

Research article

DETECTION OF SEVERAL VIRULENCE PROPERTIES, ANTIBIOTIC RESISTANCE AND PHYLOGENETIC RELATIONSHIP IN *E. COLI* ISOLATES ORIGINATED FROM COW MASTITIS

CENGİZ Seyda^{1*}, DİNÇ Gökçen², SÖĞÜT Mehtap Ünlü³¹Department of Microbiology Faculty of Veterinary Medicine, University of Atatürk, Erzurum;²Department of Medical Microbiology Faculty of Medicine, University of Erciyes, Kayseri;³High School of Health, University of Ondokuz Mayıs, Samsun

(Received 21 February; Accepted 09 September 2014)

Cow mastitis caused by *Escherichia coli* (*E. coli*) exhibits various local and systemic clinical signs at varying degrees of severity. The aim of this study was to elucidate the virulence properties, antibiotic resistance and phylogenetics of 56 *E. coli* strains. Of all the studied strains, 12 were positive for hemolytic properties and 38 were positive for biofilm production. Additionally, 55 of the strains were positive for multiple resistances in bacteriological tests. PCR analysis revealed that 42 strains carried the *traT* gene, 20 strains had the shiga toxin gene (*stx1-stx2*), and 8 strains carried the intimin gene (*eae*), but all strains were negative for aerobactin gene (*aer*). All strains encoding shiga toxin genes were also positive for *stx1*, but only 4 strains were positive for *stx2*. There were no significant differences in virulence genes between antibiotic-resistant and antibiotic-susceptible strains. The random amplified polymorphic DNA-polymerase chain reaction patterns revealed the existence of 13 main groups with 4 subgroups of *E. coli*. In this study, *E. coli* strains causing intramammary infections and originating from various sources might show resistance against common antibiotics. Pathogenicity of *E. coli* that cause clinical mastitis, and prognosis of the infection might be predicted by obtaining the *traT* gene. Additionally, antibiotic resistance should be investigated at the genomic level to detect the relationship between virulence factors and antibiotic resistance. In field conditions, development of antibiotic resistance is the main cause of mastitis treatment failure. Thus, antibiotic resistance profiles in herds should be monitored and effective antibiotics should be administered.

Key words: *Escherichia coli*; virulence properties; antibiotic resistance; phylogenetic-relationship; cow mastitis

INTRODUCTION

Mastitis reduces milk yield, increases health cost and makes milk less suitable for both consumption and processing [1]. Pathogenic microorganisms that most frequently

Corresponding author: e-mail: seydacengiz@atauni.edu.tr

cause mastitis can be divided into two groups based on their source: environmental and contagious. Today, while the use of antibiotics has reduced the frequency of contagious mastitis pathogens, environmental bacteria, especially coliform bacteria continue to be the cause of mammary infections.

Escherichia coli, environmental bacteria, mainly cause infection and inflammation of the mammary gland in dairy cows around parturition and during early lactation with striking local and sometimes severe systemic clinical symptoms. This environmental type of mastitis does not only reduce the productivity of a high-yielding dairy herd, but also can cause severe clinical symptoms, characterized by local mammary symptoms. Although, several virulence factors, which contribute to the occurrence of intramammary infections, are defined for *E. coli*, none of these factors are considered specific. Investigations have focused on several factors: the *traT* gene which is involved in serum resistance and protection against bacterial inactivation, aerobactin gene which enables iron use by bacteria, toxin structures (Shiga toxins, CNF1, CNF2, labile toxins, stable toxin) and intimin gene which determine bacterial pathogenesis through various toxins [2, 3]. Besides these virulence properties, biofilms can be defined as communities of microorganisms attached to a surface.

Antibiotic resistance frequently develops due to excessive and inappropriate antibiotic use in herds and becomes an important problem for management. The various genes encode both single and multiple antibiotic resistance. It has been reported that plasmids carrier genes may promote bacterial antibiotic resistance and can frequently be transmitted from one bacterium to another (even of different species) via horizontal gene transfer. There are large variations in levels of antibiotic resistance in *E. coli* strains isolated from mastitis [3, 4]. Random Amplified Polymorphic DNA (RAPD), which allows typing of bacterial isolates, is the commonly preferred method for the molecular investigation of bacteria in epidemiological studies. RAPD PCR is based on genetic linkage and mapping between species [5,6].

