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Araştırma Makalesi ***Research Article***

Determination of Ampicillin Resistant Enterococci (ARE) Isolated From Canine and Feline Rectal Swabs

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Abstract

Enterococci species, which are normal inhabitants of the gut flora of healthy animals and human, began to be recognized as an important pathogen in both human and veterinary medicine due to the acquired resistance profiles. The aim of the study is to examine the diversity of ampicillin resistance enterococci (ARE) species in cats and dogs, their antimicrobial susceptibility profiles and to determine some of the virulence related genes; *ace, gelE, efaA, agg* and *esp.* For this purpose, rectal swabs from companion animals were collected and processed for ampicillin resistant enterococci isolation. One hundred fifty seven swab samples (86 canine and 71 feline) were examined. ARE were isolated from 18 canine and 18 feline samples. All isolates identified as *E. faecium* by PCR. Antimicrobial susceptibilities of the isolates were determined by disk diffusion method. The isolates were resistant to ampicillin, penicillin, tetracycline (100%), followed by rifampicin and erythromycin (97%), streptomycin (92%), gentamicin (81%), ciprofloxacin (61%), nitrofurantoin (19%). Only two of *E. faecium* isolates were resistant to vancomycin and one to chloramphenicol. Multidrug resistance (resistance ≥4 antimicrobials) observed in all isolates. Virulence genes *ace, agg* and *esp* were not detected in any of the tested isolates. The *efaA* and *gelE* genes detection rates were, 13.8% and 11.1% respectively. The ARE isolation rate among pet animals was 22.9%. Screening of antimicrobial resistant enterococci among companion animals would be useful to detect any emerging antimicrobial resistance problem related with public health.

Özet

Köpek ve Kedi Rektal Svablarından İzole Edilen Ampisilin-Dirençli Enterokokların (ARE) İncelenmesi

Sağlıklı hayvanlar ve insanların barsak florasının bir parçası olan Enterekok türleri, kazanılmış direnç profilleri nedeniyle hem insan hem de hayvan hekimliğinde önemli birer patojen olarak tanınmaya başlanmıştır. Bu çalışmada, kedi ve köpeklerde ampisiline dirençli enterokok (ARE) türlerinin dağılımını, antimikrobiyal duyarlılık profillerini ve bu izolatların virülans ile ilişkili *ace, gelE, efaA, agg* ve *esp* genleri incelendi. Bu amaçla evcil hayvanlardan (86 köpek, 71 kedi) toplanan yüz elli yedi svap örneği ampisilin dirençli enterokok izolasyonu yönünden incelendi. ARE 18 kedi ve 18 köpek örneğinden izole edildi. İzolatların antimikrobiyal duyarlılıkları disk difüzyon metodu ile belirlendi. Bütün izolatlar PCR ile *E.faecium* olarak saptandı. İzolatlar ampisilin, penisilin ve tetrasiklin (%100), rifampisin ve eritromisin (%97), streptomisin (%92), gentamisin (%81), siprofloksasin (%61) ve nitrofurantoin'e (%19) dirençliydi. Sadece iki izolat vankomisine, bir izolat kloramfenikole dirençliydi. Bütün izolatlarda çoklu-antibiyotik direnci (direnç ≥4 antimikrobiyal) saptandı. Virülens genleri *ace, agg* ve *esp* test edilen hiçbir izolatta saptanmadı. *efaA* ve *gelE* genlerinin saptanma oranları sırasıyla %13,8 ve %11,1 di. Pet hayvanlarında ARE izolasyon oranı %22,9 olarak saptandı. Evcil hayvanlar arasında antimikrobiyal dirençli Enterokokların taranması halk sağlığı ile ilişkilidir. Ayrıca hayvan ve insanlarda antimikrobiyal direnç sorununu tespit etmek ve zamanında önlem alabilmek konusunda yapılan çalışmanın aydınlatıcı olabileceği düşünülmektedir.

Introduction

Enterococci are commensal bacteria of the intestinal microbiota in humans and animals. However, they are also one of the most prevalent zoonotic pathogens and cause opportunistic bacteremia, endocarditis, urinary tract infections (UTI) and surgical wound infections (Kataoka et al., 2013; Kwon et al., 2012; Lopes et al., 2006). Last two decades enterococci have become as an important cause of nosocomial infections and strains of enterococci have been acquired with resistance to

ampicillin (ampicillin-resistant enterococci [ARE]), high-level aminoglycoside and glycopeptides (vancomycin-resistant enterococci [VRE]) (Billström et al., 2008, Lester et al., 2008; Sava et al., 2010; Toledo-Arana et al., 2001). Enterococcal infections are mostly caused by *Enterococcus faecalis* and *E. faecium*. *E. faecalis* is the most common species associated with clinical infection while *E. faecium* poses the higher antibiotic resistance threat (Billström et al., 2008; Damborg et al., 2009; Rathnayake et al., 2012).

