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Research Article

Investigation of *esp* Gene Presence from *Enterococcus faecalis* Strains Isolated from Cats

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ABSTRACT

In this study, 130 rectal swab samples taken from cats were involved to enterococci isolation in Adnan Menderes University Faculty of Veterinary Medicine Department of Microbiology. The isolated enterococci were identified by species specific PCR. Multiple drug resistancy genes and enterococcal surface proteins (Esp) were investigated in identified strains. The isolation studies revealed that out of 130 samples, 64 (50%) enterococci were identified as being 38 (29%) of them are *Enterococcus faecalis* and 26 (21%) of them are *Enterococcus faecium.* A total of 8 (21%) strains were detected as carrier of phosphorilation enzyme coding gene and none of the isolates were detected as esp gene carrier. By this research it is confirmed that, the cats which have close contact with humans, have the risk for exposing the carriage of enterococci, and it is cleared that this situation have to be estimated for examinations and routine laboratory analyses.

Key words: *E. faecalis, E. faecium,* Identification, Cat, PCR, Esp gene

INTRODUCTION

Enterococci are highly responsible for nosocomial infections such as endocarditis, bacteremia, urinary tract infections, or neonatal sepsis (Schaberg *et al*., 1991). *E. faecalis* is the most common species for enterococcal infections. *E. faecalis* causes 80-90% of infections and *E. faecium* follows it with 10-15% (Murray, 1990).

Enterococci most commonly form infections through the urinary system, blood, endocardium, abdomen, bile ducts, burn injuries, and devices that remain in the patient (such as intravascular catheters) (Jett *et al*., 1994). Enterococci cause otitis externa and rarely urinary tract infections on cats and dogs. *E. durans*, *E. hirae* and *E. villorum* species cause diarrhea in dogs, cats, foals and calves. *E. hirae* has been reported to cause suppurative inflammation in a pediatric enteropathy, liver bile duct, and pancreatic duct. (Moellering, 2000).

The enterococcal surface protein (Esp) identified in *Enterococcus faecalis* (Shankar *et al*., 1999) and *E. faecium* (Baldassarri *et al*., 2001; Willems *et al*., 2001) strains isolated from patients involved in nosocomial infections is associated with an increase in virulence, colonization (Shankar *et al*., 2001) and biofilm formation (Toledo-Arana *et al*., 2001). However, the esp gene, which encodes Esp, as also detected in 40% of *E. faecalis* isolates obtained from healthy humans (Waar *et al*., 2002).

The relationship between the presence of the esp gene, and antibiotic susceptibility among *E. faecalis* isolates from patients with bacteremia has been investigated in previous studies (Vergis *et al*., 2002).

Most enterococci naturally show resistance to antimicrobials such as β-lactams, clindamycin, and at low concentrations, aminoglycosides and fluoroquinolones. Naturally, enterococci are sensitive to ampicillin and vancomycin, but they develop resistance when exposed to various antibiotics. Also; resistance may develop to tetracyclines, macrolides, glycopeptides (vancomycin and teicoplanin) and β-lactams as well as chloramphenicol and high levels of aminoglycosides (Çetinel, 2008; Aktaş *et al*., 2009). The infections caused by these bacteria, which are multiple resistance to virtually every useful antibiotic, have begun to attract attention in recent years (Herman *et al*., 1991).

High-level gentamicin resistance (HLGR) in enterococci is usually due to the presence of bifunctional AAC(60)-APH(200) aminoglycoside-modifying enzyme (Ferretti *et al*., 1986). HLGR Enterococcus has caused concern with respect to antimicrobial therapy for severe enterococcal infections, such as endocarditis, because synergism with cell wall-active antimicrobials, such as

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ampicillin or vancomycin is no longer anticipated (Chow *et al*., 1997; Chow, 2000). The distribution of drug-resistant enterococci in companion animals has also been studied (Butaye *et al*., 2001; Simjee *et al*., 2002), due to concern regarding transfer of these strains from animals to humans or contamination among animals in hospitals, but it is not known whether HLGR has a significant association with the presence of the esp gene in enterococci of animal origin.

In our study, it was aimed to investigate the relationship between the presence of the esp gene and antibiotic susceptibility in *Enterococcus* sp. isolates obtained from cats.

