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ANTIBIOTIC RESISTANCE IN ENTEROCOCCI ISOLATED FROM URINE WITH EMPHASIS TO HIGH-LEVEL RESISTANCE TO BETA-LACTAMS AND AMINOGLYCOSIDES(*)

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SUMMARY

From urine specimens of pediatric patients, 100 enterococcal strains (88 *E. faecalis,* 10 *E. faecium,* 1 *E.avium,* 1 *E. casseliflavus*) were isolated. In disk diffusion tests most of the isolates (75-95) were found to be resistant to tetracycline, gentamicin and amikacin and the numbers of resistant strains varied from 2 to 29 for other 16 antibiotics. The most effective antibiotics were quinolones. None of the strains produced beta-lactamase. High-level resistance, singly or in combination, were detected in 5 strains for penicillin (100 µg/ml), in 4 strains for ampicillin (50 µg/ml), in 11 strains for gentamicin (500 µg/ml), in 15 strains for streptomycin (2000 µg/ml), in 1 strain for tobramycin, amikacin and netilmicin (2000 µg/ml). Twenty-two strains had high-level resistance to at least one aminoglycoside. Vancomycin resistance was not detected.

ÖZET

İdrardan izole edilen enterokoklarda antibiyotik direnci: beta-laktamlara ve aminoglikosidlere yüksek düzeyde direnç.

Çocuk hastaların idrar kültürlerinde 100 enterokok suşu izole edilmiştir (88 *E. faecalis,* 10 *E. faecium,* 1 *E.avium,* 1 *E.casseliflavus).* Disk difüzyon yöntemiyle suşların çoğu (75-95) tetrasikline, gentamisine ve amikasine dirençli bulunmuş, diğer 16 antibiyotiğe dirençli suş sayıları 2-29 arasında değişmiştir. En etkili antibiyotikler kinolonlar olmuştur. Suşlar beta-laktamaz oluşturmamıştır. Bazılarında birine, bazılarında birkaçına olmak üzere suşların 5'i penisiline (100 µg/ml), 4'ü ampisiline (50 µg/ml), 11'i gentamisine (500 µg/ml), 15'i streptomisine (2000 µg/ml), 1'i tobramisin, amikasin ve netilmisine (2000 µg/ml) yüksek düzeyde dirençli bulunmuştur. 22 suş aminoglikosidlerden en az birine yüksek düzeyde direnç göstermiştir. Vankomisine dirençli suşa rastlanmamıştır.

INTRODUCTION

The increase of infections caused by enterococci is striking in recent years. In a survey in U.S. hospitals, they were reported as the second leading cause of nosocomial infections exceeded in frequency only by *E.coli* and outnumbering the third and fourth-ranked organisms *P. aeruginosa* and *S. aureus* (14). It was reported that isolation of *Enterococcus* species from urinary infections had risen from 4 % in 1971 to 12.6 % in 1990 (4) or to 13 % in 1989 (7) in hospitalized patients. Antibiotic resistance of enterococci has become another focus of interest.

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Urinary tract infections are frequently caused by enterococci and are the most common enterococcal infections in humans. Intraabdominal or pelvic wound infections, bacteremia with or without endocarditis are among other common enterococcal infections (14). Mortality in enterococcal bacteremia and endocarditis has generally been high (e.g. 44 %) (12). Urinary tract infections which are frequently related to instrumentation or anatomical abnormality were blamed as the usual sources of enterococcal bacteremia and endocarditis. In a research, the most common (25 %) entry site was found to be urinary tract for *E. faecalis* bacteremia (5). It was also reported that there is no correlation between species and source of the specimen in enterococcal infections (1). Thus, the strains isolated from urinary tract infections may be regarded as representatives of overall strains in a region.

Enterococci are intrinsically resistant and tolerant to beta-lactam antibiotics such as penicillins and cephalosporins. One of the reasons for this resistance is the low affinity of these antibiotics for PBPs of enterococci, and PBP 5 has been reported to play the crucial role in this kind of resistance (13). Another mechanism of enterococcal resistance to beta-lactam antibiotics is beta-lactamase production which was not reported until 1983 (17). MICs of penicillin (2-8 µg/ml) for *E. faecalis* may be 10 to 100 times of those for streptococci (16). *E. faecium* strains are usually more resistant (MIC 16-32 µg/ml). Low-level resistance to aminoglycosides is also an inherent property of enterococci and it is due to low uptake of these antibiotics by bacteria (16). When a cell wall inhibitor such as a beta-lactam antibiotic or vancomycin is used together with an aminoglycoside, cell wall inhibitor enhances the uptake of the aminoglycoside by changing the permeability of the cell wall and thus enhances the bactericidal effect of the aminoglycoside (15). The emergence of high-level aminoglycoside resistance in enterococcal strains together with high-level beta-lactam resistance and the increasing reports of vancomycin resistant enterococci create great difficulty for clinicians to cure serious enterococcal infections such as bacteremia and endocarditis caused by such strains.

