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RESEARCH ARTICLE

Investigation on Antimicrobial Resistance Levels of *Escherichia coli* Strains Isolated from Bovine Fecal Samples and Comparison with Guidelines of the Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST)

Recep KALIN[1*], Burcu KARAGÜLLE[2], Mehmet Nuri AÇIK[3], Murat KARAHAN[4], Yasin ÖZTÜRK[5], Burhan ÇETİNKAYA[2]

[1]*Department of Microbiology, Faculty of Veterinary Medicine, Sivas Cumhuriyet University, Sivas, Türkiye*
[2]*Department of Microbiology, Faculty of Veterinary Medicine, Fırat University, Elazığ, Türkiye*
[3]*Department of Microbiology, Faculty of Veterinary Medicine, Bingol University, Bingol, Türkiye*
[4]*Department of Microbiology, Faculty of Veterinary Medicine, Hatay Mustafa Kemal University, Hatay, Türkiye*
[5]*Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Necmettin Erbakan University, Konya, Türkiye*

ABSTRACT

This study was carried out to investigate the antibiotic resistance levels of *Escherichia coli* isolates of bovine origin and to compare the results with CLSI and EUCAST guideline values. For this purpose, 97 *E. coli* strains isolated from fecal samples of cattle in 12 different farms were tested against 32 antibiotics by using the disk diffusion method. The zone diameters of 13 antibiotics examined within the scope of the study were compared according to the CLSI and EUCAST 2020 guidelines, and their consistency levels were evaluated statistically. The highest resistance rates in *E. coli* isolates were determined against tetracycline (68%), streptomycin (63.9%), ampicillin (58.8%), and doxycycline (50.5%) antibiotics. On the other hand, the isolates were found to be highly susceptible to amikacin and cephalosporin group antibiotics. When CLSI and EUCAST guidelines were compared, it was found that there were statistically significant differences between the resistance rates of nitrofurantoin, gentamicin, and amikacin. Only 10 (10.3%) of the isolates were detected to be susceptible to all the antibiotics tested, whereas 17.5% were resistant to 10 or more antibiotics. The results of this study showed that *E. coli* isolates of bovine origin were highly resistant against antibiotics used in the field for a long period, especially the number of isolates with multiple antibiotic resistance was striking. It was concluded that due to substantial inconsistencies between the CLSI and EUCAST guidelines for some antibiotics such as amikacin, nitrofurantoin, and gentamicin, there is an urgent need to execute necessary updates in both guidelines.

Keywords: Antibiotic resistance, Bovine, CLSI, *Escherichia coli*, EUCAST

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Sığır Dışkı Örneklerinden İzole Edilen *Escherichia coli* Suşlarının Antimikrobiyal Direnç Düzeylerinin Araştırılması ve Klinik Laboratuvar Standartları Enstitüsü (CLSI) ve Avrupa Antimikrobiyal Duyarlılık Testi Komitesi (EUCAST) Kılavuzlarıyla Karşılaştırılması