MATERIALS AND METHODS

Specimen collection

A total of 130 rectal swabs taken from the cats that was brought to the veterinary clinics in Aydın and İzmir for our research were brought to the Routine Diagnostic Laboratory of the Department of Microbiology, Department of Veterinary Medicine, Adnan Menderes University, in the cold chain. The local animal ethics committee (ADU-HADYEK) approved this study with document no: 64583101/2013/103.

Isolation and identification of enterococci

Samples were incubated at 37ºC for 24 hours with bloody agar. At the end of this period, Gram staining method and catalase test were applied to colonies. Catalase test negative colonies were regarded as *Streptococcus* sp. and inoculated into bile esculin agar (Enterococcocel agar) for identification of Enterococci. The petri dishes were incubated at 37ºC for 24 hours. After that, black colonies were selected and inoculated into brain heart infusion agar media. *Enterococcus* sp. isolates were tested for oxidase test, PYR test, 6.5% NaCl reproduction test and identified by genus level. The identified colonies were passaged to storage medium for identification and stored at -20 °C for PCR tests.

DNA isolation

DNA isolations of strains were conducted via genomic DNA extraction units (Fermentas®) appropriate to procedure. The DNAs has been kept in cryo tubes in deep freeze at -20°C.

Primers

The primer pairs used for the detection of Enterococcus species, aminoglycoside resistance genes and the presence of the esp gene are shown in Table 1.

Positive control

E. faecalis ATCC 29212 and *E. faecium* ATCC 700221 strains were used during the isolation-identification and PCR studies in our study.

PCR

Identification of *E. faecalis* and *E. faecium* was carried out by a PCR assay to detect $_{\text{ddlE. faecalis}}$ and $\text{ddl}_{E. faecium}$, respectively, with primer pairs previously described by Dutka-Malen *et al*. (1995). PCR was performed on according to the method by using a DNA thermal cycler (Eppendorf® Mastercycler Personel). The cycles used were 94°C for 2 min for the first cycle; 94°C for 1 min, 54°C for 1 min and 72°C for 1 min for the next 30 cycles and 72°C for 10 min for the last cycle (Dutka-Malen *et al*., 1995).

Gentamicin resistance genes were detected by a PCR assay as described elsewhere (Chow *et al*., 1997). Amplification conditions were as follows; initial denaturation at 94°C for 20s, 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30s, and extension at 72°C for 30s, followed by an elongation step at 72°C for 30s. PCR amplifications to detect the esp gene were performed using the primer pair Esp 11 and Esp 12 previously described (Vergis *et al*., 2002). Amplification conditions were as follows; initial denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min.

Detection of the amplification product

The 10 µl amplified products were detected by staining with 0.5 µg/ml ethidium bromide after electrophoresis at 80 Volt for 40 min in 2% agarose gel.

PCR analysis revealed that *Enterococcus* sp. were identified as 941 bp for *E. faecalis* and 550 bp for *E. faecium* in the identification of *E. faecalis and E. faecium* species, respectively. For detection of the broad aminoglycoside resistance, 369 bp for aph (2'')-Ia, 867 bp for aph (2'')-Ib, 444 bp for aph (2'')- Ic and 641 bp size for aph (2'')- Id, 933 bp for Esp11 and Esp12 amplicon sizes were examined.

Table 1: Primer pairs used in the study and expected amplicon size

Primer	Oligonucleotide pairs (5'-3')	Target gene	Size (bp)	References
$\text{ddl}_{E. faecalis}$	F-ATCAAGTACAGTTAGTCTT R-ACGATTCAAAGCTAACTG	D-Ala Ligase	941	Dutka-Malen *et al*., 1995
$\text{ddl}_{E. faecium}$	F-GCAAGGCTTCTTAGAGA R-CATCGTGTAAGCTAACTTC	D-Ala Ligase	550	
aph(2'')-Ia1	F-GAGCAATAAGGGCATACCAAAAATC R-CCGTGCATTTGTCTTAAAAAACTGG	Gentamicin resistance genes	369	Chow *et al*., 1997
aph(2'')-Ib1	F-TATGGATCCATGGTTAACTTGGACGCTGG R-ATTAAGCTTCCTGCTAAAATATAAACATCTCTG	Gentamicin resistance genes	867	
aph(2'')-Ic1	F-TGACTCAGTTCCCAGAT R-AGCACTGTTCGCACCAAA	Gentamicin resistance genes	444	
aph(2'')-Id1	F-GGTGGTTTTTACAGGAATGCCATC R-CCCTCTTCATACCAATCCATATAACC	Gentamicin resistance genes	641	
Esp 11 Esp 12	F-TTGCTAATGCTAGTCCACGACC R-GCGTCAACACTTGCATTGCCGAA	Esp	933	Vergis *et al*., 2002

