BF-1822
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RESEARCH ARTICLE Indian Journal of Animal Research

Presence of Enterococci in Cats with Urinary Tract Infections and Antibiotic Resistance Profiles

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10.18805/IJAR.BF-1822

ABSTRACT

Background: Bacterial urinary tract infection (UTI) is one of the most common diseases in cats caused by an imbalance between the natural host defence mechanisms of the urinary tract and virulent bacteria. This study investigated the prevalence and antimicrobial susceptibility of enterococci isolated from cats presenting urinary tract problems to veterinary clinics in Istanbul.

Methods: 100 urine samples were collected from cats by cystocentesis and plated on blood agar. Isolates found catalase and oxidase negative plated on Enterococcocel agar. After incubation, the isolates formed black colonies were tested by PCR using *E. faecium* and *E. faecalis*-specific primers. Then the antibiotic susceptibility of the isolates was determined.

Result: Eight of the 100 isolates that formed black colonies on Enterococcosel agar were identified as *Enterococcus* spp. After PCR tests, six isolates were classified as *E. faecalis*, one isolate was classified as *E. faecium* and one isolate that did not form a band was classified as *Enterococcus* spp. As a result of the Kirby-Bauer disc diffusion method used to determine the antibiotic susceptibility of the isolates, most resistance was found to cephalothin (100%) and penicillin, erythromycin, clindamycin and tetracycline (87.5%). While vancomycin resistance was not observed in any isolate, the lowest resistance was to enrofloxacin and ciprofloxacin (33.3%). Multiple antibiotic resistance was observed in all (100%) isolates. The presence of multi-resistant enterococci in cats represents a significant health threat. Bacterial identification and antimicrobial susceptibility testing should always be performed in urinary tract infections to guide definitive diagnosis and treatment.

Key words: Antibiotic resistance, Cat, *Enterococcus*, Urinary tract infection.

INTRODUCTION

Enterococci are widely distributed in nature in soil, water, plants, birds, insects and mammals. They are also part of the gastrointestinal and genital tract microbiota of many animals and humans. Although generally harmless, they are important opportunistic pathogens for humans and animals. Enterococci can cause many infections, including urinary tract infections (UTI), urogenital infections, endocarditis, meningitis, mastitis and intra-abdominal and wound infections. The two most common species isolated from clinical samples are *Enterococcus faecium* and *Enterococcus faecalis* (Kırkan *et al.*, 2019).

Urinary tract infection (UTI) occurs when bacteria colonise normally sterile parts of the urinary tract. It most commonly affects the bladder. Bacterial colonisation can be superficial on the mucosa or deeper in the mucosa/submucosa (Parmar *et al.*, 2021b). Bacterial UTI is much less commonly diagnosed in cats than in dogs and is estimated to affect 1–3% of cats during their lifetime (Chew *et al.*, 2014; Parmar *et al.*, 2021a). Although the disease can occur at any age, it is more common in middle-aged (2-6 years), overweight, diabetic and inactive cats. Most UTIs are caused by bacteria, usually from pathogens in the host's enteric or distal urogenital flora. As in humans and dogs, the most common bacterial pathogen isolated from feline UTI is *E. coli*. However, due to the high resistance of enterococci to antimicrobial drugs, their prevalence in chronic UTI or recurrent infections is much higher than that of other bacteria (Dorsch *et al.*, 2019; Banik *et al.*, 2016).

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How to cite this article: Orujov, P., Celik, B., Kekec, A.I. and Diren Sigirci, B. (2024). Presence of Enterococci in Cats with Urinary Tract Infections and Antibiotic Resistance Profiles. Indian Journal of Animal Research. doi: 10.18805/IJAR.BF-1822.

Submitted: 21-05-2024 **Accepted:** 23-09-2024 **Online:** 16-10-2024

Although enterococci are part of the microbiota of animals and humans, their ability to become multidrug resistant makes them a potential health threat. Studies have reported the transmission of pathogenic and/or antimicrobial-resistant bacteria from pets to their owners. Therefore, the surveillance of drug-resistant pathogens in pets living with us at home is crucial for public health and veterinary medicine. While enterococci attract attention with their natural resistance to many antibiotics, such as penicillin, cephalosporin, quinolone and aminoglycosides used in the treatment of infections caused by Gram-positive organisms, it has also been found that they can generate new antibiotic resistance and can transfer this resistance *via* plasmids (Tumpa *et al.*, 2022; Kataoka *et al.*, 2014; Dotto *et al.*, 2018).

