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Antimicrobial susceptibility patterns of environmental and hospital isolations of enterococci in Aydın

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Abstract: The aim of the present study was to evaluate antimicrobial susceptibilities of enterococci isolated from water, soil, waste, and food samples to determine resistance mechanisms and their transferability, and to test clonality of these environmental enterococci. The results were compared with the susceptibility patterns of hospital enterococci reported in a previous study. Samples from 9 environmental sources were cultured, and species were identified using 16S rRNA sequencing. Antimicrobial susceptibilities were determined by the agar dilution method. The minimum inhibitory concentrations were tested for vancomycin, tetracycline, teicoplanin, ampicillin, gentamicin, erythromycin, rifampicin, clindamycin, chloramphenicol, and ciprofloxacin. Resistance genes and transposons were determined by polymerase chain reaction. The transferability of resistance was tested by conjugation. A total of 57 enterococci were isolated. All erythromycin-resistant enterococci had *ermB* and all tetracycline-resistant isolates had *tetM* genes. Macrolide and tetracycline resistances were transferable by conjugation to *Enterococcus faecalis* JH2-2. Transconjugants were confirmed by pulsed-field gel electrophoresis analysis. Our study showed the existence of antimicrobial resistance among bacteria isolated from environmental samples. All isolates were resistant to at least one antibiotic tested. Transferability of resistance genes in environmental enterococci showed the importance of environmental resistance for public health and the potential of resistance gene dissemination. Although the sample collection times of environmental and hospital isolates were different, the possibility exists that the difference in susceptibility patterns may be the result of differences in antimicrobial pressure in hospitals and in the environment, and this should be studied further.

Key words: Enterococci, environmental resistance, antibiotic resistance, conjugation, quinolone resistance, antimicrobial pressure

1. Introduction

Enterococci are gram-positive, catalase-negative, nonspore-forming, facultative anaerobic bacteria. Large amounts of enterococci are found in stool, which makes their spread easy in nature and in the environment (1). Different species of enterococci are able to adapt better to different environments. *Enterococcus faecium* and *E. faecalis* are quite common in the human intestinal flora. *E. faecium* is found within animal sources, and *E. mundtii* and *E. casseliflavus* are more often found in vegetable sources (2). Enterococci are naturally resistant to low levels of beta-lactam antibiotics and aminoglycosides. *E. faecalis* is also naturally resistant to lincosamides. In addition to intrinsic resistance to beta-lactams and aminoglycosides, acquisition of resistance to streptogramins, macrolides, and vancomycin limits therapeutic options for infections due to enterococci (3). Use of antibiotics in treatment and as a growth promoter causes antibiotic pressure on the bacterial gut flora of humans and animals, which results in selection of antibiotic-resistant bacteria. Antibiotic-resistant floral bacteria disperse in nature and cause dissemination of antibiotic-resistant floral bacteria into the environment. Accumulation of active antibiotics discarded from humans and animals without modifications in nature increases the risk of the development of resistance among environmental bacteria. Reconsumption of these antibiotic-resistant bacteria from the environment with food increases dissemination of resistance genes in the gut (4,5). The aim of the present study was to evaluate antimicrobial susceptibility of enterococci isolated from water, soil, waste, and food samples. Among the resistant strains, antimicrobial resistance mechanisms, transferability of resistance genes, and clonality were also studied.

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2. Materials and methods

2.1. Isolation and preidentification of enterococci samples

Samples were collected from different soil and water samples, animals, raw vegetables, and fruits that may represent enterococci populations. Sampling was done in 9 stations predetermined and cultured on m-Enterococcus Agar (Difco) plates. These samples were from the Menderes River, irrigation canals, agricultural soil, nonagricultural soil, the Nazilli and Umurlu waste management centers, fecal samples from farm animals, thermal springs in Ilıca and Sarayköy, garbage trucks, and vegetables and fruits from local farmers' markets. The samples from soil and raw vegetables and fruits were homogenized. Upon arrival to the laboratory, at least 10 g of sample was diluted 6 times with phosphate-buffered saline (PBS). One milliliter of these solutions was incubated for 24 h at 37 °C on m-Enterococcus Agar. Distinct colonies were transferred for 24 h at 44 °C on Enterococcosel Agar (Difco). Feces and cecal samples (at least 1.5 g) were diluted 6 times with PBS and mixed carefully. Each of the diluted PBS samples for inoculation was done to spread 100 µL of this PBS solution on m-Enterococcus Agar (6).