RESULTS

Isolation results

In our study, 130 cattle rectal swab samples were collected and subjected to identification of Enterococcus species. Samples were incubated at 37ºC for 24 hours with blood agar and then stained by Gram staining method. Gram positive colonies were tested for catalase reaction. Catalase test negative colonies were inoculated into Enterococcocel Agar and incubated at 37ºC for 24 hours. Enterococcoci susceptible black colonies were selected for oxidase test, PYR test, 6.5% NaCl reproduction test. As a result of these biochemical tests, 64 (50%) *Enterococcus* sp. was identified from 130 rectal swab samples.

PCR results

A total of 38 (59%) of enterococci were identified as *E. faecalis* and 26 (41%) of enterococci were identified as *E. faecium* because of PCR by using $ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$ primer pairs. Identification rates for *Enterococcus* sp. are shown in Table 2. The electrophoresis image of *E. faecalis* and *E. faecium* strains is shown in Figure 1.

In our study, it was determined that 8 (21%) of 38 *E. faecalis* strains were positive for aph (2")-Ic gene and had the enzyme of phosphorylation as a result of the PCR study for the detection of aminoglycoside resistance by using aph(2'')-Ia, aph(2'')-Ib, aph(2'')-Ic ve aph(2'')-Id primer pairs. Aminoglycoside resistance was not detected in 26 *E. faecium* strains. In addition, Esp resistance gene was not detected in 38 *E. faecalis* and 26 *E. faecium* strains isolated in this study.

DISCUSSION

Although enterococci are found at high levels in the gastrointestinal tract, they are lesser normal flora candidates in the skin, oral cavity, lower respiratory tract and urogenital systems. Despite having low virulence, opportunistic pathogenic characters are at the forefront with the breakdown of the immune system of the host, and they can often be found as primary or secondary in endogenous and exogenous infections such as intraabdominal or urinary tract infections, meningitis, endocarditis, skin and soft tissue infections (Schouten *et al*., 1999; Ustaçelebi *et al*., 1999; Devriese *et al*., 2006).

The prevalence of enterococci, especially nosocomial infections, is increasing, and in some cases, they are regarded responsible for nosocomial bacteraemia. (Lautenbach *et al*., 1999; Patterson, 2000).

Enterococci are reported to be the second most common cause of nosocomial infections in various countries. *E. faecalis* constitutes 85-95% of enterococci isolated from clinical specimens and 5-10% of *E. faecium* is the most common infectious species. (Teixeria *et al*., 2003).

Özseven *et al*. (2011) found that *E. faecalis* in 60 (48%) and *E. faecium* in 61 (49%) of 124 enterococci strains isolated from urine samples; Baylan *et al*. (2011) reported that 59 (64.8%) of isolates were identified *as E. faecalis* and 31 (34.1%) were *E. faecium* isolates from urine specimens taken from the study. Vural *et al*. (2014) reported that 103 (55.1%) of 187 enterococcal strains isolated from urine specimens identified *E. faecalis* and 74 (39.5%) *E. faecium.*

Yazgı *et al*. (2003) identified 67 faecalis in *E. faecalis* and 45 faecium *E. faecium* in 116 enterococcal strains isolated from 163 rectal swab samples. Kaçmaz *et al*. (2003) found that *E. faecalis* and 5 (19%) of 62 enterococcal strains isolated from various clinical specimens identified *E. faecium* as 20% (74%); Ergin *et al*. (2013) reported that 47 enterococcal cultures isolated from urine specimens were *E. faecalis* in 21 (44.7%) samples and *E. faecium* in 18 (38.3%) samples.

