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Determination of antibiotic resistance and resistance plasmids of clinical *Enterococcus* species

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To determine the antibiotic resistance pattern and resistance plasmids, we studied 23 antibiotic-resistant clinical isolates of *Enterococcus* spp. which caused infection in Bayındır-Ankara Hospital, Turkey. Biochemical and physiological identification tests were applied by the Vitek system and compared with the results of protein profiles by SDS-PAGE. From 23 isolates, 20 were identified as *E. faecalis*, 2 as *E. faecium* and 1 as *E. gallinarum*. Twenty four antibiotics belong to 10 different groups were used in susceptibility tests. Multiple antibiotic resistance was determined in 10 of 23 *Enterococcus* spp. Overall resistance to the used antibiotics was 47.3% and low level resistance was 16.6%. Among the isolates tested, 8.7% demonstrated high level gentamicin resistance, 17.4% demonstrated high level streptomycin resistance, and 43.5% demonstrated penicillin resistance. High level vancomycin resistant *Enterococcus* spp. rate was 34.8%, and 60.9% exhibited low level resistance to vancomycin and teicoplanin. They contain plasmids which varied in numbers between 1 and 11 and the plasmid sizes ranged from 2.08 to 56.15 kb. In curing experiments with acriflavine, two different plasmids were shown in different molecular sizes of 33.49 and 13.6 kb while the first determined glycopeptide and penicillin resistance, the second one determined either glycopeptide or penicillin resistance in two different *E. faecalis* strains. On the other hand, a 22.58 kb plasmid, determining kanamycin resistance, was detected in an *E. faecium* strain. After the curing experiments, an elimination of 37.17 and 44.47 kDa protein bands was shown in *E. faecium* EFA1 and *E. faecalis* EFA13 in SDS-PAGE, respectively. This survey indicates the increase of antibiotic-resistant enterococci, especially to vancomycin in our hospital isolates.

Key Words——antibiotic resistance; *Enterococcus*; plasmids

Introduction

Enterococci have emerged as the third most common cause of nosocomial infections, requiring bactericidal antimicrobial therapy (Tenover et al., 1993). Approximately 85–90% of enterococcal infections are attributed to *E. faecalis* and 5–10% to *E. faecium* (Heaton et al., 1996). Antibiotic resistance among these organisms is being described with increased frequency worldwide and is narrowing the therapeutic options for treatment of enterococcal infections (Handwerger et al., 1992). Correct species identification, optimization of antibiotic susceptibility tests, and determination of the genetic basis of antibiotic resistance are important for preventing this resistance and developing novel antibiotic utilization policies especially for third

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world countries. In recent years enterococci, resistant to multiple antimicrobial agents, including vancomycin, β-lactams and aminoglycosides have been detected (Tenover et al., 1993). Plasmid mediated antibiotic resistance has been observed in both vancomycin, and aminoglycoside resistant and also in β-lactamase producing enterococcal isolates (Bozdogan and Leclercq, 1999; Leclercq et al., 1988, 1989; Schouten et al., 1999; Simjee and Gill, 1997). Of these antibiotic-resistant nosocomial pathogens that were detected in Turkey, only two vancomycin resistant *Enterococcus* species have been determined till now (Basustaoglu et al., 2001; Colak et al., 2002). This survey is the third report of vancomycin and multiple antibiotic-resistant enterococci in Turkey. The aim of this study is to determine the antibiotic susceptibility and resistance plasmids of clinical *Enterococcus* species isolated from Bayındır-Ankara Hospital in Turkey.