The aims of this study were to determine biofilm and hemolysis structures, *traT* gene, aerobactin gene, shiga toxin, intimin gene and to identify genetic similarities using RAPD PCR in 56 *E. coli* strains isolated from cows with mastitis.

MATERIAL AND METHODS

A total of 56 *E. coli* strains isolated from cows with mastitis were investigated. The strains were passaged on blood agar before being incubated at 37°C for 24-48 hours. The strains, with confirmed biochemical properties (catalase, oxidase, carbohydrate fermentation tests, IMVIC, indole, metil red, voges-proskauer, and citrate tests), were stored for conventional investigations at -20°C. DNA extraction was conducted for molecular analysis according to the manufacturer's protocol based on a filtration system with spin colon (Qiamp DNA mini kit-Qiagen).

Detection of biofilm production

Biofilm production was detected with the Congo red agar method [7], where bacteria were cultured for single colonies and incubated at 37°C for 24 hours. Black colonies were evaluated as slime factor positive, while colorless or pink colonies were evaluated as slime factor negative.

Antibiogram test

Antibiotic susceptibility tests were performed by the Agar Disc Diffusion method, according to the Clinical Laboratory Standards Guide [8]. *E. coli* strains were grown on Trypticase Soy Agar (TSA) for 24 hours at 37°C, and then used to prepare the respective inocula. The cells were suspended in saline solution. Turbidity was adjusted to 0.5 McFarland standard (about 10⁸ CFU/mL), and used as the inoculum for the antibiotic susceptibility tests. Resistance was evaluated according to CLSI (2013) as susceptible (S), intermediate resistant (I) or resistant (R).

PCR reactions

The protocol reported by Abd El-Razik *et al.* (2010) was modified and optimized for identification of *E. coli* isolates. All reactions were carried out in a final volume of 50 µl. Volumes of 200 ng of extracted DNA template, 5 µM primer (specific primer for *E. coli*; Table 1) and 25 µl of Taq PCR Master Mix (Taq PCR Master Mix Kit, Cat no 201443, Qiagen®) were added to a 0.5 ml microcentrifuge tube. A pre-PCR step at 95°C for 2 minutes was applied. A total of 35 PCR cycles were run under the following conditions: denaturation at 94°C for 45 seconds, annealing for 45 seconds, and extension at 72°C for 45 seconds. After the final cycle, the preparation was kept at 72°C for 10 minutes to complete the reaction. The PCR products were analyzed by electrophoresis on 1.5% agarose.

PCR detection of virulence genes

PCR mixes were prepared for four *E. coli* virulence genes: traT (outer membrane protein), aer (aerobactin), stx (shiga toxin) and eae (intimin).

The traT PCR assay was carried out in a total volume of 50 µl of mixture containing PCR buffer (Promega, Madison, WI, USA), 2.5 mM of MgCl₂, 250 µM each of the deoxynucleoside triphosphates, 0.5 µM each of the virulence gene-specific primers, 1.25 U of Taq polymerase (Promega) and 5 µl of template DNA. The amplification conditions included 25 cycles of denaturation at 95°C for 2 minutes, primer annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. The final extension step was 10 minutes at 70°C [10].

For aer, the PCR assay was carried out in a total volume of 25 µl of mixture containing PCR buffer, 1.5 mM of MgCl₂, 200 µM each of the deoxynucleoside triphosphates, 0.4 µM each of the virulence gene-specific primers, 1 U of Taq polymerase and 5 µl of

template DNA. Following pre-denaturation at 94°C for 60 seconds, the amplification conditions included 30 cycles of denaturation at 94°C for 60 seconds, primer annealing at 63°C for 30 seconds, and extension at 70°C for 90 seconds. The final extension step was 5 minutes at 70°C [11].

For *stx1* and *stx2*, PCR assay was carried out in a total volume of 50 µl of mixture containing PCR buffer, 2 mM of MgCl₂, 200 µM each of the deoxynucleoside triphosphates, 0.25 µM each of the virulence gene-specific primers, 1 U of Taq polymerase (Promega) and 5 µl of template DNA. The amplification conditions included 35 cycles of the first denaturation step at 94°C for 10 minutes, denaturation at 94°C for 60 seconds, primer annealing at 60°C for 60 seconds, and extension at 72°C for 60 seconds. The final extension step was 7 minutes at 72°C [12].