Enterococci are intrinsically resistant to some antibiotics such as cephalosporins, quinolones, aminoglycosides, clindamycin and erythromycin where the resistance genes are located on the chromosome, or they possess acquired resistance to several antimicrobial agents such as aminoglycosides, β-lactams and glycopeptides which are located on plasmids or transposons and to be able to spread these resistance genes to other species (Mannu et al., 2003; Rathnayake et al., 2012, Rodrigues et al., 2002). On the other hand, the presence of virulence factors in enterococci increase their pathogenicity and some of them related to antimicrobial resistance directly. Enterococci' several virulence molecules have been defined like aggregation substance *(agg)*, endocarditis antigen *(efaA)*, enterococcal surface protein *(esp)*, gelatinase *(geIE)* and collagen-binding cell wall protein *(ace)* (Billström et al., 2008; Fisher and Phillips, 2009; Gülhan et al., 2007; Lopes et al., 2006; Ratnayake et al., 2012; Toledo-Arana et al., 2001).

Dogs and cats, carry ARE in their gastrointestinal tract might transfer these resistant bacteria to humans because of the close physical contact that occurs between pets and their owners. Companion animals are the potential source of antimicrobial resistant bacteria because of extensive use of antimicrobials in those animals. In veterinary perspective, AREs are usually resistant to all antimicrobial agents to generally use in dogs and cats like ampicillin, amoxicillin with clavulanic acid, first generation cephalosporins, sulphonamides and fluoroquinolones. In human perspective, one of the limited treatment option is penicillins alone or combine gentamicin in ARE infections. Vancomycin is the last choice for life-treating human infections which is forbidden to use in animals in EU (Bagcıgil et al., 2012; Damborg et al., 2009; Guardabassi et al., 2004; Lopes et al., 2006).

The aim of the present study was to determine antimicrobial susceptibility profiles of ampicillin resistant enterococci from dogs and cats' rectal swab samples and the occurrence of five virulence determinants in those isolates.

Materials and Methods

Sampling

Samples were collected from domestic dogs and cats that visited Istanbul University, Faculty of Veterinary Medicine, Department of Internal Medicine, Small Animal Clinics in Istanbul. Totally, one hundred fifty seven swab samples (86 canine and 71 feline) were examined which were 75 of them from patients with clinical signs, such as feline infectious peritonitis (FIP), urinary tract infection (UTI), upper respiratory tract infections (URI), icterus, constipation, vomiting, etc., and 82 of them from clinically healthy animals. The fecal samples were removed directly from the rectum with sterile swabs and placed into tubes containing Amies transport medium without charcoal (Copan Diagnostics, USA) and transported to the microbiology laboratory for the detection of ampicillin-resistant enterococci.

Isolation and Identification

All swab samples were streaked onto plates of Enterococcosel agar (BD, Maryland, USA) supplemented with 32 µg/mL of ampicillin, and the plates were incubated for 24 h at 37°C under aerobic conditions (Damborg et al., 2009). When the black pinpoint colonies were observed after incubation, presumed ampicillin-resistant enterococci were determined at genus level and identification of *E. faecium* and *E. faecalis* species were carried out by PCR (Kariyama et al., 2000; Ke et al., 1999).

Antimicrobial Susceptibility

Antimicrobial susceptibilities of the isolates were determined by disk diffusion method according to the standard recommendations of the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012). Disks with the following antimicrobials were used: ampicillin (10µg), chloramphenicol (30µg), ciprofloxacin (5µg), erythromycin (15µg), nitrofurantoin (300µg), norfloxacin (10µg), penicillin (10U), rifampicin (5µg), tetracycline (30µg), vancomycin (30µg) (Oxoid). High level aminoglycoside resistance (gentamicin and streptomycin) evaluated according to growth in gentamicin (600µg/ml) and streptomycin (1000µg/ml) (CLSI, 2012). *E. faecalis* ATCC 29212 and *S. aureus* ATCC 29213 were used as control strains.

Detection of Virulence Genes

Colonies from pure cultures were picked up from agar plates and cultured overnight at 37°C in Brain Heart Infusion Broth, then 100 µl of culture mixed with 100 µl 6% Chelex-100 (BioRad, California, USA) and heated at 99°C for 10 min. The suspension was centrifuged at 12000 g for 1.5 min and 100 µl of the supernatant was

transferred to a new tube and used as a template DNA (Martin et al., 2009).