Materials and Methods

Bacterial isolates and their identifications. The following standard strains were used in this study: *E. faecium* CDC NJ-1, *E. gallinarum* CDC NJ-4 and *E. faecalis* ATCC 51299, all obtained from Jana M. Swenson (Centers for Disease Control, Atlanta, GA, USA). Twenty-three antibiotic-resistant isolates were selected from 64 clinical isolates of enterococci. They were obtained from various body sites of different patients that were hospitalized at the Bayındır-Ankara Hospital and none of the isolates were clonally related. These were named in order from EFA1 to EFA23. Isolates were reidentified as enterococci by using bile esculin utilizing, Group D antigen (Shield Diagnostics) and growth in 6.5% NaCl. All strains were tested further by fermentation tests with a Vitek System (Biomeriux) to differentiate the species level (Tenover et al., 1993; Vincent et al., 1991; Willey et al., 1993). Pigment production and motility were tested as previously described (Facklam and Collins, 1989; Facklam et al., 1989, 1997; Vincent et al., 1991).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate whole cell proteins as previously described (Laemmli, 1970). The bacterial cells were collected from sencronized Brain Heart Infusion broth cultures and the total cell proteins were extracted by the SDS-sample buffer. The samples were applied in the same protein contents (Esen, 1978) into wells, and the electrophoresis was carried out at 30 mA for 3 h. Protein bands were stained with Coomassie Brilliant blue.

Susceptibility testing. Twenty-four antibiotics belonging to 10 different groups were used for the antibiotic susceptibility test. Vancomycin, gentamicin, ampicillin, penicillin, streptomycin, tetracycline, ciprofloxacin and nitrofurantoin antibiotics were used to determine their MIC in Vitek (Tenover et al., 1993; Vincent et al., 1991). A disk diffusion test was performed by using Mueller-Hinton agar according to the National Committee for Clinical Laboratory Standards guidelines for enterococci (NCCLS, 2001a, b). The disks (Oxoid) and their contents were as follows: vancomycin 30 μg; teicoplanin 30 μg; bacitracin 0.04 IU; erythromycin 15 μg; chloramphenicol 30 μg; rifampin 5 μg; tetracycline 30 μg; gentamicin 120 μg; tobramycin 10 μg; kanamycin 30 μg; streptomycin 10 μg; amikacin 30 μg; ampicillin 10 μg; penicillin 10 IU; meropenem 10 μg; oxacillin 1 μg; ampicillin-clavulonate 30 μg; nalidixic acid 30 μg; ofloxacin 1 μg; norfloxacin 10 μg, trimethoprim-sulfomethoxazole 30 μg, and cephalothin 30 μg (NCCLS, 2001a, b; Tenover et al., 1993).

Isolation and analysis of plasmid DNA. Plasmid DNA was isolated by the procedure of Anderson and McKay (1983), separated by 0.7% agarose gel electrophoresis, and stained with ethidium bromide (Meyers et al., 1976). Supercoiled ccc DNA (Sigma Chem., Co., D5292) was used as the standard DNA marker in agarose gel electrophoresis. Curing experiments were carried out by the treatment of enterococci with the mutagen acriflavine to eliminate plasmid-mediated resistance (Akcelik et al., 2001; Leclercq et al., 1989; Uttley et al., 1988). Acriflavine concentration was adjusted according to the minimal inhibition of bacterial growth.

Results and Discussion

Identification results

Vitek correctly identified the two standard strains *Enterococcus faecium* CDC NJ-1 and *Enterococcus faecalis* ATCC 51299 but identified *Enterococcus gallinarum* CDC NJ-4 as an *E. faecium* strain. On the other hand, Vitek identified 20 isolates as *E. faecalis* and 3 as *E. faecium*. But according to the motility and pigment production tests, *E. gallinarum* EFA-5 was a motile and non-pigmented strain.

There was an apparent similarity when the biochemical and physiological identification results were con-

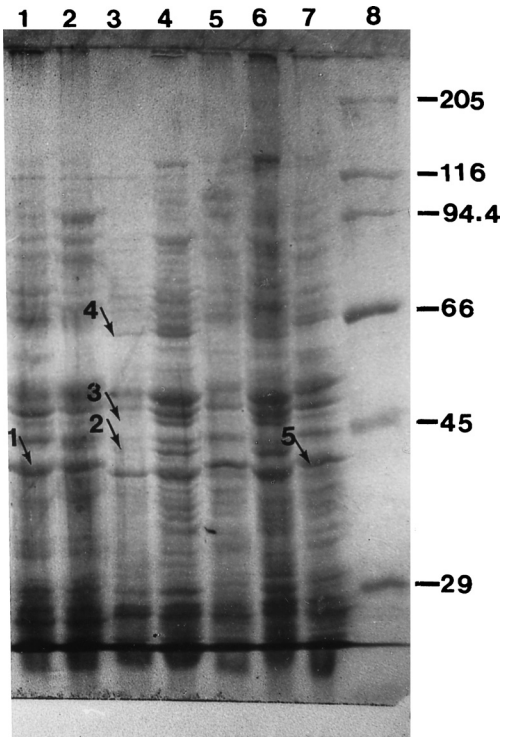


Fig. 1. Protein profiles of *Enterococcus* species by SDS-PAGE.