ÖZ

Bu çalışma, sığır orijinli *Escherichia coli* izolatlarının antibiyotik direnç düzeylerinin araştırılması ve sonuçların CLSI ve EUCAST kılavuz değerleri ile karşılaştırılması amacıyla yapılmıştır. Bu amaçla 12 farklı işletmedeki sığırların dışkı örneklerinden izole edilen 97 *E. coli* suşu, disk difüzyon yöntemi kullanılarak 32 antibiyotiğe karşı test edilmiştir. Çalışma kapsamında incelenen 13 antibiyotiğin zon çapları CLSI ve EUCAST 2020 kılavuzlarına göre karşılaştırıldı ve tutarlılık düzeyleri istatistiksel olarak değerlendirildi. *E. coli* izolatlarında en yüksek direnç oranları tetrasiklin (%68), streptomisin (%63,9), ampisilin (%58,8) ve doksisiklin (%50,5) antibiyotiklerine karşı saptanmıştır. Öte yandan, izolatların amikasin ve sefalosporin grubu antibiyotiklere karşı yüksek duyarlı olduğu saptanmıştır. CLSI ve EUCAST kılavuzları karşılaştırıldığında nitrofurantoin, gentamisin ve amikasin direnç oranları arasında istatistiksel olarak anlamlı farklar olduğu görüldü. İzolatların sadece 10'unun (%10,3) test edilen tüm antibiyotiklere duyarlı olduğu, %17,5'inin ise 10 ve daha fazla antibiyotiğe dirençli olduğu saptandı. Bu çalışmanın sonuçları sığır orijinli *E. coli* izolatlarının uzun süredir sahada kullanılan antibiyotiklere karşı oldukça dirençli olduğunu göstermiştir, özellikle çoklu antibiyotik direncine sahip izolatların sayısı dikkat çekicidir. Amikasin, nitrofurantoin ve gentamisin gibi bazı antibiyotikler için CLSI ve EUCAST kılavuzları arasındaki önemli tutarsızlıklar nedeniyle, her iki kılavuzda da gerekli güncellemelerin acilen yapılması gerektiği sonucuna varılmıştır.

Anahtar Kelimeler: Antibiyotik direnci, CLSI, *Escherichia coli*, EUCAST, Sığır

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ORCID ID; *RK: 0000-0002-9173-9550, BK: 0000-0002-6628-4515, MNA: 0000-0002-1908-5898, MK: 0000-0002-7024-1649, YÖ: 0000-0002-9612-0677, BÇ: 0000-0001-6347-1684*

*Corresponding author e-mail: recep.kalin@gmail.com

INTRODUCTION

Increasing resistance to antibiotics used in the control and treatment of bacterial infections in humans and animals has become a global problem threatening public health. Unfortunately, the increase in antimicrobial resistance makes it difficult to control bacterial infections and leads many drugs available in the market to lose their effectiveness (Prestinaci et al. 2015). Antibiotics used widely for the control and treatment of diseases in food animals have an important role in the emergence of resistance as they can be transferred to humans through food chain (Vidovic and Vidovic 2020). Also, antibiotic resistance genes in the intestinal bacteria of animals can pass to humans by direct contact with animal or through animal products. The number and diversity of antibiotic resistant genes may be increased in parallel with the increase in the number of resistant bacteria (Liu et al. 2016). The shedding of bacteria carrying resistance genes with feces of animals and humans makes it possible to transfer these genes to non-resistant microorganisms in the environment. Fecal contamination of surface waters, rivers, wetlands and even drinking waters plays an important role in the spread of this resistance (Bengtsson-Palme et al. 2018).

Escherichia coli (*E. coli*), one of the most important members of the normal intestinal microbiota, plays a significant role in the contamination of environment with human and animal feces (Odonkor and Ampofo 2013). Although *E. coli* can be found as harmless and commensal microorganism in the gastrointestinal system of humans and animals, it may cause many important intestinal and extraintestinal infections (Stromberg et al. 2018). The World Health Organization (WHO) has described *E. coli* among nine pathogens that are recognized globally as the most worrying in terms of antibiotic resistance (WHO, 2014). *E. coli* is thought to be an excellent tool for monitoring antimicrobial resistance in food and environmental samples, due to the fact that it acquires antimicrobial resistance readily and has a very broad host spectrum. In addition, changes in antibiotic resistance in *E. coli* are considered as an early warning of the possibility of developing resistance in other pathogenic bacteria and therefore this agent has been used as an indicator (van den Bogaard and Stobberingh 2000). Although *E. coli* is naturally susceptible to many antibiotics used in the market, it can take resistance genes from other bacteria through horizontal gene transfer, as well as transfer its resistance genes to other bacteria in the environment (Blake et al. 2003). For this reason, antimicrobial resistance in *E. coli* is considered as one of the major concerns for both human and animal healthcare. The increase in the prevalence of multiple antibiotic resistant *E. coli*, especially in the last 20 years, has reached important levels which puts forward severity of the problem. Therefore, determination of antimicrobial resistance in *E. coli* is of great importance in terms of developing effective measures to tackle this problem.

