Antimicrobial Resistance and Virulence Characteristics in Enterococcus Isolates from Dogs [1]

Yılmaz BOYAR 1, Özkan ASLANTAŞ 1, Süheyla TÜRKYILMAZ 2

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1 Department of Microbiology, Faculty of Veterinary Medicine, Mustafa Kemal University, TR-31040 Hatay - TURKEY
2 Department of Microbiology, Faculty of Veterinary Medicine, Adnan Menderes University, TR-09010 Aydın - TURKEY

Abstract

Antimicrobial resistant enterococci are among the leading causes of nosocomial infections. Transmission of antimicrobial-resistant enterococci from animals to humans has been shown. For this reason, continuous monitoring of antimicrobial resistance in different animal species is of importance both for animal and human health. In this study, it was aimed to investigate the antimicrobial resistance profiles, resistance mechanisms implicated and virulence traits of 107 enterococci isolated from 125 rectal swab samples taken from dogs. The highest resistance rate was determined against tetracycline (65.4%), followed by ciprofloxacin (19.6%), erythromycin (19.6%), chloramphenicol (8.4%) and ampicillin (3.7%). Fourteen (12.1%) enterococci showed multidrug resistance (MDR) phenotype. The tetM gene was predominantly detected among tetracycline isolates. Of 21 erythromycin resistant isolates, 18 harbored the ermB gene. The frequently detected virulence genes was ccf (54.2%), efaAfs (52.3%), cpd (45.8%) and gelE (44.9%). These results indicate that high level of antimicrobial resistance and virulence genes exist among enterococci from dogs and pose a potential public health concern.

Keywords: Dog, Enterococcus spp., Antimicrobial resistance, Virulence genes

INTRODUCTION

Although enterococci are known as commensal agents residing on intestinal microbiota of animals and common in environmental sources, they emerged as an important agents of nosocomial and community-acquired infections due to their ability for developing high level of antimicrobial resistance [1,2]. Overuse and misuse of antimicrobials in pet animals also increases the likelihood of development of resistance to these agents [3-5]. Enterococci can develop antimicrobial resistance mainly by two mechanisms: (i) target mutation and (ii) horizontal gene transfer which includes transformation, conjugation and transduction [6]. Furthermore, enterococci may serve as a reservoir for transfer...
Enterococci have the ability to produce numerous virulence factors playing an important role of pathogenesis of diseases caused by these bacteria [9]. These virulence factors include gelatinase (gelE), enteroococcal surface protein (esp), aggregation substance (aggA), cytolysin (cyIA, cyIB, cyIM), cell wall adhesins (efaAni and efaAmni), sex pheromones (cpd, cob, ccf, eep) [9].

Pet animals are owned by people at increasing rates in Turkey, and this has made these animals a member of the family. Pet animals are known to transfer antimicrobial-resistant bacteria to humans due to their close physical contact with humans [4]. This situation requires continuous surveillance to track antimicrobial resistance among commensal, zoonotic and pathogenic bacteria from these animals, and to monitor changes in antimicrobial resistance over time. Therefore, the objective of this study was to investigate the prevalence of Enterococcus spp. and their antimicrobial resistance profiles and to determine the antimicrobial resistance and virulence genes by polymerase chain reaction (PCR).

MATERIAL and METHODS

Sampling

A total of 125 rectal swab samples from dogs admitted to private veterinary clinics in Hatay were collected from January 2014 to June 2014 for different purposes (medical check up, vaccination, treatment etc.). Rectal swabs were transferred to laboratory in Stuart transport medium. Age of dogs was ranging from two month to 13 year (median age 2 years), including 17 breeds. Among the dogs sampled, 61.6% (n=77) were males and 38.4% (n=48) were females. The study was approved by the Animal Ethical Committe of Mustafa Kemal University (2013/7-6).