Lane 1, *E. faecalis* EFA3; Lane 2, *E. faecalis* ATCC51299; Lane 3, *E. faecium* CDC NJ-1; Lane 4, *E. faecium* EFA23; Lane 5, *E. gallinarum* EFA5; Lane 6, *E. faecium* EFA1; Lane 7, *E. gallinarum* CDC NJ-4; Lane 8, molecular weight standard mixture (Sigma Chem. Co., MW-SDS-6H), (in kilodalton, kDa). The molecular weights of distinctive protein bands, marked from 1 to 5 were 40.35, 42.67, 47.24, 66.28 and 40.98 kDa, respectively.

firmed by the SDS-PAGE protein profiles. Depending on the difference and similarity in the protein bands of three different standard strains, distinctive protein bands were selected and compared with the 23 enterococcal isolate as shown in Fig. 1. Protein bands of 40.35 and 47.24 kDa molecular weight were both similar in *E. faecium* and *E. faecalis* and used to differentiate these two species from *E. gallinarum*. Protein bands of 42.67 and 66.28 kDa molecular weight were detected in *E. faecium* strains and used to separate this species from *E. faecalis* and *E. gallinarum* strains. On the other hand, a 40.98 kDa molecular weight protein band was used to differentiate *E. gallinarum* species from *E. faecalis* and *E. faecium* strains. Only *E. gallinarum* EFA-5 and *E. gallinarum* CDC NJ-4, identified as *E. faecium* by Vitek, were correctly reidentified as *E. gallinarum* by the similar results obtained in the SDS-PAGE protein profiles, motility and pigment production tests.

Antibiotic susceptibility test results

Multiple antibiotic-resistant enterococci are becoming an increasing problem throughout the world (Lavery et al., 1997). However, several multiple antibiotic-resistant *Enterococcus* species have been detected, probably because of the drug-using policies of the vancomycin antibiotic; there could not be any detection of VRE (vancomycin resistant enterococci) except in a few studies in Turkey (Basustaoglu et al., 2001; Colak et al., 2002). A wide variety of antibiotic susceptibility was tested for detection of the increased frequency of antibiotic resistance pattern. Table 1 shows the resistance percentages of 24 antibiotics from the 10 different groups among 23 *Enterococcus* species. The susceptibility tests indicated that multiple antibiotic resistances among enterococci were increasing in our nasocomial isolates of Bayındır-Ankara Hospital from Turkey. Multiple antibiotic resistance was determined in 10 of the 23 *Enterococcus* spp. According to these results, *E. faecium* EFA1 exhibited high level β-lactam resistance, quinolone, tetracycline resistance and aminoglycoside resistance except gentamicin and streptomycin resistance. Especially *E. faecium* EFA23 demonstrated the multiple antibiotic resistance to 8 different groups of antibiotics except glycopeptide and tetracycline. Surprisingly, these two multiple antibiotic-resistant *E. faecium* EFA1 and EFA23 isolates were not resistant to either vancomycin or teicoplanin.