In this paper the results of disk diffusion susceptibility tests for 19 antibiotics of 100 *Enterococcus* strains isolated from urine specimens and identified at species level were reported together with beta-lactamase production, and high-level resistance to penicillins, vancomycin and aminoglycosides.

MATERIALS AND METHODS

Enterococci isolated from urine specimens of pediatric patients in a three-months period (Dec 92-Feb 93) were identified by morphology, catalase production, haemolytic properties, growth in esculine-bile agar and 6.5 % NaCl containing agar at genus level (3). API 20 Strep identification strips (bioMérieux) were used for the identification at species level of 100 strains. Susceptibilities to antibiotics shown in table 1 were determined by disk diffusion tests on Mueller-Hinton agar. High-level resistance was also investigated to antibiotics shown on table 2 by agar dilution tests.

Antibiotic susceptibility tests were performed and evaluated according to NCCLS proposals. 5 ml Mueller-Hinton broth was inoculated from 4 to 5 colonies in tryptic soy agar, incubated at 37°C for 3 to 4 hours, and the turbidity was adjusted to McFarland No 0.5 tube. The surface of Mueller-Hinton agar plates were spread with this suspension, antibiotic disks were applied and the diameters of inhibition zones were recorded after 24 hours incubation at 37°C. For agar dilution tests a drop containing 10^4 CFU of a strain was placed on

Mueller-Hinton agar containing appropriate concentrations of penicillins or vancomycin. In agar dilution tests for aminoglycosides, 10^6 CFU/drop bacterial suspension and brain-heart infusion agar were used. Any growth in 24 hours at 35°C was evaluated as a sign of resistance to the indicated concentration of antibiotic. Beta-lactamase production was investigated by nitrocefin test (BBL-Cefinase disks).

RESULTS

Out of 100 enterococcal isolates, 88 were identified as E. faecalis, 10 as *E. faecium*, 1 as *E.avium* and 1 as *E. casseliflavus.*

By disk diffusion tests, most of the strains were found to be resistant to tetracycline, gentamicin and amikacin as shown in table 1. The numbers (and percentages) of resistant strains varied from 2 to 29 for other antibiotics. Eight strains classified as resistant to vancomycin by disk diffusion tests would be classified as intermediate if their inhibition zones were only 1 mm wider. For this reason they were subjected to tube dilution tests for vancomycin and MIC values were found to be 1 μg/ml for 5 and 0.5 μg/ml for remaining 3 strains.

No beta-lactamase activity was detected in any strain although the number of resistant strains to amoxycillin/clavulanic acid was 7 less than those which gave inhibition zones indicating resistance to ampicillin/amoxycillin.

In agar dilution tests, 2 strains were found highly-resistant only for beta-lactam antibiotics, 17 strains only for aminoglycosides and 5 strains for both beta-lactams and aminoglycosides (Table 2). No strain grew on 4 μg/ml vancomycin containing agar. The most resistant strain was a *E. faecalis* (no.39) which was found to be resistant to 12 antibiotics except chloramphenicol, vancomycin and quinolones by disk diffusion tests and highly-resistant to penicillin, ampicillin, tobramycin, amikacin, netilmicin and gentamicin. It was the only strain with high-resistance to tobramycin, amikacin and netilmicin. On the other hand, results suggest that high-level resistance is more common in *E. faecium* strains since 6 of 10 *E. faecium* and 18 of 88 *E. faecalis* strains was found to be highly resistant to one or more of 2 beta-lactam or of 5 aminoglycoside antibiotics to which this kind of resistance was searched ($p<0.02$).

Table 1. Results of disk diffusion tests for 100 Enterococcus strains.

	Erythromycin	Penicillin G	Ampicillin/Amoxycillin	Ampicillin/Sulbactam	Amoxycillin/Cl.acid	Mezlocillin	Carbenicillin	Gentamicin	Tobramycin	Amikacin	Netilmicin	Chloramphenicol/ Thiamphenicol	Tetracycline	Ofloxacin	Ciprofloxacin	Nitrofurantoin	Norfloxacin	Pefloxacin	Vancomycin
Sensitive	50				88	76	78	11	61	3	49	89	24	93	56	93	82	16	
Moderately susceptible		78	84	85	3	8	6							1	37				51
Intermediate	34							11	20	2	22	2	1			5	10	57	41
Resistant	16	22	16	15	9	16	16	78	19	95	29	9	75	6	7	2	8	27	8[x]

[x] by agar dilution tests MIC ≤ 1 μg/ml.

Table 2. High-level resistance determined by agar dilution tests in 100 Enterococcus strains.