2.2. Identification of enterococci

Identification of bacteria was done by 16S rRNA sequencing. First, the total DNA was extracted from isolates by the phenol-chloroform method (7). 16S rRNA genes were amplified by PCR using universal 16S primers. To amplify the 16S rRNA gene, universal primers S16S20 5' AGA GTT TGA TCC TGG CTC AG 3' and 16S1390 5' GAC GGG CGG TGT GTA CAA 3' were used (8,9). PCR experiments were carried out under the following selected conditions: 2.5 U Taq polymerase (Fermentas), 10X Taq buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2 mM MgCl2, 0.4 pmol primers, 0.2 mM dNTP, and 2 µL template sample DNA in a final volume of 30 µL. Amplification was obtained with an initial denaturation step at 94 °C for 10 min followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. All amplified 16S rRNA fragments were sequenced (Macrogen, http://dna.macrogen.com/eng/). The sequences were compared to the gene bank at www.blast.ncbi.nlm.nih.gov and were identified by sequence homology.

2.3. Minimum inhibitory concentration testing

Antimicrobial susceptibilities were tested using the agar dilution minimum inhibitory concentration (MIC) method (10). Inoculum was prepared by 1/10 dilution of 0.5 McFarland bacterial solution. Bacteria (5×10^4 cfu/mL) were transferred from a 96-well plate to agar with antibiotics using a multipoint inoculator. Susceptibilities of all isolates to vancomycin (Sigma), tetracycline (Applichem), teicoplanin (Aventis), ampicillin (Sigma), gentamicin (Sigma), erythromycin (Applichem), rifampicin (Sigma), clindamycin (Sigma), chloramphenicol (Applichem), and ciprofloxacin (Biopharma) were tested.

2.4. Investigation of antibiotic resistance genes and transposons

The presence of known resistance genes and the presence of transposons were tested by polymerase chain reaction (PCR). *ermB*, *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetM*, *tetK*, *tetO*, *tetS*, *tetQ*, and *tetW* resistance genes were studied by PCR in all resistant enterococci using specific primers as described previously. The tetracycline resistance genes in enterococci is carried by transposon *Tn916*, and the erythromycin resistance gene is carried by transposons *Tn917* and *Tn1545*. The presence of these transposons was investigated by PCR using specific primers (11,12).

2.5. Transferability of resistance genes

The transferability of resistance determinants was tested by conjugation using *E. faecalis* JH2-2 as the recipient strain. *Enterococcus faecalis* JH2-2 is resistant to rifampicin and fusidic acid and susceptible to erythromycin and tetracycline. Mobility of *tetM* and *ermB* resistance genes was studied using the conjugation method. In brief, 500-µL cultures of both donor and recipient strains in the exponential phase (OD_{600} = 0.5–0.8) were mixed and centrifuged. The pellet was resuspended in 100 µL of broth and inoculated onto a BHI agar plate. After 24 h of incubation at 37 °C, the grown bacteria were resuspended and inoculated onto selective agar with antibiotics. The antibiotics used for the selection of transconjugants were rifampin (100 mg/mL), fusidic acid (50 mg/mL), and either erythromycin, tetracycline, or chloramphenicol (each at 10 mg/mL).