The PCR assay to detect the intimin (*eae*) gene was performed in a total volume of 50 µl of mixture containing PCR buffer, 1.5 mM of MgCl₂, 250 µM each of the deoxynucleoside triphosphates, 0.5 µM each of the virulence gene-specific primers, 1.25 U of Taq polymerase and 5 µl of template DNA. The amplification conditions included 25 cycles of denaturation at 94°C for 30 seconds, primer annealing at 50°C for 45 seconds, and extension at 70°C for 90 seconds. The extension time was ramped for an additional 3 seconds per cycle, followed by a final extension step of 10 minutes at 70°C. The PCR products were analyzed by electrophoresis on 1.5% agarose gel, after which the gel was stained with ethidium bromide and photographed [13].

Oligonucleotide sequences encoding virulence genes of the *E.coli* isolates and expected band sizes are displayed in Table 1.

Table 1. Oligonucleotide sequences of the primers used in the detection of virulence properties

Target		Oligonucleotide sequences (5'-3')	PCR product (bp)	References
<i>E. coli</i>	F	GCTTGACACTGAACATTGAG	662	Abd El-Razik et al. (2010)
	R	GCATTATCTCTTCCGCATT		
<i>eae</i>	F	ATA TCC GTT TTA ATG GCT ATC T	425	Güler and Gündüz (2007)
	R	AAT CTT CTG CGT ACT GTG TTC A		
<i>aer</i>	F	TACCGGATTGTCATATGCAGACCGT	602	Oliveira et al. (2011)
	R	AATATCTTCCCTCCAGTCCGGAGAAG		
<i>traT</i>	F	GATGGCTGAACCGTGGTTATG	307	Kaipainen et al. (2002)
	R	CACACGGGTCTGGTATTTATGC		
<i>Stx1</i>	F	ATAAATCGCCATTCGTTGACTAC	180	Fitzmaurice, (2003)
	R	AGAACGCCCACTGAGATCATC		
<i>Stx2</i>	F	GGCACTGTCTGAAACTGCTCC	255	Fitzmaurice, (2003)
	R	TCGCCAGTTATCTGACATTCTG		

Genotyping of isolates

The extracted DNA was genotyped using the modified ERIC PCR (5'-AAG TAA GTG ACT GGG GTG AGC G-3') reaction described by Versalovic *et al.*[14]. The RAPD PCR assay was carried out in a total volume of 25 µl of mixture containing PCR buffer, 2.5 mM of MgCl₂, 200 µM each of the deoxynucleoside triphosphates, 25 pM of primer, 2.5 U of Taq polymerase and 5 µl of template DNA. Following pre-denaturation for 4 minutes at 94°C, the thermal cycling programme consisted of denaturation for 1 minute at 94 °C, annealing for 1 minute at 40°C, 40 cycles of extension for 3 minutes at 72°C, and the final polymerization for 7 minutes at 72°C. The PCR products were analyzed by electrophoresis through 1.5% agarose gel, after which the gel was stained with ethidium bromide and photographed.

Similarity coefficients for pairs of tracks were calculated using a 70-80% similarity coefficient, with strains grouped using the unweighted pair group method with arithmetic averages (UPGMA).

RESULTS

Twelve of the isolates were positive for hemolytic properties, while 48 of the strains were positive for biofilm production.

The antibiogram test results indicated multiple resistances in 56 strains, as vancomycin susceptibility was found in only one strain, while most of the strains (n=48) were resistant to danofloxacin, 54 of the strains were susceptible to amoxicillin-clavulanic acid. Interestingly, a remarkable resistance for enrofloxacin (n=23), which was the commonly preferred antibiotic against Gram-negative mastitis pathogens, was detected. On the other hand, 41 of the strains were susceptible trimethoprim-sulphonamide, which was less preferred in clinical cases (Table 2).

Table 2. Antimicrobial resistance profiles of *Escherichia coli* isolates

	Resistant R	Moderate I	Susceptible S
Ampicillin	10	8	38
Amox-Clav.	-	2	54
Danofloxacin	48	6	2
Enrofloxacin	23	30	3
Oxytetracycline	29	16	11
Tetracycline	21	2	33
Trim-Sulfa.	9	6	41
Vancomycin	55	-	1

PCR results

The PCR results showed that all isolates produced bands of 662 bp, and phenotypic identifications was confirmed by PCR (Figure 1).