The aim of this study was to isolate, identify and highlight the importance of *Enterococcus* species in domestic cats. At the same time, it aimed to reveal antibiotic resistance profiles by performing antimicrobial susceptibility tests.

MATERIALS AND METHODS

Between April and August 2023, 100 cats with urinary tract problems were brought to private practices and Istanbul University-Cerrahpaşa Veterinary Faculty Hospital for urine samples taken by cystocentesis. The sex, age, breed and neuter status of the cats were recorded. Thirty-one cats are female, 69 are male, five are less than one year old, 63 are between 1-7 years old, 26 are older than seven years and six are unknown. Looking at the breeds of the cats, 79 were defined as Mixed, eight were defined as British Shorthair, six were Scottish, three were Siamese, two were Persians, one was Chinchilla and one was described as Angora. Twenty-nine cats were sterilised, 11 were non-sterilised and the status of 60 was unknown.

Isolation and identification

The urine samples were brought to the Routine Diagnostic Laboratory of the Microbiology Department of Istanbul University-Cerrahpaşa Faculty of Veterinary Medicine under a cold chain and were examined immediately without waiting. 100 µl of the samples were plated on 5% sheep Blood Agar and incubated under microaerobic conditions (7% CO_2 added to the culture conditions) at 37°C for 24 hours. Catalase-negative, oxidase-negative Gram-positive cocci were considered suspect *Enterococcus* spp. Isolates were then plated on Enterococcosel Agar and the black colonies were considered *Enterococcus* spp. Isolates were stored at -80°C in Tryptic Soy Broth containing 50% glycerol until use (Arda, 2011).

DNA extraction

DNA was extracted from fresh cultures grown on blood agar using a 2% Chelex-100 solution (Tumpa *et al.*, 2022).

Amplification

Multiplex polymerase chain reaction was used to identify Enterococcus spp isolates with *Enterococcus faecium* and *Enterococcus faecalis*-specific gene regions, according to the method reported by Tumpa *et al.* (2022).

Electrophoretic separation and detection of DNA

The PCR products obtained at the end of the cycles were electrophoresed in an agarose gel (1%). Electrophoresis was continued at 100 volts for 60 min. Isolates forming a 550bp band were identified as *E. faecium* and isolates forming a 941bp band were identified as *E. faecalis*. Samples that did not form bands were considered to be *Enterococcus* spp..

Antimicrobial susceptibility testing

According to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2018), antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (MHA). The antibiotic susceptibilities of the isolates were evaluated against amoxicillin/clavulanic acid (30 µg), penicillin (10 U), ampicillin (10 µg), ampicillin/sulbactam (20 µg), vancomycin (30 µg), cephalothin (30 µg), enrofloxacin (5 µg), marbofloxacin (5 µg), ciprofloxacin (5 µg), gentamicin (10 µg), erythromycin (15 µg), clindamycin (5 µg), oxytetracycline (30 µg) and sulfamethoxazole/trimethoprim (25 µg). Isolates resistant to three or more antibiotic classes were considered to have multidrug resistance (MDR) (Magiorakos *et al.*, 2011).

Statistical analysis

The results were analysed with the SPSS 13.0 programme. The Chi-squared test was used for the comparisons of sex, age, breed and sterility status groups with respect to bacterial growth. Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

UTIs in cats are relatively rare events due to various host defence mechanisms. Cats with bacterial UTIs have been reported to have anatomical, metabolic, or functional problems in the bladder or urethra or to have undergone urinary tract instrumentation (*e.g.*, urinary catheterisation) that facilitates bacterial shedding and colonisation of the urinary tract (Chew *et al.*, 2014). In this study, we aimed to determine the rate of bacterial UTI and the antimicrobial resistance profiles of enterococcal species isolated from samples in cats with urinary tract problems brought to the IU-C Veterinary Faculty Animal Hospital or private clinics around Istanbul between April and August (2023).

Isolation and identification

In 43 out of 100 urine samples, growth was observed plated on blood agar. A single organism was isolated in 38 of these, two in 4 patients and all three in one patient. It was found that 23 of the 49 organisms were Gram-positive cocci. After catalase and oxidase testing, ten isolates were found to be catalase and oxidase negative and were identified as suspected *Enterococcus* spp. Eight of the ten isolates that formed black colonies on Enterococcosel agar were identified as *Enterococcus* spp.