In this study, a total of 64 *Enterococcus* sp. was identified from 130 rectal swab samples examined from the cats. 38 (59%) of faecal enterococci isolates were identified as *E. faecalis* in the identification of isolates using multiplex PCR technique using species-specific primers; 26 isolates (41%) were identified as *E. faecium.*

The isolation rate of *E. faecalis* was found to be 44-74% and the isolation rate of *E. faecium* was 19-49% in previous studies (Kaçmaz *et al*., 2003; Yazgı *et al*., 2003; Baylan *et al*., 2011; Özseven *et al*., 2011; Ergin *et al*., 2013). It was observed that the total isolation rates of enterococci examined in this study were relatively high, consistent with other studies. It has been demonstrated that the excess of isolation rates constitutes a risk for people who have cat-owned pets.

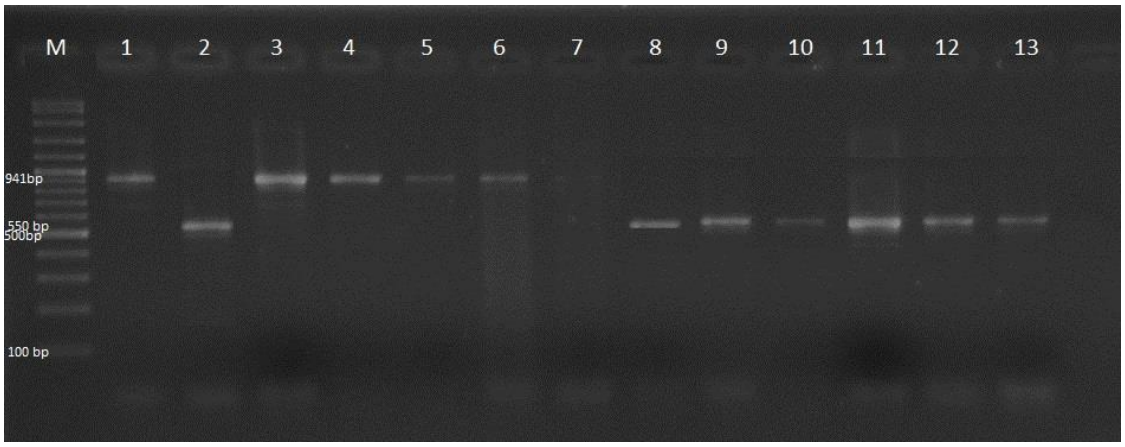

Fig. 1: The electrophoresis image of *E. faecalis* and *E. faecium* strains. M:100 bp DNA ladder, 1: *E. faecalis* ATCC 29212 positive control, 2: *E. faecium* ATCC 700221 positive control, 3-7: *E. faecalis* positive samples, 8-13: *E. faecium* positive samples.

Table 2: Identification rates of *Enterococcus* sp.

Strain (n:64)	Identification number	Identification rate
E. faecalis	38	59
E. faecium	26	41

A significant number of enterococci are noted for their natural resistance to many antimicrobial agents used in the treatment of Gram-positive bacterial infections (Güçkan *et al*., 2013). It has been found that enterococci have antibiotic resistance with new mechanisms and they can transfer through these resistance plasmids (Moellering, 2000), as well as natural resistance to many antibiotics such as penicillins, cephalosporins, quinolones and low level aminoglycosides.

Recently, resistance to many antibiotics including beta-lactams, aminoglycosides, erythromycin, clindamycin, and trimethoprim/sulfamethoxol have been found to be increased from the most commonly used antimicrobial agents in the treatment of Gram-positive bacterial infections. Partial resistance to beta-lactams is due to the low affinity of penicillin-binding proteins for these agents. Aminoglycosides exhibit low levels of resistance to these agents since macrolides and linkozamides do not pass enough through the cell wall. In vitro, trimethoprim / sulfamethoxol sensitive specimens are also resistant to this agent because they can use sources of exogenous folate (Ulusoy, 1999; Güçkan *et al.*, 2013).