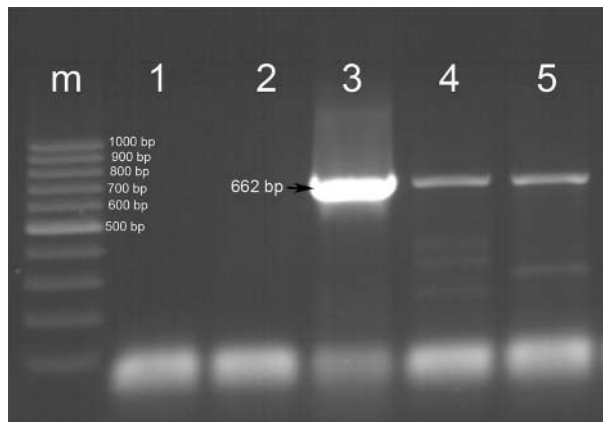


Figure 1. PCR results of *E.coli* isolates. M: marker (100 bp); 1: Negative control (*Staphylococcus aureus*); 2: Negative control (*Klebsiella pneumoniae*); 3-5: *E.coli* isolates

Specific PCR analysis showed that 42 (75.0%) strains carried the *traT* gene, 20 (35.7%) had the shiga toxin gene, 8 (14.2%) carried the intimin (*eae*) gene, but all strains were negative for aerobactin gene. All strains encoding shiga toxin genes were also positive for *stx1*. However, only 4 strains were positive for *stx2*. The isolates were jointly evaluated for antibiotic susceptibility and virulence properties. However, there were no significant differences in virulence genes between antibiotic-resistant and antibiotic-susceptible strains.

More strains were resistant to two or more antibiotics than to just one. These strains also had more virulence factors than those strains with single antibiotic resistance.

Genotyping results

RAPD PCR patterns were grouped by using cluster analysis with 70% similarity analysis and the unweighted pair group method using arithmetic averages (UPGMA). Cluster analysis of RAPD PCR patterns obtained for *E. coli* revealed thirteen main groups. These 13 groups included nine unique and four subgroups (Figure 2).

The number of strains in each group (G) ranged between two and eight, while the numbers of sub-groups of strains varied between two and five. Similarities in antibiotic resistances and virulence factors were not detected among profiles. Virulence properties and antibiotic resistance were evaluated for each profile. Variations in profile G4 and G8 were obtained according to virulence factors. Similarly, the same variations were also found in the sub-group of profile G5 (G5A). Variations were observed in profiles for antibiotic resistances other than G2, G3A, G3B, G13C, G13D and G13E. Profiles

G2, G3B and G13C had resistance against danofloxacin only, whereas profile G8 and profile G13D were resistant to most of the antibiotics. The strains in profile G7 were susceptible to all antibiotics with a various degree (I to S).

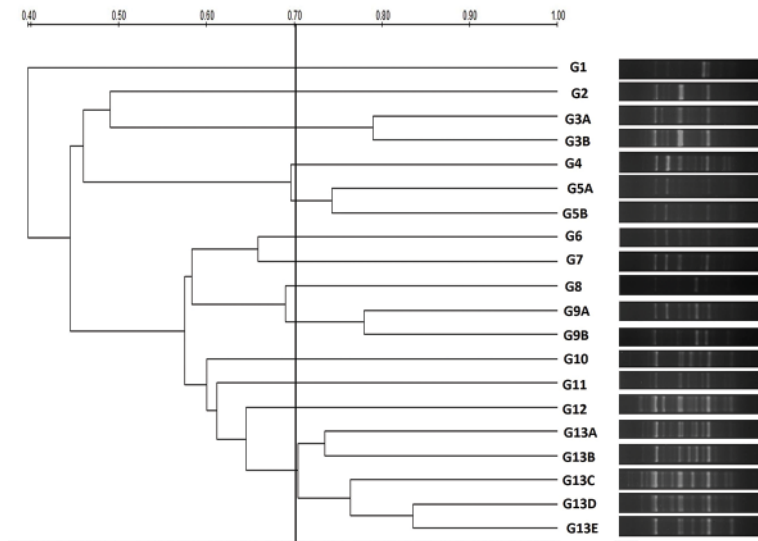


Figure 2. Cluster analysis and RAPD-PCR results of *E. coli* isolates

DISCUSSION

E. coli is an environmental mastitis pathogen, which causes persistent and repeated intramammary infections and has variable bacterial virulence factors. Therefore, determination of the virulence factors is important to identify the infection character. Of the virulence factors, traT, aerobactin, shiga toxin, intimin, and biofilm formation were evaluated in addition to RAPD profiles of the strains.