Eight isolates identified as *Enterococcus* spp. were tested by PCR using *E. faecium* and *E. faecalis*-specific primers. Six isolates were interpreted as *E. faecalis* and one as *E. faecium*. In contrast, one isolate that did not form a band was determined to be *Enterococcus* spp. As a result, *E. faecalis* was identified in five urine samples from seven cats, *E. faecalis* and *E. faecium* in one and *Enterococcus* spp. in one. In this study, no significant difference was found between sex, age, breed and sterility status groups with respect to bacterial growth statistically ($p>0.05$). The distribution and rates of enterococci isolated from cats with bacteriuria according to sex, age, breed and sterility status are shown in Table 1.

Although studies in previous years have reported that bacterial UTI in cats is not common, it has recently been reported that the prevalence has increased from 1% to 38% (Amphaiphan, 2021). Eggertsdottir *et al.* (2011) collected urine by cystocentesis from 108 healthy cats in

Norway. They found that only 1% had bacterial growth and reported that contamination was sporadic when urine was collected by cystocentesis. D'Aout *et al.* (2022), in England, examined 2712 urine samples and found that 425 (15.7%) were culture-positive. Fonseca *et al.* (2021) detected bacteriuria in 10% of feline urine samples. Aurich *et al.* (2022) reported that uropathogenic bacterial growth was observed in 38.5% of cats in Germany and attributed the high prevalence to the higher number of female cats. Tumpa *et al.* (2022) showed bacteriological growth at 36% in Croatia and Amphaiphan *et al.* (2021) at 38% in Thailand. In this study, bacteriological growth was observed in 43 (43%) of the urine samples taken by cystocentesis from 100 cats. This bacteriological prevalence is much higher than the recent reports in Istanbul (Dokuzeylül, 2015, 2019). Although Eggertsdottir *et al.* (2011) attributed the low prevalence to the fact that the samples were taken by cytosynthesis, in this study all samples were taken by cytosynthesis too. It is known that all of the cats sampled in this study were brought to clinics with various urinary tract problems. Similar high prevalence rates exist due to urinary tract defense mechanisms, effects of other diseases and/or treatment (*e.g*. catheterization, perineal urethrostomy) as reported in many studies and supports the work done in recent years. Since there are no specific clinical signs of UTI, the high prevalence found in this study shows the importance of bacteriological culture in animals with urinary tract problems.

The majority of UTIs are caused by bacteria, with bacteriuria usually resulting from pathogens in the host's own enteric or distal urogenital flora. The majority (>85%) of UTIs are caused by a single bacterial pathogen (Dorsch *et al.*, 2019). In both dogs and cats, *Escherichia coli* is the most commonly isolated pathogen from the urinary tract (Dorsch *et al.*, 2019; Hernando *et al.*, 2021; Thompson *et al.*, 2011). The second most common isolate was found to be *Enterococcus* species, with detection rates ranging from 5% to 55% (Seidel *et al.*, 2022). In a study conducted in Norway in 2015 by Lund *et al.* (2015), 82 bacteria were isolated from the urine of 71 cats and *Enterococcus* was found to be the third most frequently isolated bacterial species. In a 5-year retrospective study in Germany, Teichmann-Knorrn *et al.* (2018) isolated enterococci in 15.2% of cat urine and stated that they were the 3rd most frequently isolated bacteria. In the study by Aurich *et al.* (2022), also conducted in Germany, the most frequently isolated bacteria were *Enterococcus* spp., with 11.9%. In a study conducted in England in the same year, enterococci were found in cat urine at a similar rate (14.9%) (D'Aout *et al.*, 2022). Another study conducted in England mentioned the prevalence of *Enterococcus* species by reporting that the second most common organism isolated from feline urine was *Enterococcus* species and that *Enterococcus* spp. was isolated from 40 of 171 cats (23.2%) (Fonseca *et al.*, 2021). Similar to the high prevalence in England, *Enterococcus* spp. was isolated from 22.2% of feline urine in the Spanish study. It was found to be the second most commonly isolated bacterium after E. coli (Hernando *et al.*, 2021). As in Europe, *Enterococcus* has been identified as the 2nd most frequently isolated pathogen from cat urine in Far Eastern countries such as Korea (Moon *et al.*, 2022) and Taiwan (Lien and Wang, 2020). As a result of a retrospective study conducted in America, they showed that enterococci, with its ability to cause disease in healthy young or middle-aged cats, is not only an opportunistic organism but also a true uropathogen (Kukanich and Lubbers, 2015). In studies conducted in our country, Dokuzeylül *et al.* (2015) reported that bacterial growth was observed in 26.2% of urine samples from 61 cats with lower urinary tract infection (LUTI). They noted that the most commonly isolated agents were *E. coli* and

Table 1: Distribution of cats from which enterococci were isolated according to sex, age, breed and sterility status.