Molecular detection of resistance genes and phenotypic methods such as antibiogram tests are mostly used for the examination of antimicrobial resistance levels. Disc-diffusion method, which allows to test many antibiotics at the same time, is one of the most frequently used phenotypic tests in clinical microbiology laboratories (Boyen et al. 2010). The two most popular guidelines used worldwide in evaluating disk diffusion test results are the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and The Clinical Laboratory Standards Institute (CLSI). However, studies have reported that there are inconsistencies between EUCAST and CLSI standards in evaluating antibiotic susceptibilities. (Kassim et al. 2016, Sánchez-Bautista et al. 2018). Therefore, revised guidelines have been published since 2014 to regulate the clinical limit values of both guidelines.

This study was carried out to investigate the levels of antimicrobial resistance in *E. coli* field strains isolated from fecal samples of clinically healthy cattle (ages were 1-5) located in Bingol province and the surrounding areas. The results obtained here were evaluated by comparing with the criteria announced in EUCAST and CLSI guidelines.

MATERIALS AND METHODS

Bacteria strains

A total of 97 *E. coli* strains isolated from fecal samples collected from 12 different cattle farms located in Bingol province and its surrounding were used in the study. Fecal samples (received directly from the rectum or after fresh defecation) were taken in sterile stool collection container and transported immediately to the laboratories under cool conditions (at 4 °C) for culture. The samples (1 g) were aseptically transferred to 9 ml of Tryptic Soy Broth and were then incubated at 37°C for 18-24 h under aerobic atmosphere for pre enrichment. Following pre-enrichment, a loopful of broth was inoculated onto MacConkey Agar (Merck, 105465) and Eosin Methylene Blue Agar (Merck, 101347) and, was incubated under the same conditions. Isolates identified as *E. coli* by conventional methods were also confirmed by Polymerase Chain Reaction (PCR) using a pair of primers specific to this species (Acik et al. 2004).

Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were conducted by Kirby-Bauer disc diffusion method according to the 2020 guidelines published by the Clinical and Laboratory Standards Institute (CLSI, 2020). Before the antimicrobial susceptibility tests, all the isolates were cultivated in Mueller-Hinton Agar (HIMEDIA, M173) and incubated at 37 °C for 16-18 h under aerobic conditions. *E. coli* isolates were suspended with physiological saline and adjusted to 0.5 McFarland standard by densitometer. Then, the suspension was poured onto Mueller-Hinton Agar and spread over the entire surface with the help of a sterile swab. All the isolates were tested by Disc diffusion method against 32 different antibiotics listed in Table 1 and Figures 2 and 3.

Since the zone diameter ranges for 13 antibiotics listed in Table 1 were included in both EUCAST and CLSI guidelines, only these antibiotics were evaluated comparatively (CLSI, 2020; EUCAST, 2020). Because the zone diameters of nine antibiotics presented in Figure 1 were only included in the CLSI guideline, the results of these antibiotics were evaluated according to CLSI criteria, but the comparison with EUCAST data was not possible. On the other hand, due to the absence of zone diameters in both CLSI and EUCAST guidelines for 10 antibiotics listed in Figure 2, no sensitivity assessment was made. Nevertheless, evaluation was carried out by dividing them into four groups according to the zone parameters as 10 mm and below, 11-15 mm, 16-20 mm and 21 mm and above.

Statistical Analyses

Data were analyzed with SPSS (Statistical Package for the Social Sciences) Version 22.0. The zone diameter breakpoints were determined according to both CLSI 2020 and EUCAST 2020 guidelines to categorize them as either susceptible, intermediate or resistant. Cohen's Kappa statistics were conducted to determine the proportion of agreement over and above chance between CLSI 2020 and EUCAST 2020 guidelines. The p-value less than 0.05 indicated that the agreement was significantly different from "0" and it was not due to chance.