Isolation and Identification

Rectal swabs were subjected to pre-enrichment procedure in Enterococcal Broth (BD, USA) at 37°C for 24 h. Following enrichment procedure, a loopful of broth was streaked on Vancomycin Resistant Enterococci (VRE) agar (Oxoid, CM0985, UK) plates with and without vancomycin (6 mg/L). Plates were incubated at 37°C for 24 h and one typical colony randomly selected and passaged to blood agar (Merck, 110886, Germany) supplemented with 5% defibrinated sheep blood for obtaining pure culture. These isolates were presumptively identified as Enterococcus spp. by Gram staining and catalase test. Identification of the isolates was done by 16S rRNA sequencing by using universal primers [10,11]. The PCR products were sequenced from both ends and compared with published nucleotide sequences in GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov/) and a similarity score of 97% or higher was accepted as criterion for establishing species-level identification.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the isolates was determined using disk diffusion method in accordance with Clinical Laboratory Standards Institute guidelines (CLSI) [12] on Mueller Hinton Agar (MHA) plates. Antibiotic disks used were: ampicillin (10 μg), chloramphenicol (30 μg), vancomycin (30 μg), erythromycin (15 μg), tetracycline (30 μg), teicoplanin (30 μg), ciprofloxacin (30 μg), trimethoprim-sulfamethoxazole (1.25 μg /23.75 μg) and gentamicin (120 μg). Enterococcus faecalis ATCC29212 was used as control strain. The isolates resistant to at least three different antimicrobial classes were deemed as multidrug resistant (MDR).

Detection of Antimicrobial Resistance Genes

Presence of antimicrobial resistance genes responsible for phenotypic resistance in disk diffusion assay was investigated by PCR. Erythromycin and tetracycline resistant isolates were screened for the presence of tetK, tetL, tetM, tetO, tetS, emmA, emmB and mefA/E [13]. High level gentamicin resistant isolates for aac(6)-le-aph(2)-la, aph(2)-lb, aph(2)-lc, aph(2)-Id, aph(3)-IIa and ant(4)-Ia [14], chloramphenicol resistant isolates for cat [15], vancomycin resistant isolates for vanA, vanB, vanC1,2, vanD, vanE and vanG [16]. Following resistant reference strains were used as controls: E. faecium BM4147 (vanA), E. faecalis V583 (vanB), E. gallinarum BM 4174 (vanC1), E. casseliflavus ATCC 25788 (vanC2), S. pyogenes BM137 (tetM, emrB), S. pyogenes UR1092 (ermA), S. aureus R-16794 (tetK).

Detection of Virulence Genes

The genes responsible for the expression of gelatinase (gelA), cytolysin (cyIA, cyIM and cyIB), cell wall adhesins (efaAni and efaAmni), enterococcal surface protein (esp), sex pheromones (cpd, cob, cad, ccf and eep) and the aggregation substance (aggA) were investigated as previously described by Eaton and Gasson [17], Marques and Suzart [18] and Shankar et al.[19].

RESULTS

Isolation and Identification of Enterococcus spp.

Overall, 107 (85.6%) Enterococcus spp. were isolated from 125 rectal swab samples. E. faecalis was the most frequently detected species (96; 89.7%), followed by E. faecium (9; 8.4%), E. hirae (1; 0.9%) and E. durans (1; 0.9%).

Antimicrobial Resistance Profiles of Enterococcus spp.

Of 107 enterococci isolates, 15 (14.02%) were susceptible to all antimicrobial tested. None of the isolates were resistant to vancomycin, teicoplanin and high level gentamicin genes to other resident bacteria [4,5,6].
Various rates of resistance were observed to tetracycline (70; 65.4%), ciprofloxacin (21; 19.6%), erythromycin (21; 19.6%), chloramphenicol (9; 8.4%) and ampicillin (4; 3.7%). MDR were detected among 14 (13.1%) enterococci. MDR to six, five, four and three antimicrobials was detected in one (0.9%), one (0.9%), five (4.7%) and six (5.6%) isolates, respectively (Table 1).

**Presence of Resistance Genes Among Resistant Enterococci**

Antimicrobial resistant isolates were screened for the presence of resistance genes acquired by horizontal gene transfer. Of the 70 tetracycline resistant isolates, tetM was the most common, found in 45 isolates (64.3%), both tetL and tetM in 12 (17.1%), tetO in 4 (5.7%) isolates, tetK in 2 (2.9%) and tetL in 2 (2.9%) isolates, which encode proteins involved in ribosomal protection and efflux, respectively. Of 21 erythromycin resistant isolates, 18 harbored ermB gene. cat gene was detected only two resistant isolates (Fig.1, Fig. 2).