High levels of aminoglycoside resistance (AME); expresses the resistance against gentamycin, streptomycin and kanamycin antibiotics. The fact that enterococci are naturally resistant to aminoglycosides at a low level necessitates the combined use of an antibiotic that inhibits cell wall synthesis and an antibiotic group of aminoglycosides to produce a synergistic effect in the treatment. However, because of the resistance development achieved through plasmids and transposons, the occurrence of high level aminoglycoside resistance has been abolished from the synergistic effect obtained using agents inhibiting cell wall synthesis in combination with aminoglycosides (Shepard *et al*., 2002). For this reason, it is important to determine AME resistance. In our study PCR was performed with specific primers of genes belonging to the aminoglycoside resistance group. Sequence analysis of the PCR products revealed that the amplified regions were aminoglycoside resistance gene regions. The aph(2'')-Ic1 gene region responsible for the phosphorylation enzyme coding from the aminoglycoside resistance was amplified by PCR with specific primers in the region of 444 bp amplicon size.

High levels of resistance to one or more aminoglycosides in enterococci have been reported at increasing frequency (Gordon *et al*., 1992; Strausbaugh *et al*., 2000). Baykan (2001) found gentamicin resistance in enterococcal strains isolated from urine specimens in 52%; Kart *et al*. (2010) found that gentamicin resistance was 80% in enterococcal strains isolated from patients in infant clinics and neonatal intensive care unit; Özseven *et al*. (2011) reported that 44% of enterococci strains were produced from various clinical specimens; Kalaycı *et al*. (2011) found that enterococci isolated from urine samples showed gentamicin resistance of 51.2%.

Berzeg (2005) found that 8% of *E. faecalis* strains and 68% of *E. faecium* strains were resistant to gentamicin resistance in enterococci strains isolated from various clinical specimen; Aral *et al*., (2011) reported that 16% of *E. faecalis* strains and 60% of *E. faecium* strains; Iraz *et al*. (2012) reported that 42% of *E. faecalis* strains and 69% of *E. faecium* strains; Altun *et al*. (2013) reported that 44% of the *E. faecalis* strains and 71% of the *E. faecium* strains; Güçkan *et al*. (2013) report that 44% of isolated *E. faecalis* strains and 40% of *E. faecium* strains were resistant to gentamicin.

In this study, none of *E. faecalis* and *E. faecium* strains examined was found to be resistant to gentamicin. The rate of results is lower than those found in previous studies. High-level resistance of gentamicin in enterocytes has been reported to be frequently due to bifunctional enzymes (Harada *et al*., 2005). In the enterococcal strains we have obtained no genes encoding bifunctional enzymes. Therefore, it is concluded that gentamicin resistance is not seen.

Conclusions

In our study, a total of 64 enterococcal strains, 38 *E. faecalis* and 26 *E. faecium*, were isolated and identified from 130 rectal swab samples taken from cats in Aydın and İzmir provinces. The result of examining these strains in terms of determinants involving the development of multiple drug resistance genotype; it was determined that they had 12.5% of the aph(2'')-Ic1 gene regions causing the acquired antibiotic resistance.

Although enterococci are low virulent bacteria, they remain important for nosocomial infections. Because of their natural or acquired resistance against many antibacterial agents, the enterococci have increased rapidly among the hospital infections in recent years. As in the rest of the world, the widespread and uncontrolled use of antibiotics in Turkey provides a selection for the benefit of resistant bacterial strains. The investigations indicate an increase in resistance to antibiotics in clinically isolated bacteria isolated from Turkey. Especially after the 1980s, researchers who have developed resistance mechanisms in antibiotics against various types of enterococci, which restrict the use of available antibiotics in infectious diseases, have attracted the attention of researchers. The ability of multiple resistant enterococci to transmit vancomycin and penicillin resistance properties to streptococci and staphylococci by carrying plasmids carrying the broadest resistance from these bacteria, and the potential for spread of these infections, are highly risky. Therefore, it is necessary to systematically determine ways to protect infection risk factors, preparative factors, resistance detection and screening methods.

The reason for the accumulation of these bacteria, which cause nosocomial infections, on their attention in veterinary medicine is probably due to the lack of intensive care units. On the other hand, it has been shown with this study that close contact with people is a risk for enterococcal carriage, and it is necessary to take this into consideration in laboratory and routine laboratory analyses. There is a need for further study of veterinary medicine in relation to multiple drug resistance of enterococci.

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