The traT gene encodes the bacterial outer membrane protein [3], which provides resistance against complement and protects the bacteria against phagocytosis. On the other hand, Nemeth et al. (1991) ignore the correlation between phagocytosis and traT gene. However, this correlation was not evaluated in the presented study [15]. Although, the rates of traT gene vary depending on the country, it is the most commonly obtained virulence factor in *E. coli* strains. Likewise, traT gene prevalence in previous studies varied from 36% to 62% [10, 16]. In the current study, a higher rate (75%) was detected in the observed strains. This result supported the previous findings about higher presence rate of traT gene and variations in the prevalence depending on origin.

Aerobactin with its low molecular weight and high affinity to iron molecules is present in pathogenic and nonpathogenic *E. coli* strains. The presence of the aerobactin gene was evaluated in previous studies [10, 17, 18]. Although, Lin et al. (1998) defined the

gene in all strains of *E. coli*, some researchers [10, 18] suggested smaller rates (4-11%) of aerobactin gene presence. Conversely, none of the strains carried the gene in the current study. This variation was associated with geographical changes as stated in previous studies [17, 19].

Shigatoxin producing *E. coli* strains (STEC) are responsible for food-born outbreaks, and cattle and other ruminants are the most important reservoirs of STEC. The shigatoxin (stx) is accepted as the additional virulence factor such as intimin, which provides attaching of STEC to the intestinal mucosa. Additionally, the STEC strains carry *eae* gene for intimin. In the current study, *stx1*, *stx2*, and *intimin* genes were evaluated. In the previous studies, researchers also stated variations in the presence rates for these genes [5, 20, 21, 22]. Although, Momtaz et al. (2012) and Kobori et al. (2004) stated a higher presence rate, other researchers [18, 23, 24] could not detect the gene in *E. coli* strains. In the here presented study, *stx* gene presence was higher than in previous studies, as *intimin* was lower. The significant difference between the reports was associated with the origin of the strains [20].

Bacteria, which have biofilm producing properties, can be protected from immune system cells and deactivated the antibiotics [25, 26]. Researchers stated biofilm production in *E. coli* strains in previous reports [18, 27, 28]. Friedman et al. (2013) classified the biofilm production property of the strains as strong and medium, and found medium level production in one third of the 55 strains. Moreover, some researchers suggested higher rates (85.7%) of biofilm producing positive strains [28]. In the current study, a higher positivity (67.8%) was detected in biofilm production. The variety in biofilm positivity might be associated with the infection status (clinical and subclinical) and severity (acute, subacute or chronic) of cases the strains were isolated from.

Antibiotic resistance is an actual problem for public and animal health. Excessive and inappropriate antibiotic use leads to the development of resistance against most of the antibiotics. *E. coli* is an environmental mastitis pathogen, which causes clinical signs in mammary glands in cows and its resistance against antibiotics depends on herd management. In previous studies, various antibiotic resistance profiles and rates in *E. coli* strains were stated in previous studies especially for oxytetracycline [29, 30, 31, 32], penicillin [22, 32, 33, 34], macrolides [21, 35, 36], trimethoprim-sulphamethoxazole [6, 36, 37], fluoroquinolones [35, 36] and cephalosporins [38]. In the presented papers, resistance profiles of *E. coli* strains were evaluated for vancomycine, danofloxacin, oxytetracyline, enrofloxacin, amoxicillin-clavulanic acid, trimethoprim-sulphamethoxazole, ampicillin and tetracycline. According to the results, innate resistance of *E. coli* against vancomycine was confirmed. A remarkable resistance was detected against fluoroquinolones (danofloxacin and enrofloxacin). Fluoroquinolones and tetracyclines are the most preferred antibiotics in cow mastitis due to Gram-negative pathogens. Thus, this result was associated with the wide use of these antibiotics and blind treatment of mastitis in cows. Although, penicillins (ampicillin and amoxicillin-clavulanic acide) and trimethoprim-sulphamethoxazole

were especially recommended for the treatment of mastitis due to Gram-positive pathogens [35, 36, 39, 40], a significant susceptibility was defined in this study. This result might be associated with the *in vitro* design of the study.