All of the 23 isolates were resistant to bacitracin, oxacillin and nalidixic acid. 65.2% of the 23 isolates were resistant to tetracyclines. Resistance to β-lactam group antibiotics was 43.5% for penicillin, and 8.7% for ampicillin. The HLGR (high level gentamicin resistance) rate of the aminoglycoside group was 8.7% and the HLSR (high level streptomycin resistance) rate was 17.4% among isolates. Only in *E. gallinarum* EFA5 and *E. faecium* EFA23, both the HLGR and the HLSR were detected respectively. There was a correspondence between the MIC and disc diffusion test results in the standard strains, but the automated antimicrobial testing system, Vitek, had difficulties detecting low level VRE. Although the disc diffusion test results classified *E. gallinarum* CDC NJ-4 as intermediate to vancomycin with zone size of 15 mm and *E. faecalis* ATCC 51299 as resistant with the zone size of 16 mm, as did the CDC laboratory, Vitek classified these standard strains as resistant to vancomycin with the MIC of >32 μg/ml. The first report of high level vancomycin resistant enterococci with a VanA phenotype from

Table 1. Antibiotic resistance of 23 enterococcal strains according to the disc diffusion and MIC tests.

Antibiotic group / Antibiotic	Resistance (%)			
	S	I	R	MS
1. Glycopeptides				
Vancomycin (Van)[c]	8.7	56.5	34.8	0
Teicoplanin (Tec)[a]	34.8	65.2	0	0
A	21.7	60.9	17.4	0
2. Polypeptides				
Bacitracin (Bac)[a]	0	0	100	0
A	0	0	100	0
3. Macrolides				
Erythromycin (Ery)[a]	0	60.9	39.1	0
Chloramphenicol (Chl)[a]	74	13	13	0
Rifampin (Rif)[a]	8.7	17.4	73.9	0
A	27.6	30.4	42	0
4. Tetracyclines				
Tetracycline (Tet)[c]	34.8	0	65.2	0
A	34.8	0	65.2	0
5. Aminoglycosides				
Gentamicin (Gen)[c]	91.3	0	8.7	0
Tobramycin (Tob)[a]	34.8	43.5	21.7	0
Kanamycin (Kan)[a]	0	34.8	65.2	0
Streptomycin (Str)[c]	82.6	0	17.4	0
Amikacin (Amk)[a]	0	4.3	95.7	0
A	41.7	16.5	41.8	0
6. β-Lactams				
Ampicillin (Amp)[c]	91.3	0	8.7	0
Penicillin (Pen)[c]	56.5	0	43.5	0
Meropenem (Mem)[a]	52.2	8.7	21.7	17.4
Oxacillin (Oxa)[a]	0	0	100	0
Ampicill.-Clavul (AMC)[a]	91.2	0	4.4	4.4
A	58.2	1.7	35.7	4.4
7. Nitrofurantoins				
Nitrofurantoin (Nit)[b]	100	0	0	0
A	100	0	0	0
8. Quinolones				
Nalidixic acid (Nal)[a]	0	0	100	0
Ofloxacin (OFX)[a]	34.8	0	21.7	43.5
Ciprofloxacin (Cip)[b]	65.2	17.4	17.4	0
Norfloxacin (NOR)[a]	0	43.5	56.5	0
A	25	15.2	48.9	10.9
9. Metabolite analogs				
Trimet.-Sulfomet. (SXT)[a]	47.8	0	47.8	4.4
A	47.8	0	47.8	4.4
10. Cephalosporins				
Cephalothin (Cef)[a]	4.4	0	73.9	21.7
A	4.4	0	73.9	21.7
B	36.1	12.5	47.3	4.1

S, susceptible; I, intermediately resistant; R, resistant; MS, moderately susceptible.

[a] Only disc diffusion. [b] MIC+disc diffusion. [c] Only MIC was tested.

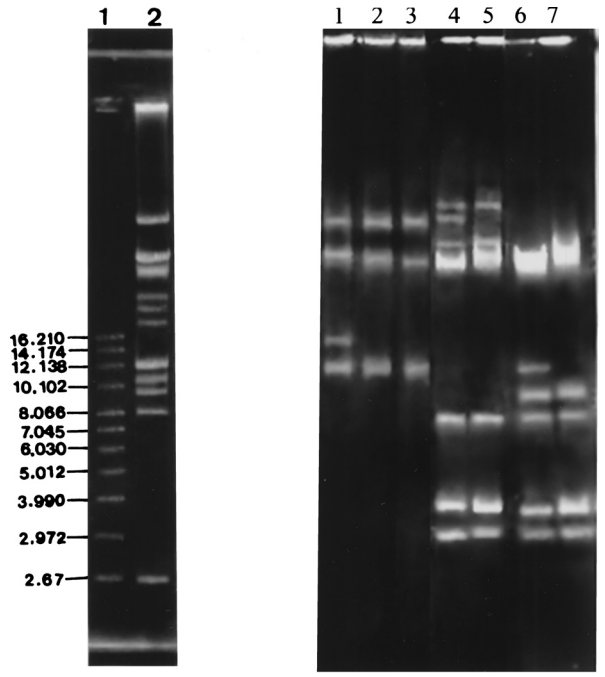
A, group average (%); *B*, overall resistance (%).