RESULTS

The highest antibiotic resistance rate was obtained against tetracycline (68%), which was followed by streptomycin (63.9%), ampicillin (58.8%) and doxycycline (50.5%) in the evaluation of susceptibility test findings of 97 *E. coli* isolates to 22 antimicrobial drugs by taking the CLSI 2020 guideline values into consideration. On the other hand, the isolates were found to be highly susceptible to amikacin and cephalosprin group antibiotics (cefaperazone, ceftazidime, cefepime) (Table 1, Fig. 1). When CLSI and EUCAST guidelines were compared, statistically significant differences were found between the resistance rates against nitrofurantoin, gentamicin and amikacin antibiotics (Table 1). The kappa value for ampicillin was measured as 1, while the kappa values for cefepime, cefocyte, ciprofloxacin, levofloxacin and chloramphenicol were above 0.9 (Table 1). Only 10 (10.3%) of the isolates were determined to be susceptible to all the antibiotics tested, whereas 17.5% were found to be resistant to 10 or more antibiotics. Meanwhile, one isolate was noticed to show resistance to 17 of 22 antibiotics tested (Fig. 3).

Among 10 antibiotics, the zone diameters of which were not provided in the CLSI and EUCAST guidelines, the percentage of isolates producing a zone diameter of 21 mm and above against enrofloxacin was 77.3%. On the other hand, a zone diameter of 10 mm and below was obtained in 94.8% of the isolates against tilmicosin (Fig. 2).

Table 1. Resistance rates of *E. coli* isolates to various antibiotics, concordance and kappa statistical data between the CLSI and EUCAST guideline data.

Tablo 1. *E. coli* izolatlarının çeşitli antibiyotiklere direnç oranları, CLSI ve EUCAST kılavuz verileri arasındaki uyum ve kappa istatistiksel verileri

	CLSI (%) n = 97			EUCAST (%) n = 97			Concordance (%)	Kappa, (95 % CI)
	R	I	S	R	I	S		
Ampicillin	58.76	0.00	41.24	58.76	0.00	41.24	100.00	1
Amoxicillin Clav.	24.74	17.53	57.73	35.05	0.00	64.95	82.47	0.674 (0.566-0.762)
Cefepime	9.28	5.15	85.57	11.34	3.09	85.57	97.94	0.919 (0.759-1.000)
Ceftazidime	8.25	2.06	89.69	9.28	4.12	86.60	96.91	0.806 (0.628-0.906)
Cefoxitin	21.65	1.03	77.32	22.68	0.00	77.32	98.97	0.971 (0.902-1.000)
Ciprofloxacin	13.40	10.31	76.29	23.71	3.09	73.20	98.97	0.975 (0.910-1.000)
Levofloxacin	17.53	5.15	77.32	22.68	6.19	71.13	96.91	0.926 (0.827-1.000)
Norfloxacin	16.49	4.12	79.38	25.77	0.00	74.23	90.72	0.748 (0.624-0.900)
Amikacin	6.19	8.25	85.57	28.87	0.00	71.13	77.32	0.393 (0.247-0.523)
Gentamicin	16.49	1.03	82.47	47.42	0.00	52.58	69.07	0.366 (0.224-0.487)
Chloramphenicol	44.33	0.00	55.67	45.36	0.00	54.64	98.97	0.979 (0.930-1.000)
Nitrofurantoin	14.43	16.49	69.07	8.25	0.00	91.75	77.32	0.360 (0.140-0.584)
Trimethoprim-Sulfa.	42.27	2.06	55.67	42.27	0.00	57.73	97.94	0.959 (0.913-1.000)