**Characterization of Virulence Genes**

The percentage of virulence genes among the tested

<table>
<thead>
<tr>
<th>Resistance Phenotype*</th>
<th>E. faecalis</th>
<th>E. faecium</th>
<th>E. durans</th>
<th>E. hirae</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP, TE, E, CIP, C</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>TE, E, CIP, C</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP, TE, E</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>TE, E, CIP, C</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TE, E</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIP</td>
<td>9</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>3</td>
<td>41</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>2</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Susceptible</td>
<td>19</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>96</td>
<td>9</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* AM: ampicillin, TE: tetracycline, CIP: ciprofloxacin, E: erythromycin, C: chloramphenicol

![Fig 1. Agarose gel electrophoresis of tetracycline and macrolide resistance genes. Lane 1: 100 bp molecular marker, Lane 2: ermB and tetL, Lane 3: ermB and tetM, Lane 4: tetM, Lane 5: ermB, tetL and tetM](image1)

![Fig 2. Agarose gel electrophoresis of cat gene detected in chloramphenicol resistant isolates. Lane 1: 100 bp molecular marker, Lane 2-3: cat gene](image2)
strains is shown in Fig. 3. One hundred and four (97.2%) isolates carried one or more virulence genes. efaA was not detected in any of the isolates. The efaA gene, encoding cell wall adhesin in enterococci, was found in 52.3% of the isolates. Sex pheromone genes, ccf, cpd, cob and eep, were detected in 54.2%, 45.8%, 16.8% and 24.3% of the isolates, respectively. Frequency of cyIA, cyIM and cyIB genes were observed in 8.4%, 7.5% and 5.6%, respectively. The gelE, esp and aggA genes were observed in 44.9%, 33.6% and 21.5% of the isolates, respectively.

DISCUSSION

Antimicrobial resistance is a major concern in human and animal health throughout the world. Even tough it is known that enterococci are ubiquitous microorganisms found different habitats including gastrointestinal tract of human and animals, enterococci recently emerged as a leading cause of multiresistant, nosocomial infections in humans [20]. A recent study showed that possible transmission of resistant enterococci species between dog, human and hospital environments [35].

Among 107 enterococcal isolates, E. faecalis (89.7%) was the most frequently isolated species followed by E. faecium (8.4%). In contrast, E. faecium was reported to be most common species isolated from dogs in previous studies carried out in Turkey [23,24]. E. faecalis has been reported to be predominantly isolated from urinary tract infections of both dogs KuKanich and Lubbers [25] and humans [26] as a causative agent.

Increased use of antimicrobials in pet animals lead to the emergence of resistant bacteria [1]. The rate of antimicrobial resistance observed among enterococci in this study was lower than those reported of Türkyılmaz et al.[23] in Aydın, but higher than findings of Boynukara et al.[24] in Van, suggesting that resistance rates for enterococci vary regionally and influenced by antibiotic usage. However, Boynukara et al.[24] did not mention the origin of the dogs in their study. Resistance rate to tetracycline (65.4%), which is a widely used in veterinary field in Turkey, was higher than those from findings (41.1%) of Boynukara et al.[24], but lower than findings (70.3%) of Türkyılmaz et al.[23]. The erythromycin resistance rate (19.6%) was similar to findings (21.1%) of Boynukara et al.[24]. But, Türkyılmaz et al.[23] reported higher erythromycin resistance rate (69.2%). In previous studies, no acquired vancomycin resistance mediated by transferable vanA or vanB gene was reported in Turkey among enterococci [23,27,28]. Similarly, no resistance was observed against this agent in the current study.

However, intrinsic resistance encoded by vanC gene has been reported among E. gallinarum and E. casseliflavus species from dogs [23,27,28]. Resistance to ciprofloxacin, which is a clinically important drug, was found high (19.6%), in contrast to findings of Boynukara et al.[24], who reported a resistance rate of 6.7%. The rate of chloramphenicol resistance (8.4%) found in this study was comparable to findings (12.1%) of Türkyılmaz et al.[23]. Ampicillin is a drug of choice to be used for the treatment of multi-drug resistant enterococcal infection in combination with aminoglycosides when the isolates did not exhibit high level aminoglycoside resistance [29]. Furthermore ampicillin resistant enterococci are usually resistant to other classes of antimicrobials used in dogs [30]. Even tough overall prevalence of ampicillin resistance was low (3.7%), detection of ampicillin resistance poses a significant risk for both human and animal health, and further investigation are, therefore, necessary to determine the true prevalence of ampicillin resistant enterococci in dogs using selective media. In a recent study, Celik et al.[31] reported higher prevalence (20.9%) of ampicillin resistant enterococcus using selective media.