Turkey was reported by Basustaoglu et al. (2001), and then Colak et al. (2002) characterized the first outbreak of vancomycin resistant enterococci caused by 20 different vanA-positive *Enterococcus faecium* isolates. According to our survey, the resistance rate to glycopeptide antibiotics was 17.4%. 56.5% of 23 isolates exhibited intermediate vancomycin resistance, 65.2% exhibited intermediate teicoplanin resistance and 34.8% was resistant to vancomycin except AFA5 *E. gallinarum* isolates because of its intrinsic resistance according to the NCCLS documents (2001a, b). These resistance rates were quite high and indicate the increase of vancomycin resistant *Enterococcus* species. As shown in Table 1, overall resistance to the antibiotics that were used was 47.3% and the sum of moderate resistance and intermediate resistance rate was 16.6%.

Plasmid analysis and curing experiment results

According to the plasmid patterns, it was established that *Enterococcus* isolates contained plasmids, which were varied in number from 1 to 11 and in different molecular sizes. Especially multiple antibiotic resistant *E. faecium* EFA23 had a broad range of plasmid patterns, up to 11 (Lavery et al., 1997). The diversity in plasmid sizes ranged from 2.08 to 56.15 kb as shown in Fig. 2A, Lane 2.

In the curing experiments with acriflavine, two different plasmids were shown in different molecular sizes of 13.6 and 33.49 kb. A 33.49 kb plasmid was eliminated which determined resistance to both glycopeptide and penicillin in *E. faecalis* EFA13h mutant. On the other hand, a 13.6 kb plasmid was eliminated in *E. faecalis* EFA14a mutant that determined either glycopeptide or penicillin resistance (Fig. 2B, Lane 5 and 7). *E. faecalis* EFA14 was resistant to both penicillin (MIC$\geq$16 μg/ml) and vancomycin (MIC$>$32 μg/ml). However, *E. faecalis* EFA14a mutant was resistant to neither penicillin nor vancomycin and lost a 13.6 kb plasmid after curing with acriflavine. In addition, while *E. faecalis* EFA13 was a vancomycin (MIC$>$32 μg/ml) and penicillin (MIC$\geq$16 μg/ml) resistant isolate, *E. faecalis* EFA13h mutant lost its penicillin and vancomycin resistance and a 33.49 kb plasmid after curing experiments. *Enterococcus* strains are resistant to penicillin through the expression of low-affinity penicillin-binding protein PBP5 (Williamson et al., 1983), and are resistant to vancomycin most often through the action of resistance operons encoding vanA and vanB types of re-



(a) (b)

Fig. 2. Ethidium bromide-stained agarose gels of plasmid DNA and eliminated plasmids of mutants of enterococcal strains.

(A) Lane 1, supercoiled ccc DNA molecular weight markers (Sigma Chem., Co., D5292), (in kilobase pairs, kb); Lane 2, *E. faecium* EFA23. (B) Lane 1, *E. faecium* EFA1; Lane 2, *E. faecium* EFA1e mutant; Lane 3, *E. faecium* EFA1h mutant; Lane 4, *E. faecalis* EFA13; Lane 5, *E. faecalis* EFA13h mutant; Lane 6, *E. faecalis* EFA14; Lane 7, *E. faecalis* EFA14a mutant.