Because antibiotic resistance and virulence genes could be carried on the same chromosomal structures or plasmids, these virulence factors (aerobactin and traT genes) and antibiotic resistance were evaluated together in previous studies [3, 5, 15]. According to the results, the strains, which had multiple antibiotic resistances, had more virulence genes than the strain, which had single antibiotic resistance. This finding was compatible with the previous studies [3, 15]. However, antibiotic resistance was not evaluated at the genomic level in the presented study. In future, the genomic antibiotic resistance should be detailed together with the virulence factors.

RAPD helps to understand the epidemiology, ecology, tracking outbreaks, and spreading of the microorganisms [41, 42, 43]. It allows dendrograms (i.e. tree diagrams) to visualize and quantify the relationship between strains of bacterial species [44, 45]. In the presented study, strains were divided into 13 main and 4 subgroups using RAPD. This result showed that, *E. coli* strains which caused intramammary infections might be originating from various sources.

In conclusion, *E. coli* strains from intramammary infections and originating from various sources might show resistance against common antibiotics. The traT gene was the most common virulence factor, whereas the distribution of other factors was lower. In future studies, the relationship between clinical findings and traT gene should be evaluated. Pathogenicity of *E. coli* that cause clinical mastitis and prognosis of the infection might be predicted by obtaining of traT gene. Additionally, antibiotic resistance should be investigated at the genomic level to detect the relationship between virulence factors and antibiotic resistance. In field conditions, development of antibiotic resistance is the main cause of failure in mastitis treatment. Thus, antibiotic resistance profiles in herds should be clarified and controlled antibacterial protocols must be established.

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DETEKCIJA NEKOLIKO FAKTORA VIRULENCIJE, REZISTENCIJE NA ANTIBIOTIKE I FILOGENETSKIH ODNOSA IZOLATA *E. COLI* POREKLOM OD KRAVA SA MASTITISOM

CENGİZ Seyda, DİNÇ Gökçen, SÖĞÜT Mehtap Ünlü

Mastitis kod krava, izazvan bakterijom *Escherichia coli* (*E. coli*) ima različite lokalne i sistemske kliničke simptome čiji stepen izraženosti značajno varira. Cilj ispitivanja je bio da se ustanove virulentni faktori, rezistencija na antibiotike kao i filogeneza 56 sojeva *E. coli*. Od svih ispitivanih sojeva, 12 je bilo pozitivno na hemolitičku aktivnost a 38 je proizvodilo biofilm. Pored ovoga, 55 sojeva je bilo pozitivno na multiplu rezistenciju u bakteriološkim testovima. PCR analiza je pokazala da je 42 soja posedovalo traT gen, 20 sojeva je posedovalo shiga toksin gen (stx1-stx2), a 8 sojeva je posedovalo intimin gen (eae), ali su svi ispitivani sojevi bili negativni na aerobaktin gen (aer). Svi sojevi koji su posedovali shiga toksin gene bili su pozitivni i na stx1, ali je samo 4 soja bilo pozitivno na stx2 gen. Nije bilo značajnih razlika u genima virulencije između antibiotik-rezistentnih i antibiotik-osetljivih sojeva. Polimorfna DNK-PCR je pokazala da postoji 13 osnovnih grupa sa 4 podbrupe *E. coli*. U ovoj studiji, *E. coli* sojevi

koji su izazivali intramamarne infekcije i koji su poticali iz različitih izvora pokazali su rezistenciju na uobičajene antibiotike. Patogenost *E. coli* koja izaziva kliničke forme mastitisa kao i prognoza infekcije može da se predvidi na osnovu podataka o prisustvu traT gena. Pored ovoga, rezistencija na antibiotike treba da se ispita na genetskoj osnovi u cilju detekcije povezanosti između faktora virulencije i rezistencije na antibiotike. U terenskim uslovima, razvoj rezistencije na antibiotike je osnovni uzrok neuspešne terapije mastitisa. Može da se zaključi da je u stadu neophodno obavljati monitoring profila rezistencije na antibiotike uz primenu efikasnih antibiotika.