Colonies grown on selective agar were incubated onto agar containing the same concentrations of antibiotics. All transconjugants were stored at –80 °C for further study. All transconjugants were compared to *E. faecalis* JH2-2 by pulsed-field gel electrophoresis (PFGE) to discriminate contaminants from transconjugants (3).

2.6. PFGE analysis

Clonal relations between enterococci and the transferability of resistance determinants were shown by PFGE (13).

3. Results

A total of 57 enterococci were isolated from environmental samples collected in Aydın, Turkey. Of 57 enterococci, 33 (58%) were *E. faecium*, 4 (7%) were *E. casseliflavus*, 6 (11%) were *E. hirae*, 4 (7%) were *E. durans*, 4 (7%) were *E. faecalis*, 4 (7%) were *E. mundtii*, and 2 (4%) were *E. avium* (Table 1).

The MIC ranges for these antibiotics and MIC_{50} and MIC_{90} values were as indicated in Table 2. Tetracycline

Table 1. Samples were collected from different areas, animals, and raw vegetables and fruits that represent enterococci populations.

Source	Distribution of enterococci strains						
	E. faecalis	*E. faecium*	*E. casseliflavus*	*E. durans*	*E. avium*	*E. mundtii*	*E. hirae*
Water and soil samples	4 (7%)	21 (37%)	4 (7%)	4 (7%)	2 (4%)	1 (2%)	3 (5%)
Animal samples		9 (16%)					2 (4%)
Vegetable and fruit samples		3 (5%)				3 (5%)	1 (2%)
Total	4 (7%)	33 (58%)	4 (7%)	4 (7%)	2 (4%)	4 (7%)	6 (11%)

(0.5 μg/mL) had the lowest MIC_{50} levels, followed by teicoplanin (1 μg/mL), erythromycin (1 μg/mL), vancomycin (2 μg/mL), ampicillin (2 μg/mL), gentamycin (2 μg/mL), ciprofloxacin (2 μg/mL), rifampicin (4 μg/mL), chloramphenicol (8 μg/mL), and clindamycin (128 μg/mL). Resistance rates for vancomycin, teicoplanin, gentamicin, chloramphenicol, tetracycline, ampicillin, erythromycin, rifampicin, clindamycin, and ciprofloxacin were 0%, 0%, 0%, 0%, 16%, 9%, 11%, 63%, 95%, and 33%, respectively. Of 57 enterococci, 13 (23%) were of intermediate resistance to vancomycin; 6 (46%) of these were *E. faecium*, 4 (31%) were *E. casseliflavus*, 2 (15%) were *E. mundtii*, and 1 (8%) was *E. durans*. All enterococci were resistant to at least one antimicrobial tested. None of the strains had resistance to vancomycin, teicoplanin, gentamicin, and chloramphenicol (Tables 2 and 3).

All erythromycin-resistant enterococci had the *ermB* gene, and all tetracycline-resistant isolates had the *tetM* gene. The tetracycline-resistant enterococci had *Tn*916, and 3 of the 6 erythromycin-resistant isolates had *Tn*917. The remaining 3 strains resistant to erythromycin were negative for *Tn1545* and *Tn917*. All erythromycin-resistant strains were tested for their ability to transfer erythromycin resistance to *E. faecalis* JH2-2. Among 6 isolates with *ermB*, 3 were positive for resistance transfer and transconjugants were stored for further study. Only 3 erythromycin-resistant strains with *Tn*917 were positive for resistance transfer. Conjugation experiments showed that only 1 tetracycline-resistant strain among 9 was able to transfer tetracycline resistance to *E. faecalis* JH2-2. Conjugation experiments showed transferability of erythromycin and tetracycline resistance to *E. faecalis* JH2-2. All transconjugants were analyzed by PFGE and restriction profiles were compared to *E. faecalis* JH2-2. No difference was observed between transconjugants and recipient strain *E. faecalis* JH2-2.