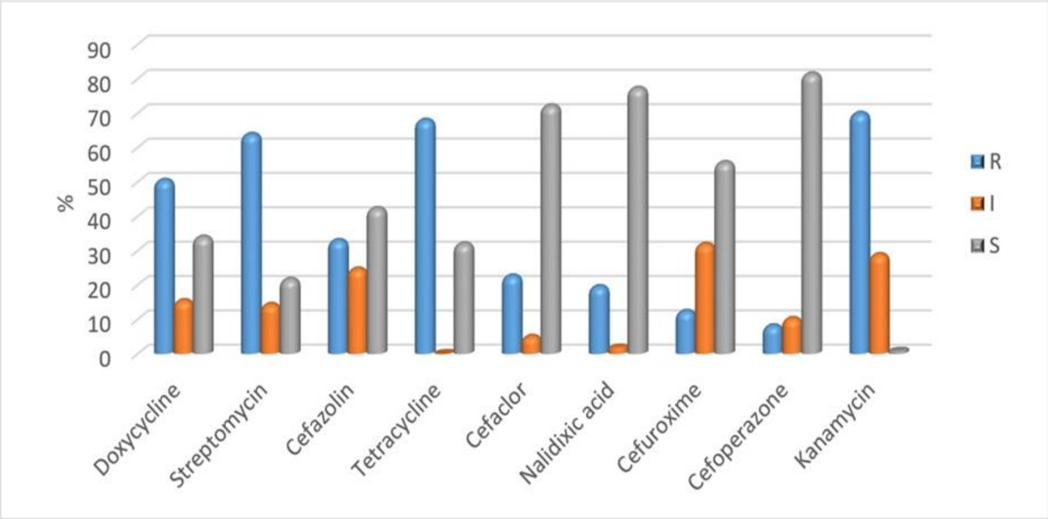

Figure 1: Resistance rates of *E. coli* isolates to various antibiotics according to CLSI 2020 guidelines
Şekil 1: CLSI 2020 kılavuzuna göre *E. coli* izolatlarının çeşitli antibiyotiklere direnç oranları

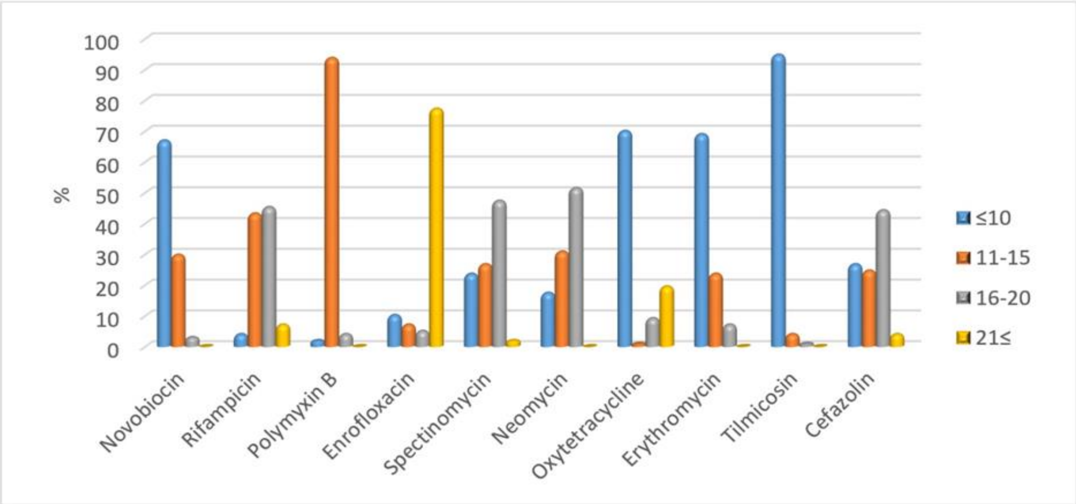

Figure 2: Zone diameter ranges of antibiotics that were not available in the CLSI and EUCAST 2020 guidelines
Şekil 2: CLSI ve EUCAST 2020 kılavuzlarında bulunmayan antibiyotiklerin zon çapı aralıkları