Presence of *esp*, *agg*, *ace*, *gelE* and *efaA* genes were investigated as previously described by Mannu et al. (2003) and Shankar et al. (1999). *E. faecalis* ATCC 29212, *E. faecium* ATCC 6057 and *E. faecalis* NCDO 581 (from a collection of Prof. Dr. Mustafa Akçelik, Faculty of Sciences, Department of Biology, Ankara University, Ankara, Turkey) were used as positive and negative controls.

Results

Identification of the Isolates

A total of 157 fecal samples were examined. *Enterococcus* spp. were isolated from 37 (23.5%) of the 157 fecal samples. Isolates confirmed by genus – specific PCR and 36 (22.9%) of them were determined as *Enterococcus* spp. PCR with *E. faecium* species-specific primers showed that all those isolates belonged to this species.

Antimicrobial Susceptibility

Ampicillin resistance was confirmed in all isolates. The resistance patterns are summarized in Table1. All *E. faecium* isolates were resistant to ampicillin, penicillin, tetracycline (100%), followed by rifampicin and erythromycin (97%), streptomycin (92%), gentamicin (81%), ciprofloxacin (61%), nitrofurantoin (19%). Only two *E. faecium* isolates were resistant to vancomycin and one to chloramphenicol. Multidrug resistance (resistance ≥4 antimicrobials) observed in all isolates (Table 2).

Table 1. Antimicrobial susceptibilities of isolates.
Tablo 1. İzolatların antimikrobiyal duyarlılıkları.

Antimicrobial Agent	Susceptible Number (%)	Intermediate Number (%)	Resistant Number (%)
Ampicillin (10 µg)	0 (0)	0 (0)	36 (100)
Chloramphenicol (30µg)	27 (75)	8 (22)	1 (3)
Ciprofloxacin (5µg)	1 (3)	13 (36)	22 (61)
Erythromicin (15µg)	0 (0)	1 (3)	35 (97)
Gentamicin HLAR(500 µg/mL)	7 (19)	0 (0)	29 (81)
Nitrofurantoin (300µg)	12 (33)	17 (47)	7 (19)
Penicillin (10 U)	0 (0)	0 (0)	36 (100)
Rifampisin (5µg)	1 (3)	0 (0)	35 (97)
Streptomycin HLAR(1000µg/mL)	3 (8)	0 (0)	33 (92)
Tetracicylin (30µg)	0 (0)	0 (0)	36 (100)
Vancomycin (30µg)	33 (92)	1 (3)	2 (6)

The Virulence Determinants

All strains were analyzed for the presence of several known virulence determinants by PCR. Virulence genes *ace, agg* and *esp* were not detected in any of the tested isolates. PCR for *efaA* gene which is encoding for a cell wall adhesin, gave positive results in 5 out of 36 *E. faecium*. Four of the isolates harbored gene for *gelE*. In three isolates both *efaA* and *gelE* genes were detected.

Discussion

Domestic animal feces may represent an important source of microorganisms potentially pathogenic for both owners and the community (Cinquepalmi et al., 2013). Although *E. faecium* strains are resistant to vancomycin and ampicillin more often than *E. faecalis* strains, the relative proportion of infections caused by these species has not dramatically changed in recent years (Huycke et al., 1998). In fact, antimicrobial resistance of this organism is a serious problem in public health. Many of the *E. faecium* isolates tested in the current study were resistant to a wide range of antibiotics, such as ampicillin, penicillin, tetracycline, rifampicin and erythromycin; they were also resistant to aminoglycosides at a high level, from which one can predict that there is resistance to synergism between cell-wall-active agents (ampicillin, penicillin, and vancomycin) and aminoglycosides. It is considered that, as it reduces the number of possible treatments available for enterococcal infections. In this study; the high frequency of rifampicin resistance observed. This finding is clinically important because this antibiotic can be used as a second-line drug for treatment of enterococcal infections in humans (Cetinkaya et al., 2000).

Table 2. Antibiotic resistance patterns for multidrug resistant strains.
Tablo 2. Çoklu-dirençli suşların antibiyotik direnç profilleri.

Number of the Isolates	Resistance Profile
1	AMP, ERY, PEN, TET
1	AMP, CIP, ERY, GEN, PEN, RIF, TET
7	AMP, ERY, GEN, PEN, RIF, STR, TET
1	AMP, CIP, ERY, GEN, NIT, PEN, RIF, TET
1	AMP, ERY, GEN, NIT, PEN, RIF, STR, TET
2	AMP, CIP, ERY, GEN, NIT, PEN, RIF, STR, TET
14	AMP, CIP, ERY, GEN, PEN, RIF, STR, TET
5	AMP, ERY, PEN, RIF, STR, TET
1	AMP, CIP, NIT, PEN, RIF, STR, TET
1	AMP, CHL, CIP, ERY, GEN, PEN, RIF, STR, TET
2	AMP, CIP, ERY, GEN, NIT, PEN, RIF, STR, TET, VAN