Table 2. Susceptibilities (S: susceptible, I: intermediate, R: resistant), MIC_{50} values (μg/mL), MIC_{90} values (μg/mL), and MIC ranges (μg/mL) of ampicillin, vancomycin, teicoplanin, erythromycin, clindamycin, gentamicin, tetracycline, rifampicin, chloramphenicol, and ciprofloxacin detected for all isolates.

Antibiotics	S (%)	I (%)	R (%)	MIC_{50}	MIC_{90}	Range
Ampicillin	52 (91)	--	5 (9)	2	4	0.5–32
Vancomycin	44 (77)	13 (23)	0	2	8	0.5–16
Teicoplanin	57 (100)	0	0	1	2	0.25–4
Erythromycin	22 (38)	29 (51)	6 (11)	1	2	0.12–128
Clindamycin	3 (5)	--	54 (95)	>128	>128	2–128
Gentamicin	57 (100)	--	--	2	16	0.12–32
Tetracycline	48 (84)	0	9 (16)	0.5	32	0.25–128
Rifampicin	18 (32)	3 (5)	36 (63)	4	16	≤0.06–16
Chloramphenicol	56 (98)	1 (2)	0	8	8	4–16
Ciprofloxacin	25 (44)	13 (23)	19 (33)	2	4	0.25–16

Table 3. Antimicrobial susceptibilities of *Enterococcus* spp. isolated from environmental samples. S = susceptible, I = intermediate, R = resistant.

Antibiotics	*E. faecium* (n = 33) (%)			*E. faecalis* (n = 4) (%)			*E. casseliflavus* (n = 4) (%)			*E. mundtii* (n = 4) (%)			*E. avium* (n = 2) (%)			*E. hirae* (n = 6) (%)			*E. durans* (n = 4) (%)		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Ampicillin	29 (87.9)	0 (0.0)	4 (12.1)	4 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	3 (75.0)	0 (0.0)	1 (25.0)	2 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)
Vancomycin	27 (81.8)	6 (18.2)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	2 (50.0)	2 (50.0)	0 (0.0)	0 (100.0)	2 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	3 (75.0)	1 (25.0)	0 (0.0)
Teicoplanin	33 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)
Erythromycin	10 (30.3)	18 (54.6)	5 (15.1)	2 (50.0)	2 (50.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	2 (50.0)	2 (50.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	5 (83.3)	1 (16.7)	0 (0.0)	1 (25.0)	2 (50.0)	1 (25.0)
Clindamycin	3 (9.1)	0 (0.0)	30 (89.9)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)
Gentamicin	33 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)
Tetracycline	27 (81.8)	0 (0.0)	6 (18.2)	4 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	3 (75.0)	0 (0.0)	1 (25.0)	1 (50.0)	0 (0.0)	1 (50.0)	6 (100.0)	0 (0.0)	0 (0.0)	3 (75.0	0 (0.0)	1 (25.0)
Rifampicin	10 (30.3)	2 (6.1)	21 (63.6)	1 (25.0)	0 (0.0)	3 (75.0)	0 (0.0)	0 (100.0)	4 (0.0)	3 (75.0)	0 (0.0)	1 (25.0)	0 (0.0)	1 (50.0)	1 (50.0)	3 (50.0)	0 (0.0)	3 (50.0)	1 (25.0)	0 (0.0)	3 (75.0)
Chloramphenicol	33 (100.0)	0 (0.0)	0 (0.0)	3 (75.0)	1 (25.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)
Ciprofloxacin	12 (36.4)	5 (15.1)	16 (48.5)	3 (75.0)	1 (25.0)	0 (0.0)	0 (0.0)	3 (75.0)	1 (25.0)	3 (75.0)	1 (25.0)	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)	5 (83.3)	1 (16.7)	0 (0.0)	1 (25.0)	1 (25.0	2 (50.0)

4. Discussion

The enterococci are important agents for the dissemination of resistance genes (1,2,4). Under antimicrobial pressure, the successful resistant strains remain in the environment. The bacteria that live in the environment may be in contact with antibiotics, and their resistance levels may reflect the antimicrobials in the environment. The presence of plasmids and integrons may also increase the horizontal transfer of resistance genes among the bacterial species (14). The aim of this study was to elaborate the level of antimicrobial resistance among environmental enterococci isolates.