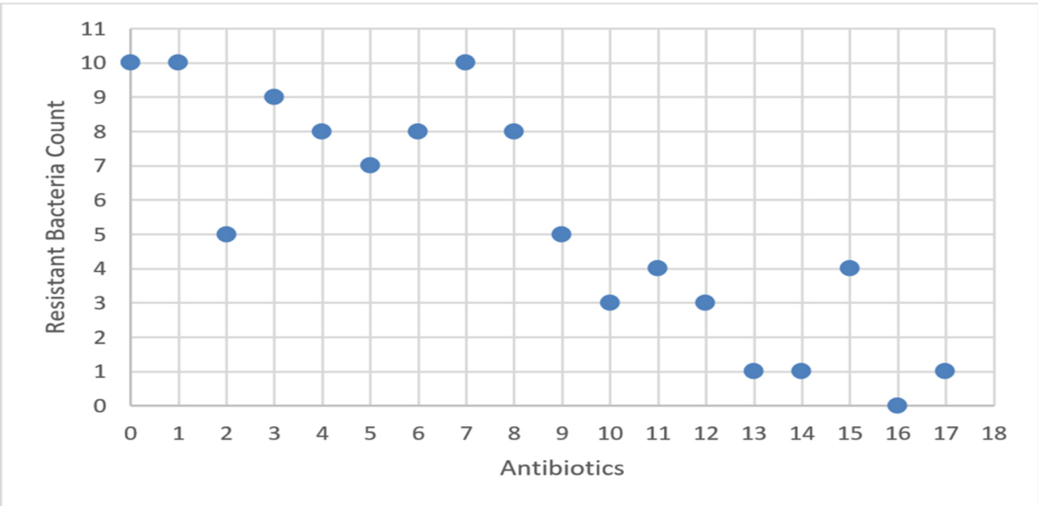

Figure 3: Number of *E. coli* isolates showing multiple antibiotic resistance
Şekil 3: Çoklu antibiyotik direnci gösteren *E. coli* izolatlarının sayısı

DISCUSSION

In this study, the most common antibiotic resistance in *E. coli* isolates was obtained against tetracycline, streptomycin and ampicillin, respectively. It should be underlined that these three antibiotics were the most preferably used antibiotics for the treatment of animal diseases in the field by both veterinarians and animal owners. Similar results concerning these antibiotics have also been reported in studies carried out in different parts of the world (Yassin et al. 2017, Aasmäe et al. 2019). High resistance rates against these antibiotics are not surprising thank to the common and long term use in animals for either treatment of prophylactic purposes in many countries. Due to the widespread and long-term use of these antibiotics for prophylactic purposes in animals in many countries, high resistance is expected. Tetracycline, which was approved for use in 1948 and used extensively for treatment and/or as feed additive, has an important place among these antibiotics. In many studies conducted in different countries, the resistance rate against tetracyclines has been reported to be rather high (Boireau et al. 2018, Aasmäe et al. 2019). In a previous study conducted to compare resistance levels in *E. coli* isolates between 2002 and 2015, an average resistance rate of 73% has been reported against tetracycline among the isolates of bovine origin and it was noted that this resistance showed a horizontal course over the years (Boireau et al. 2018). Also, it is possible that *E. coli* isolates resistant to tetracycline can generate resistance against other antimicrobial agents over time (Tadesse et al. 2012, Shin et al. 2015). A previous study has reported that 62.5% of tetracycline resistant isolates also showed multiple antibiotic resistance (Shin et al. 2015). Also, the presence of a mutual resistance has been showed between tetracycline and several antibiotics such as nalidixic acid, trimethoprim-sulfate, ampicillin and streptomycin (Tadesse et al. 2012). When the isolates resistant against 10 or more antibiotics were evaluated in this study; it was determined that all of the isolates resistant to tetracycline were also resistant to streptomycin and 94% showed resistance to ampicillin.

The resistance rate of *E. coli* to aminoglycosides shows variability according to animal species and the frequency of field use. While streptomycin resistance was reported in 68% of poultry isolates, it was detected in 18% of cattle isolates (Yassin et al. 2017). In another study carried out by Lim et al. (2010), the percentage of streptomycin resistance was found as 80.7% in isolates obtained from diarrheic cattle feces, whereas only 1.7% resistance was noted against amikacin. On the other hand, White et al. (2000) could not detect a resistance against amikacin in *E. coli* strains isolated from cattle with diarrhea. In this study, while a high resistance rate (64%) was detected against streptomycin, it was found to be as low as 6% against amikacin. The reasons for low level of resistance against amikacin might be that this antibiotic is not preferred in the treatment of animals and also it is a new generation antibiotic.