E. faecium, is more resistant to commonly used antimicrobial agents, but *E. faecalis* prevalence rate is four times higher than *E. faecium* in nosocomial infections (Mundy et al., 2000). Therefore antibiotic resistance alone can not explain the prevalence of nosocomial infections that was caused by these two microorganisms. These findings suggest that the presence of additional virulence factors that may enhance the virulence of enterococci. The virulence of enterococci is associated with several genes, including *ace* (collagen-binding cell wall protein), *agg* (aggregative pheromone-inducing adherence to extra-matrix protein), *esp* (enterococcal surface protein), *efaA* (E. faecalis antigen A) or *gelE* (gelatinase) (Mundy et al., 2000).

It appears from current study that the incidence of known virulence factors in *E. faecium* were generally low. Three strains were carrying more than one virulence determinant (*efaA* and *gelE* genes). It was previously described that *E. faecium* strains were generally free of virulence factors such as *esp*, *ace*, *agg* (Cariolato et al., 2008). This lack of virulence factors might be a reason for difference between prevalence of *E. faecalis* and *E. faecium* as nosocomial infections.

Ace is a collagen-binding protein, it has been thought that may play a role in the pathogenesis of endocarditis (Fisher and Phillips, 2009; Mannu et al., 2003). *Agg* is a pheromone-inducible surface glycoprotein and mediates aggregate formation during conjugation, thus aiding in plasmid transfer as well as adhesion to an array of eukaryotic surfaces (Eaton and Gasson, 2001; Fisher et al., 2009). In the current study; the ace gene and the aggregation substance (*agg* gene) were not present in any of the tested strains. This result is consistent with previous studies as these virulence genes have always been described only in *E. faecalis* (Duprè et al., 2003; Eaton and Gasson, 2001; Mannu et al., 2003; Nallapareddy et al., 2000). Extracellular surface protein (*esp*) is a cell-wall-associated protein that is thought to promote adhesion, colonization and evasion of the immune system, and to play some role in antibiotic resistance. It has been showed that *E. faecium* strains that carry the *esp* gene have higher conjugation rates than strains that does not possess this gene. They also demonstrate higher resistance to ampicillin, ciprofloxacin and imipenem (Billström et al., 2008; Toledo-Arana et al., 2001). In the current study; the *esp* gene was not present in any of the tested strains. These results correlates with those of Mannu et al. (2003) who did not find any of the tested strains with the *esp* gene and Eaton and Gasson (2001) who found frequent presence of the *esp* gene only in medical *E. faecium* strains. This gene presence was found only in medical *E. faecium* strains may be related to the increasing occurrence of pathogenic *E. faecium* strains.

It was hypothesized that *efaA* might be functioning as an adhesin in endocarditis. It is presumed to be involved in mechanisms that adhere to biotic and abiotic surfaces and take part in biofilm formation (Fisher and Phillips, 2009; Low et al., 2003). The specific PCR for *efaA* gene gave positive results in 5 out of 36 *E. faecium* strains. These results agree with previous studies (Eaton and Gasson, 2001; Mannu et al., 2003; Trembley et al., 2013). *efaA* gene is mostly found at similar frequencies in the *E. faecalis* and *E. faecium* strains. *efaA* gene is the only gene found in to pathogenic and nonpathogenic *E. faecium* strains, however the southern blot analyze showed that *efaA* gene from pathogenic strains were more similar to *E. faecalis efaA* gene (Eaton and Gasson, 2001).

Gelatinase is an extracellular zinc-containing metalloproteinase which it can hydrolyze gelatin,

collagen, fibrinogen, casein, hemoglobin, insulin, certain *E. faecalis* sex-pheromone-related peptides, and some other bioactive peptides, although they also have some function in biofilm formation (Fisher and Phillips, 2009; Kayaoğlu and Ørstavik, 2004). In the current study; four of the tested *E. faecium* strains were harboring gene for *gelE*. This does not agree with the findings of Mannu et al. (2003) who did not find gel production in any tested *E. faecium* strains. In other works too, *gelE* was only found in *E. faecalis* (Coque et al., 2002), while Eaton and Gasson (2001) found nine (11%) medical *E. faecium* strain with the *gelE* gene. However, Gülhan et al. (2007) in their study, found 25 (17.1%) out of the 146 *E. faecium* isolates, positive for gel production. Regional variation may cause about these differences.

In conclusion, *Enterococci* possess highly effective gene transfer mechanisms. There is a risk for transfer of resistant bacteria or/and resistance genes and virulence genes from same or different species. Because of that, screening of antimicrobial resistance and virulence determinants in enterococci among companion animals would be useful to detect any emerging antimicrobial resistance problem related with public health.

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