermidis*	*S. chromogenes*	*Staphylococcus hyicus*
Chloramphemicol	30 μg	15.7 + 5.05 (I)	14.2 ± 4.5 (I)	30 (S)	12 (R)
Tetracycline	30 μg	26.8 ± 2.78 (S)	32.5 ± 9.5 (S)	40 (S)	24.2 (S)
Clindamycin	10 μg	21.6 ± 1.85 (S)	30 ± 13 (S)	29 (S)	23.4 (S)
Penicillin	10 u	23.4 ± 11.7 (R)	25 ± 17 (R)	40 (S)	15.3 (R)
Oxacillin	1 μg	12.2 ± 3.18 (I)	24.5 ± 16.5(S)	18 (S)	21.2 (S)
Ampicillin	30 μg	22.2 ± 12.4 (S)	26 ± 15 (S)	40 (S)	18.2 (S)
Amoxycillin	10 μg	22.6 ± 13.7 (S)	14 ± 8 (R)	11 (R)	15.7 (R)
Cefuroxim	30 μg	16.2 ± 8.1 (I)	26 ± 17 (S)	41 (S)	15.4 (I)
Ceftriaxone	30 μg	17.2 ± 3.9 (I)	28.5 ± 13.5 (S)	41 (S)	16.2 (I)
Novobiocin	30 μg	14.6 ± 3.27 (R)	13.5 ± 1.5 (R)	13.2 (R)	10 (R)
Gentamicin	10 μg	25.4 ± 3.8 (S)	27 ± 5.1 (S)	31.4 (S)	24.3 (S)
Kanamycin	5 μg	9.4 ± 1.6 (R)	17 ± 7 (I)	18 (I)	13 (R)
Enrofloxacin	5 μg	32 ± 2.8 (S)	36.5 ± 2.5 (S)	35 (S)	32 (S)
Ciprofloxacin	5 μg	34.5 ± 2.3 (S)	33 ± 2.3 (S)	31.8 (S)	32.2 (S)
Sulfamethoxazole+ Trimethoprim	23.75/1.25 μg	40.6 ± 5.2 (S)	22 ± 4.3 (S)	20 (S)	17 (S)

S (sensitive); I (intermediate); R (resistant)

Table 4 presents the results of the fourth investigated farm. The performed antibioticograms of the microorganisms from this farm showed the highest resistance of the isolated pathogens to antibiotics from different groups. From this study, the resistance of a number of bacteria to the Sulfamethoxazole+Trimethoprim combination, which showed high efficiency in the pathogens from the other farms, as well as in the studies of Hristov (2018) in goats and Azzi et al. (2020) and Abed et al. (2022) in sheep, was noticed. And in this farm, all the tested strains were sensitive to Enrofloxacin and Ciprofloxacin. We also observed that many microorganisms were resistant to 4 or more antibiotics, the most resistant being *S. aureus ssp. aureus* followed by *Staphylococcus hyicus*. This is most likely due to the excessive use of antibiotic drugs on the farm and failure to complete the therapeutic courses.

Conclusions

The causative agents of subclinical mastitis in sheep show the most significant sensitivity to Ciprofloxacin and Enrofloxacin, as well as to the

Table 4. Sensitivity of the isolated bacteria from farm D to antimicrobial agents *in vitro*
Таблица 4. Чувствителност на изолираните бактерии от ферма D към антимикробни средства *in vitro*

Antimicrobial agent	Disc concentration (µg/disc)	Inhibitory zones in mm and strain sensitivity					
		Aerococ-cus viridans	*S. aureus ssp. aureus*	*S. warneri*	*S. hominis ssp. hominis*	*S. hyicus*	*Streptococ-cus sanguinis*
Chloramphemicol	30 µg	21.3 ± 2.2(R)	14 ± 4.6 (I)	20.1 ± 3.2 (S)	24.6±5.2 (S)	14.2 (I)	15 (I)
Tetracycline	30 µg	-	-	-	-	-	-
Doxycycline	30 µg	21.5 ± 0.7 (S)	19.4 ± 5.1 (S)	15.2 ± 2.4 (I)	27.4 ± 3.8 (S)	10.7 (R)	14.5 (I)
Penicillin	10 u	22 ± 7.2 (I)	8.8 ± 3.1 (R)	7.7 ± 0.6 (R)	8.1 ± 1.7 (R)	24.8 (I)	25.3 (S)
Ampicillin	30 µg	25 ± 5.5 (S)	7.1 ± 2.9 (R)	24.4 ± 3.7 (S)	23.6 ± 2.2 (S)	6.5 (R)	23 (S)
Amoxycillin	10 µg	17±5.5 (R)	9.1 ± 3.9 (R)	20 ± 2.3 (S)	20 ± 1.8 (S)	18 (R)	10.4 (R)
Cefuroxim	30 µg	9 ± 3.3 (R)	7.4 ± 1.3 (R)	21.8 ± 4.6 (S)	21.6 ± 1.3 (S)	10.6 (R)	25.3 (S)
Ceftriaxone	30 µg	5.5 ± 1.7 (R)	6.5 ± 1.5 (R)	22.5 ± 2.3 (S)	16.4 ± 3.7 (I)	13.5 (R)	17.4 (I)
Novobiocin	30 µg	10 ± 1.2 (R)	8.1 ± 2.1 (R)	12.2 ± 0.7 (R)	12.5 ± 1.4 (R)	22.9 (S)	12.7 (R)
Gentamicin	10 µg	23.5 ± 1.5 (S)	20 ± 4.7 (S)	23.1 ± 2.6 (S)	18 ± 1.8 (S)	31 (S)	18 (S)
Colistin	10 µg	10 ± 5.6 (I)	6.3 ± 0.7 (R)	15.1 ± 0.9 (S)	6.9 ± 0.8 (R)	14.6 (S)	6.4 (R)
Kanamycin	5 µg	6.7 ± 0.8 (R)	6.9 ± 1.1 (R)	8.8 ± 1.2 (R)	7.1 ± 0.9 (R)	15.3 (I)	6.3 (R)
Enrofloxacin	5 µg	28.4 ± 1.2 (S)	30.3±5.8 (S)	30 ± 5.8 (S)	27.4 ± 5.2 (S)	26.4 (S)	25.5 (S)
Ciprofloxacin	5 µg	27.2 ± 2.2 (S)	28.4 ± 4.5 (S)	28 ± 4.1 (S)	25 ± 3.8 (S)	27.7 (S)	27.8 (S)
Sulfamethoxazole+ rimethoprim	23.75/ 1.25 µg	9.8 ± 5.2 (R)	7.4 ± 1.7 (R)	9.2 ± 2.9 (R)	6.9 ± 0.5 (R)	12.7 (I)	6.4 (R)

S (sensitive); I (intermediate); R (resistant)

combination Sulfamethoxazole+Trimethoprim. Most often, these pathogens show resistance to Penicillin, Amoxycillin, Kanamycin and Colistin. The presence of resistance to second and third generation cephalosporins is unfavorable.

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