In this study, a relatively low rate of resistance was detected against cephalosporins, which also are not used frequently in animals. A total of six antibiotics from first to fourth generation of cephalosporin were evaluated in the study, and the resistance rates against first and second generation antibiotics were found to be higher than those in the third and fourth generation antibiotics. These findings showed the presence of higher resistance to antibiotics which were being used long before in the treatment of diseases. It is believed that the third and fourth generation cephalosporins were relatively new compared to others and the low rate of use in animal treatment may have prevented the development of resistance. However, it should not be neglected that the transfer of isolates resistant to cephalosporins with both environmental samples and food may lead to increased resistance against these antibiotics in humans. Therefore, observing and monitoring cephalosporin resistance in the field is thought to be important for human health.

A considerable resistance rate (44%) was obtained against chloramphenicol in the present study. The resistance rate against this antibiotic has been reported to range from 8% to 90% in *E. coli* isolates of bovine origin in previous studies conducted elsewhere in the world (White et al. 2000, Lim et al. 2010, Yassin et al. 2017). Remarkably high resistance rates against chloramphenicol were surprising due to the fact that its use in animals has been prohibited in Turkey, as well as many other countries. This suggests that the drug in question continues to be used by animal owners despite the ban. However, some studies have revealed that florphenicol, which is in the same group as chloramphenicol and used in the treatment of respiratory diseases of cattle, may play a role in chloramphenicol resistance (White et al. 2000). It has been reported that florphenicol resistance mediated by Flo gene provided enzymatic cross-resistance to chloramphenicol and has recently played an important role in the increased resistance (Tadesse et al. 2012). Although the above mentioned factors might have led to chloramphenicol resistance, it is thought that resistance mechanisms developed against other antibiotics could also have contributed to the occurrence of resistance against chloramphenicol in nearly half of the isolates tested in this study. Nevertheless, future studies are needed to reveal the factors likely to play a role in chloramphenicol resistance.

Quinolone group antibiotics are frequently used by animal owners and veterinarians in the treatment of gastrointestinal and respiratory diseases in animals. In recent years, a significant increase in resistance to quinolone group antibiotics has been observed in *E. coli* isolates of both human and animal origin (Moniri and Dastehgoli 2005, Tchesnokova et al. 2019). The fact that the resistance occurring against one of the quinolone group antibiotics causes emergence of resistance to other members of the group might accelerate this increase. The highest resistance among the five quinolone group antibiotics investigated in this study was found against enrofloxacin. Enrofloxacin is the only quinolone group antibiotic used in the treatment of gastrointestinal diseases in animals. In a previous questionnaire survey in the study area, it was reported that 5% of the farmers used enrofloxacin unconsciously and with no prescription (unpublished data). Therefore, high resistance to this drug was not considered as an unexpected finding. However, resistance rates ranging from 13% to 19% were determined against other quinolones (ciprofloxacin, norfloxacin, lovofloxacin and nalidixic acid), which are not used for therapeutic purposes in animals, in this study. This can be explained by that resistance to enrofloxacin might have generated development of resistance against other quinolone antibiotics. Indeed, the findings of a study that resistance against enrofloxacin caused resistance to ciprofloxacin and nalidixic acid supported this view (Moniri and Dastehgoli 2005). In the present study, the highest resistance subsequent to enrofloxacin was obtained against nalidixic acid, which was never used in the veterinary field. Similar to our findings, Yassin et al. (2017) reported 21.3% resistance against nalidixic acid in cattle isolates. It is thought that the transfer of plasmid-derived genes, which play a role in the resistance, from resistant bacteria to susceptible bacteria by horizontal route can have a significant effect on the occurrence of resistance to nalidixic acid (Mammeri et al. 2005). It has also been reported that the use of enrofloxacin in animals has significantly decreased susceptibility to nalidixic acid in *E. coli* isolates (Dheilly et al. 2012).