In current study, the most common resistance gene detected was the tetM in tetracycline resistant enterococci isolates. Previous studies carried out in Turkey showed predominance of tetM gene in tetracycline resistant enterococci from different sources such as meat [32], cheese [33] as well as dogs [23]. Reason of wide dissemination of tetM gene is that this gene is frequently carried by Tn946 in enterococci [34]. Main resistance mechanism against macrolides is the modification of 23S rRNA by erm methylases among enterococci [34]. In the current study, ermB was the only gene detected in macrolide resistant enterococci. Similarly, Türkyılmaz et al.[23] reported high prevalence of ermB gene. The cat gene responsible for chloramphenicol resistance was found at a very low
rate (22.2%), suggesting presence of acquired other resistance genes or mechanisms responsible for this resistance phenotype. In a study carried out by Yilmaz et al. [32], lower prevalence rate (4.8%) of cat gene among chloramphenicol resistant enterococci from meat was reported. In contrast, Türkylmak et al. [22] detected cat gene in 63.6% of chloramphenicol resistant enterococci.

Enterococci have the ability to produce various virulence factors contributing the course and severity of infection [33]. The efaA and efaAm are cell wall adhesins associated with infective endocarditis. In this study, efaA was detected in 58.3% of the isolates. efaAm was not present in any E. faecium isolate. Iseppi et al. [36] found efaA gene in 33.3% of E. faecalis isolates and efaAm gene in 50% of E. faecium isolates.

Cytolysin is a bacteriocin-type exotoxin, exerts its effects towards erythrocytes, leukocytes and macrophages [8]. clyA, clyM and clyB genes were detected in 8.4%, 7.5% and 5.6% of the isolates, respectively. A similar finding was also reported by Iseppi et al. [36], who detected these genes in 8.3%, 8.3% and 8.3% of E. faecalis isolates. In this study, the authors detected only clyM gene (7.1%) in E. faecium isolates. Gülhan et al. [37] investigated cytolytic activity of E. faecalis and E. faecium from humans and pet animals, and detected no cytolytic activity in dog isolates, except one E. faecium isolate from cat.

geE is zinc-dependent metalloendopeptidase, is capable of hydrolysing gelatine, elastin, collagen, haemoglobin [8]. Geletinase has also been reported to play an important role in the development of endocarditis and decrease neutrophil accumulation to the site of infection by splitting complement C5a fragment, which has anaphylotoxin activity [34]. In the current study, the geE was detected in 44.9% of the isolates. However, Iseppi et al. [36] reported higher prevalence rates 57.1% and 80.3% of E. faecium and E. faecalis isolates, respectively. Gülhan et al. [37] found 26.6% and 60% of E. faecium and E. faecalis isolates positive for gelatinase activity.

Aggregation substance (AS) is enterococcal surface protein that contributes the formation of mating aggregates facilitating bacterial conjugation [8]. In the current study, 21.5% of the isolates were positive for aggA gene. In contrast, Iseppi et al. [36] did not find this gene, and speculated that the isolates had a reduced capability to transfer resistance virulence genes by mating. A very low rate was also reported by Gülhan et al. [37], who detected a positivity rate of 1.6% and 6.7% for E. faecium and E. faecalis isolates, respectively.

Enterococci have the ability to produce sex pheromones (cpd, cob, ccf, cad) facilitating conjugative plasmid transfer between cells [8]. Sex pheromone genes, ccf, cpd, cob and eep, were detected in 54.2%, 45.8%, 16.8% and 24.3% of the isolates. In a recent study, higher prevalence of sex pheromones was reported among enterococci from meat samples by Yilmaz et al. [32].

The esp gene is known to be involved in biofilm formation. Furthermore, biofilm production has been shown to play an important role in the exchange of antibiotic resistance genes between cells and to increase their resistance to antibiotics [8]. The esp gene was detected in 33.6% of the isolates. In contrast, Iseppi et al. [36] did not find this gene among the isolates. Also, Oliveira et al. [39] reported a lower prevalence rate (10%) among enterococci from dogs with periodontal disease.

In conclusion, the current study reveals that dogs are colonized with potentially virulent and antibiotic resistant enterococci. Therefore, the possibility of transmission of enterococci from dogs to humans, particularly those in the risk group, can not be excluded. Also, further studies are needed to elucidate the risk of transmission to humans by analyzing the clonal relationship in enterococci from dogs and dog owners.

REFERENCES

Antimicrobial Resistance and...