Variables	*E. faecalis* / bacteriuria (+) (%)	*E. faecium* / bacteriuria (+) (%)	*Enterococcus* spp. / bacteriuria (+) (%)	Ratio to the total number of animals (%)	P value
Sex					
Male*	3/28 (10.71)	1/28 (3.57)	1/28 (3.57)	4/69 (5.79)	0.4818
Female	3/15 (20)	-	-	3/31 (9.67)	
Age					
<1	1/3 (33.33)	-	-	1/5 (20)	0.0747
1-7	1/23 (4.37)	-	1/23 (4.34)	2/63 (3.17)	
>7*	4/13 (30.76)	1/13 (7.69)	-	4/26 (15.38)	
Breed					
Mix	5/35 (14.28)	1/35 (2.85)	1/35 (2.85)	6/79(7.59)	0.0651
Persian	1/1 (100)	-	-	1/2 (50)	
Sterility					
Sterilised*	2/12 (16.66)	1/12 (8.33)	-	2/29 (6.89)	0.0846
Non-sterilised	1/5 (20)	-	-	1/11 (9.09)	
unknown	3/26 (11.53)	-	1/26 (3.84)	4/60 (6.66)	

* In one sample, *E. faecalis* and *E. faecium* were identified.

Enterococcus sp. Again, in their 2019 study, Dokuzeylül *et al.* (2019) examined urine samples from 123 cats and detected bacterial growth in 28 (22.8%). They reported the isolation of *Enterococcus faecalis* as the third pathogen, after *Staphylococcus* sp. and *E. coli*. In this study, as reported by Dokuzeylül *et al.* (2019), *Enterococcus* was detected as the third most frequently isolated agent after *Staphylococcus* sp. and *E. coli*. 18.6% of the isolates were identified as enterococci and this rate was compatible with the prevalence of studies conducted in other countries.

When looking at the distribution of enterococci species in cat urine, the most frequently isolated species has been reported to be *E. faecalis* (Litster *et al.*, 2009; Marques *et al.*, 2018; Moyaert *et al.*, 2017). Kukanich and Lubbers (2015) identified all enterococci isolated from urine samples collected from cats by cystocentesis or with a sterile catheter as *E. faecalis*. Aurich *et al.* (2022) found that 71.8% of the enterococci they isolated were *E. faecalis*, 7.6% were *E. faecium* and 20.5% were other Enterococci species. However, some studies have found *E. faecium* to be the dominant species among enterococci isolated from urine. Lien and Wang (2020) found that of the 11 (14.9%) enterococcal isolates isolated from cat urine, seven were *E. faecium* and three were *E. faecalis* and they identified one as *E. gallinarum.* Similarly, Tumpa *et al.* (2022) identified six of the seven enterococcal isolates they isolated as *E. faecium* and one as *E. faecalis*. In this study, consistent with the findings of Litster *et al.* (2009), Marques *et al.* (2018), Moyaert *et al.* (2017) and Aurich *et al.* (2022), the highest proportion of cat urine samples were isolated and identified as *E. faecalis* (75%), followed by *E. faecium* (12.5%). In total, six of the eight enterococci isolated were identified as *E. faecalis*, one as *E. faecium* and one as *Enterococcus* spp.

Determination of antibiotic susceptibility of isolates

As a result of the antibiotic susceptibility tests, the highest resistance was found against cephalothin (100%) and penicillin, erythromycin, clindamycin and oxytetracycline (87.5%). While no vancomycin resistance was observed in any isolate, enrofloxacin and ciprofloxacin had the lowest resistance (33.3%). Resistance to three or more antibiotic classes was detected in all isolates (100%) and all isolates were classified as MDR. Antibiotic resistance prevalence of isolates and Antibiotic resistance profiles of isolates are shown in Table 2 and Table 3, respectively.