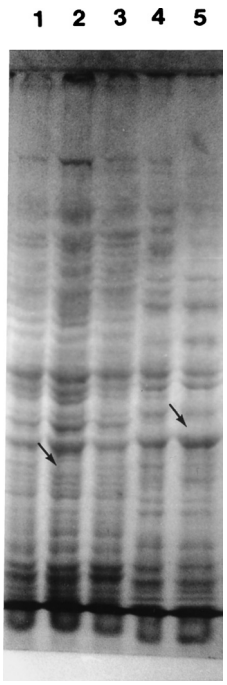


Fig. 3. Protein profiles of EFA1, EFA13, and their mutants by SDS-PAGE after curing with acriflavine.

From Lane 1 to 5; *E. faecium* EFA1, *E. faecium* EFA1e mutant, *E. faecium* EFA1h mutant, *E. faecalis* EFA13, *E. faecalis* EFA13h mutant, respectively. Arrows indicate the lost protein bands.

sistance (Handwerger et al., 1992; Lavery et al., 1997; Leclercq et al., 1989). Leclercq and colleagues described four related plasmids that mediate vancomycin resistance in *E. faecium* (Leclercq et al., 1988, 1989), and Heaton et al. (1996) designated a 41 kb PHK702 glycopeptide resistance plasmid. On the other hand, Handwerger et al. (1990) determined a 55 kb conjugate vancomycin resistance plasmid pKK100. These plasmids appear to differ from each other in their different sizes and antibioic resistance determinants. The diversity of vancomycin resistance plasmid sizes ranges from 30 to 60 kb, approximately. Our findings suggest that while 33.49 kb molecular sized plasmid determined both the vancomycin and penicillin resistance on the same genetic determinant, the 13.6 kb plasmid band determined only vancomycin or penicillin resistance because the vanA alone is in 10.5 kb (Handwerger et al., 1990; Leclercq et al., 1989).

A 22.58 kb plasmid determining kanamycin resistance, which belongs to the aminoglycoside antibiotic group, was established in the *E. faecium* EFA1 isolate (Fig. 2B, both in Lane 2 and 3). EFA1 was a multiple antibiotic-resistant isolate with kanamycin zone size of 8 mm, but *E. faecium* EFA1e and *E. faecium* EFA1h mutants lost their kanamycin resistance and a 22.58 kb plasmid. Resistance to aminoglycoside-aminocyclitol antibiotics occurs primarily as a result of three different plasmid- or transposon-encoded enzymes that modify the antibiotics (Ferretti et al., 1986). It is likely that the aminoglycoside modifiying enzyme determinant was on the 22.58 kb molecular weight plasmid. These findings suggest that a plasmid mediated aminoglycoside resistance in the presence of a 3-phosphotransferase, which demonstrates phoshorylating activity on aminoglycosides possessing a 3-hydroxyl group like kanamycin (Ferretti et al., 1986; Mederski-Samoraj and Murray, 1983).

In SDS-PAGE, which was applied after the curing experiments, elimination of 37.17 and 44.47 kDa protein bands was detected in the *E. faecium* EFA1e and *E. faecalis* EFA13h mutants, respectively (Fig. 3, Lane 2 and 5). Sequencing data available for a number of

phosphotransferases (Ferretti et al., 1986; Oka et al., 1981; Trieu-Cuot and Courvalin, 1983) reveal that the average molecular weight for these enzymes is about 30 kDa. It is considered that the protein band of 33.17 kDa is an aminoglycoside modifiying enzyme in EFA1, which designated the kanamycin resistance.

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National Committee for Clinical Laboratory Standards. (2001a) Performance standards for antimicrobial susceptibility testing; Eleventh Informational Supplement M100-S11; Table 2D. Zone diameter interpretive standards and equivalent minimal inhibitory concentration (MIC) breakpoints for *Enterococcus* spp. (M2-A7-Disk Diffusion), National Committee for Clinical Laboratory Standards, Villinova, Pa.

National Committee for Clinical Laboratory Standards. (2001b) Performance standards for antimicrobial susceptibility testing; Eleventh informational supplement M100-S11; Table 2D. MIC interpretive standards (μg/ml) for *Enterococcus* spp. (M7-A5-MIC Testing), National Committee for Clinical Laboratory Standards, Villinova, Pa.

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