The CLSI and EUCAST guidelines recommended by the World Health Organization's Global Antimicrobial Resistance Surveillance System are used to evaluate and interpret antimicrobial susceptibility tests. The comparison of these guidelines, which are updated annually, in terms of reference ranges revealed that although moderate to high level of consistencies were present for some antibiotics, significant inconsistencies for various antibiotics were noticeable. Kassim et al. (2016) compared EUCAST and CLSI 2015 guidelines for the interpretation of antibiotic susceptibilities in *E. coli* isolates, and reported that though there were medium and/or high consistencies in the vast majority of antibiotics they tested, a mark inconsistency was noted between the two guidelines in terms of amikacin and nitrofurantoin antibiotics. In addition, in a study using commercial micro-dilution method, it has been reported that the kappa value for amikacin in *E. coli* isolates was as low as 0.27 and there was a significant difference between EUCAST and CLSI guidelines (Sánchez-Bautista et al. 2018). The comparison of zone diameters of 13 antibiotics used in the present study with the reference ranges in the two guidelines showed that there was a perfect agreement with a kappa value of over 0.8 for nine antibiotics. However, in parallel with other studies, there was a serious inconsistency in nitrofurantoin and amikacin values in this study. The resistance rate of 29% was obtained against amikacin according to the EUCAST guideline, where 18 mm and above values were evaluated as sensitive, whereas this rate was found to be 6.2% according to the CLSI guideline, where 17 mm and above values were considered as sensitive. The fact that the zone diameter of 14 *E. coli* isolates was detected as 17 mm in this study was responsible for this difference. On the other hand, the significant inconsistency regarding nitrofurantoin arises from highly different zone diameters in the EUCAST (11 mm and above sensitive) and CLSI (17 mm and above sensitive) guidelines and, also the absence of intermediate values in the former. Although previous studies have reported a high consistency between the two guidelines for gentamicin, a relatively low consistency was found in this study. The reference ranges for sensitivity to gentamycin were acknowledged as 15 mm and above in the CLSI guide, and as 17 mm and above in the EUCAST guide (CLSI, 2020, EUCAST, 2020). In the current study, the gentamicin zone diameter was detected as 16 mm in 26 of *E. coli* isolates. Since this value was between the upper and lower limits of the EUCAST and CLSI guide values, a very low kappa value was calculated. Furthermore, it should not be ignored that an evaluation was made according to the 2015 and 2017 guidelines in previous studies and the reference ranges for gentamicin were updated in 2020. In the EUCAST guidelines published earlier years, the resistance limit for gentamicin was considered as 14 mm and below, while in the 2020 manual this limit was updated to 17 mm and below. It should be underlined that a significant inconsistency emerged between the two guidelines for gentamicin with the last update. In the EUCAST directives, some stricter limits have been set for some antibiotics in order to prevent inappropriate use of antibiotics and control increased antibiotic resistance, which contributed to the increase in this inconsistency.

CONCLUSION

This study revealed that *E. coli* isolates of bovine origin showed a high rate of resistance against antibiotics used in the field for a long time. Particularly, the increase in the number of multiple antibiotic resistant isolates was striking. In addition, it was thought that high resistance against antibiotics such as nalidixic acid, chloramphenicol, first and second generation cephalosporins, which were not used in the field for the treatment and prophylactic purposes, may pose a serious problem for human and animal health. This should therefore be considered in the development of strategies to combat resistance problem. Finally, it is believed that due to substantial inconsistencies between the CLSI and EUCAST guidelines for some antibiotics such as amikacin, nitrofurantoin and gentamicin, there is an urgent need to execute necessary updates in both guidelines.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: RK, MNA and BÇ contributed to the research idea, design and execution of the study. RK, BK, MNA, MK and YÖ contributed to the acquisition of data. RK, BK, MNA, MK and YÖ analysed the data. RK and MNA drafted and wrote the manuscript. MNA, RK and BÇ reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules.

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