Enterococci are naturally resistant to several commonly used antimicrobial drugs. They also have a great capacity to acquire new resistance mechanisms that can lead to multidrug-resistant isolates that can be partially treated with currently known antimicrobial agents (Kataoka *et al.*, 2014; Marques *et al.*, 2018; Scarborough *et al.*, 2020). In their study, Tumpa *et al.* (2022) reported that most enterococcal isolates were resistant to major classes of antibiotics and 80% were multidrug-resistant (MDR). They also noted that despite the high level of antibiotic resistance, no aminoglycoside resistance was found and that the one *E. faecium* isolate was susceptible to all antibiotics tested. A German study found that while 58% of enterococci isolates were MDR, most were susceptible to beta-lactam antibiotics, enrofloxacin, amoxicillin and amoxicillin-clavulanic acid. Researchers attributed the high MDR found to the fact that almost half of the animals sampled in the study had received antimicrobial treatment before sampling. They may have

Table 2: Antibiotic resistance prevalence of isolates.

Antibiotics		*E. faecalis* (N / %)	*E. faecium* (N / %)	*Enterococcus* spp. (N / %)	Total (N)	Rate (%)
P	AMC	2/33.3	1/100	1/100	4	50
	P	5/83.3	1/100	1/100	7	87.5
	AMP	2/33.3	1/100	1/100	4	50
	SAM	2/33.3	1/100	1/100	4	50
G	VA	0/0	0	0	0	0
S	KF	6/100	1/100	1/100	8	100
F	ENR	1/16.6	1/100	1/100	3	37.5
	MAR	4/66.6	1/100	1/100	6	75
	CIP	1/16.6	1/100	1/100	3	37.5
A	CN	4/66.6	1/100	1/100	6	75
	E	5/83.3	1/100	1/100	7	87.5
L	CD	5/83.3	1/100	1/100	7	87.5
T	T	5/83.3	1/100	1/100	7	87.5
Su	SXT	4/66.6	1/100	1/100	6	75

Antibiotic groups = P: Penicillin, G: Glycopeptide, S: Cephalosporin, F: Fluoroquinolone, A: Aminoglycoside, L: Lincosamide, T: Tetracycline, Su: Sulphonamide. Antibiotics= AMC: Amoxicillin/clavulanic acid (30 µg), P: Penicillin (10 U), AMP: Ampicillin (10 µg), SAM: Ampicillin/Sulbactam (20 µg), VA: Vancomycin (30 µg), KF: Cephalothin (30 µg), ENR: Enrofloxacin (5 µg), MAR: Marbofloxacin (5 µg), CIP: Ciprofloxacin (5 µg), CN: Gentamicin (10 µg), E: Erythromycin (15 µg), CD: Clindamycin (5 µg), T: Tetracycline (10 µg), SXT: Sulfamethoxazole/Trimethoprim (25 µg).

Table 3: Antibiotic resistance profiles of isolates.

Antibiotic resistance profiles	*E. faecalis*	*E. faecium*	*Enterococcus* spp.	Resistant antibiotic group (n)
SXT/KF/MAR	1	-	-	3
P/KF/E/CD/T	1	-	-	5
P/KF/MAR/CN/E/CD/T	1	-	-	6
AMC/P/AMP/SXT/KF/CN/E/CD/T	1	-	-	6
P/SAM/SXT/KF/MAR/CN/E/CD/T	1	-	-	7
AMC/P/AMP/SAM/SXT/KF/ENR/MAR/CIP/CN/E/CD/T	1	1	1	7

Antibiotics= AMC: Amoxicillin/clavulanic acid (30 µg), P: Penicillin (10 U), AMP: Ampicillin (10 µg), SAM: Ampicillin/Sulbactam (20 µg), VA: Vancomycin (30 µg), KF: Cephalothin (30 µg), ENR: Enrofloxacin (5 µg), MAR: Marbofloxacin (5 µg), CIP: Ciprofloxacin (5 µg), CN: Gentamicin (10 µg), E: Erythromycin (15 µg), CD: Clindamycin (5 µg). µg), T: Tetracycline (10 µg), SXT: Sulfamethoxazole/Trimethoprim (25 µg).

developed resistance to antimicrobials (Aurich *et al*., 2022). In this study, antimicrobial susceptibility testing was performed with 14 antibiotics from eight different antimicrobial classes. The antimicrobials were selected primarily on the basis of those commonly used in veterinary medicine to treat human enterococcal infections. Consistent with the studies by Smoglica *et al.* (2022) and Amphaiphan *et al.* (2021), this study found 100% multidrug resistance in *Enterococcus* species isolated from the urinary tract. This indicates that the potential for transmission of multidrug-resistant organisms from companion animals may be increasing and is a public health concern.