Enterococci are present normally in the intestinal flora of humans and animals. The enterococci isolated from environmental samples originated from fecal material. Oral fecal transmission of enterococci is important for dissemination of antimicrobial resistance because the gastrointestinal system, as well as the environment, give these bacteria opportunity to acquire new resistance genes (1,2,4).

Enterococcal populations may vary depending on the location from which the sample is taken. It was shown that the species that constitute enterococcal populations in a hospital and in the environment may be different. *E. faecalis* is the most commonly isolated enterococci species from hospitalized patients and hospital sewage; however, in the environment, *E. faecium* is the most common species (6). A study in a university hospital of Aydın Province showed that 69% of enterococci were *E. faecalis* and 26% were *E. faecium* (15). However, our study, done with environmental samples, showed that *E. faecium* (58%) was the most common species isolated. This did not change depending on the sample area. *E. faecium* was also the most commonly isolated bacteria from all the sample stations, including water and soil samples, animal samples, and vegetables and fruit samples. Livestock is an important sector for Aydın Province. Enterococci are less important constituents of the animal flora, which is dominated by *S. bovis* (16). However, it was shown that among enterococci isolated from meat samples, *E. faecalis* was found more frequently than *E. faecium* (17). Our study showed that in animal gut flora, *E. faecium* was the principal enterococcal species.

Enterococci are intrinsically resistant to most of the antibiotics currently used, including cephalosporins and aminoglycosides. In addition, *E. faecalis* is resistant to lincosamides. Enterococci may become resistant to streptogramins, macrolides, chloramphenicol, tetracycline, and vancomycin. The environmental isolates were resistant to at least one antimicrobial tested. The highest levels of resistances were for clindamycin, followed by rifampicin and ciprofloxacin. Ampicillin resistance was low and all isolates were susceptible to teicoplanin and gentamycin.

The resistance profile of environmental enterococci was different from the resistance profile of hospital isolates (Table 4). Although the sample collection times of the environmental and hospital isolates were different, we may still speculate some conclusions. There are different antimicrobial pressures for environmental and hospital isolates. The results of the study done by our group with these clinical enterococci showed high resistance levels to macrolides, which remained moderate among environmental isolates (15). One of the most important differences was in quinolone resistance, which was very high among environmental strains but remained low among clinical strains. This may be a clue for potential use of quinolones as a growth factor for livestock. High-level gentamycin resistance was common (38%) among clinical isolates, but the environmental isolates were all susceptible. This may indicate an absence of gentamycin pressure in the environment, which is important in hospital environments.

Our results showed that acquired resistance mechanisms can be transferred to other enterococci. This is a major risk factor for dissemination of resistance genes obtained in the environment to pathogen bacteria. Transferability of

Table 4. Comparison of antimicrobial susceptibilities of hospital enterococci and environmental enterococci isolated from Aydın area. S = susceptible, I = intermediate, R = resistant.

	Hospital enterococci (15) (n = 61)			Environmental enterococci (This study)(n = 57)		
	S%	I%	R%	S%	I%	R%
Vancomycin	100	0	0	77	23	0
Erythromycin	43	3	54	38	51	11
Clindamycin	0	0	100	5	0	95
Gentamicin	62	0	38	100	0	0
Tetracycline	46	0	54	84	0	16
Chloramphenicol	70	4	26	98	2	0
Quinolone	92	0	8	44	23	33

resistance genes in environmental enterococci shows the importance of environmental resistance for public health and the potential of resistance gene dissemination. Our study showed the existence of antimicrobial resistance among bacteria isolated from environmental samples, and all isolates were resistant to at least one antibiotic tested.

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