The antimicrobial working group of the International Society for Companion Infectious Diseases (ISCAID) has recommended amoxicillin (or amoxicillin/clavulanic acid) or trimethoprim-sulphonamides as first-line treatment for bacterial UTI in cats (Weese *et al*., 2019). Although recent studies have reported decreased susceptibility to amoxicillin, it is still one of the first-line drugs recommended for the treatment of UTI. Half of the isolates in this study were resistant to amoxicillin/clavulanic acid. This may be due to geographical distribution and differences in antimicrobial use between countries, but the results are worrying. Trimethoprim-sulfamethoxazole resistance was found to be 75% in this study, similar to studies reporting resistance of 94.3% (D'Aout *et al*., 2022) and 100% (Lien and Wang (2020). In the time since ISCAID recommendations, there has been an increase in the development of resistance to amoxicillin (or amoxicillin/clavulanic acid) and trimethoprim-sulphonamides. This highlights the need to regularly update antimicrobial resistance profiles worldwide to re-evaluate the treatment of antibiotics that have become resistant in the light of the results.

Intrinsic resistance to cephalosporins is expected in enterococci, making their use problematic. A study of antimicrobial prescriptions for cats diagnosed with urinary tract disease in the USA and Canada found that cefovecin was the most commonly prescribed antibiotic (61%). This inappropriate use is not only ineffective in treatment but may also lead to increased antimicrobial resistance (Weese *et al*., 2021). In this study, resistance to cephalothin, a cephalosporin group antibiotic, was tested and 100% resistance was observed.

Similar to EUCAST (2021), which states that ampicillin resistance is common in *E. faecium* but rare in *E. faecalis*, in this study, the only *E. faecium* isolate was resistant to ampicillin, while resistance in *E. faecalis* was 33.3%. Resistance to fluoroquinolones has been reported to range from 56 to 99% (Marques *et al*., 2018; Fonseca *et al*., 2021). While Tumpa *et al.* (2022) and Smoglica *et al.* (2022) reported high resistance to fluoroquinolones in general, Scarborough *et al.* (2020), Aurich *et al.* (2022) and Lien and Wang (2020) highlighted high resistance to enrofloxacin in particular. In this study, enrofloxacin and ciprofloxacin resistance was found to be low (37.5%), consistent with the findings of D'Aout *et al.* (2022) (40%). In studies of human urinary tract infections, ciprofloxacin resistance is relatively high (66-91%) (Tumpa *et al*., 2022). The low resistance found is encouraging, as fluoroquinolones are considered to be the most important antimicrobial class for humans. They are also of great importance for the treatment of companion animals. However, we should not forget that the use of this class of antibiotics needs to be controlled.

CONCLUSION

Since it is possible for resistant bacteria and resistance genes to be transmitted from animals to humans, the presence of multidrug-resistant enterococci in companion animals is a major threat to public health. Bacteriological culture and antimicrobial susceptibility testing should always be performed for UTIs to guide definitive diagnosis and antibiotic selection. Knowledge of the regional antimicrobial resistance status is also important for empiric antibiotic selection pending bacterial culture and antimicrobial susceptibility testing. The recent increase in resistance to antibiotics recommended for the treatment of enterococcal UTIs is of great concern. For this reason, epidemiological studies should be carried out at regular intervals in every country on cats that live with us in our homes, go to the toilet in our homes and use our beds. Bacterial UTI pathogens should be identified by bacteriological culture and then resistance profiles should be determined by antimicrobial susceptibility testing. It is, therefore, necessary to be informed about the evolving resistance status of antibiotics and to update antibiotic use protocols.

ACKNOWLEDGEMENT

Study supported by Istanbul University-Cerrahpasa, Scientific Research Projects Coordination Unit. Project number: TYL-2023-37044.

Animal welfare and ethics

Permission was taken from the Animal Use and Care Committee at the Faculty of Veterinary Medicine, Istanbul University- Cerrahpaşa (Eth No: E-72796624-604.02.01-601977).

Conflict of interest

All authors declare that they have no conflicts of interest.

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