Antibiotics	µg/ml	Resistant strains (n)	Protocol numbers of resistant strains
Vancomycin	4	0	
Penicillin	200	0	
Penicillin	100	5	34, 39, 46, 87, 88
Ampicillin	100	0	
Ampicillin	50	4	8, 15, 34, 39
Tobramycin	2000	1	39
Amikacin	2000	1	39
Netilmicin	2000	1	39
Gentamicin	500	11	7, 15, 26, 32, 34, 39, 86, 87, 88, 89, 91
Streptomycin	2000	15	2, 5, 7, 9, 14, 17, 19, 31, 34, 40, 80, 87, 88, 96, 99

E. faecium strains are underlined. Others are E. faecalis.

DISCUSSION

The importance of enterococci has increased in recent years due to the increasing frequency of enterococci in hospital infections, intrinsic or acquired resistance of these bacteria to many antibiotics, and due to the problems in therapy caused by strains with high-level antibiotic resistance.

About 85-90 % of clinical enterococcal isolates are *E. faecalis* strains which are followed by *E. faecium* (5-10 %) and less frequently by other species (14). The most common enterococcal infection is urinary tract infection. In this reserarch 100 strains were isolated from 2286 consecutive urine specimens as causative agents (4.4%). If only the specimens with a pathogen are considered, this ratio should be at least three times higher. Out of 100 enterococcal isolates 88 were identified as *E. faecalis,* 10 as *E. faecium*, 1 as *E. avium* and 1 as *E. casseliflavus.* Hasçelik et al (8) identified all of 42 strains isolated from various clinical materials in Ankara as *E. faecalis.*

As it is shown in table 1, most of the enterococcal strains isolated in this work were found resistant to amikacin, gentamicin and tetracyline by disk diffusion tests. Although 6 cephalosporin antibiotics were also used, their results were not shown since they are usually not recommended in enterococcal infections and they did not exert enough activity worthy to mention. The most active antibiotics were quinolones as a group in our study. Our results were comparable and upto a certain degree in accordance with those reported from Italy (18). More information is given below for the 8 strains found as vancomycin resistant by disk diffusion tests.

High-level antibiotic resistance to beta-lactam and aminoglycoside antibiotics has very important consequences in the therapy of enterococcal bacteremia and endocarditis. Although it may not be regarded so important for urine isolates, we determined high-level antibiotic resistance in 100 urine isolates to have an idea about the frequency of this kind of resistance in this genus in Turkey in the light of findings that urinary system is an important entry site for enterococcal bacteremia (5) and that no correlation was found between the clinical specimens and enterococcal species isolated (1). Fifteen percent of our strains were found to be highly-resistant to streptomycin, 11 % to gentamicin, 5 % to penicillin, 4 % to ampicillin and 1 % to amikacin, tobramycin and netilmicin. When the combinations of aminoglycoside resistance are taken into consideration, 11 strains were found to be highly resistant only to streptomycin, 6 strains only to gentamicin, 4 strains to both streptomycin and gentamicin, and 1 strain to gentamicin, tobramycin, amikacin and netilmicin. Thus 22 % of our strains were found to be highly resistant to at least one aminoglycoside. Two different mechanisms are known for high-level aminoglycoside resistance: one is ribosomal resistance, the second one is enzymatic modification (16). Most of the enterococci with high-level resistance to aminoglycosides are known to have enzymatic type of resistance. The most frequently encountered two enzymes modify either only streptomycin or gentamicin and other aminoglycosides except streptomycin (13). For this reason there is a great confirmity between the high-level gentamicin resistance and resistance to other aminoglycosides except streptomycin. This is not the case for our strains. Thus, our strains with high-level aminoglycoside resistance need to be investigated for the resistance mechanism. Hasçelik et al (10) reported higher ratios of high-level resistance for streptomycin and gentamicin in 20 strains isolated in Ankara in an Congress abstract but their results were highly accordant with ours in their presentation

when the number of strains were increased. Hasçelik et al (9), in another paper, reported high-level resistance in 12-24 % of *E. faecalis* strains to different aminoglycosides. Eskitürk et al (2) found 22 % high-level gentamicin resistance in 60 enterococcal isolates. These ratios for high-level resistance to aminoglycosides in Turkey are lower than most of the ratios reported in other countries. For example Watanakunakorn (21) reports that the first high-level gentamicin resistant blood isolates of *E. faecalis* was seen in USA in 1985, and the prevalence of such strains amounted to 9 % in 1985-8 and to 35 % in 1989-91, and 52 % of strains isolated in the last period had high-level resistance to at least one aminoglycoside. On the other hand Gray et al (6) reported only 8.2 % high-level gentamicin resistance in *E. faecalis* strains with none in other species.

Vancomycin is the most effective antibiotic for resistant Gram positive cocci. In serious enterococcal infections, combination of a beta-lactam and an aminoglycoside antibiotic is used due to the synergistic effects of these antibiotics. If the strain has high-level resistance to either beta-lactams or especially aminoglycosides the synergy between these two groups of antibiotics disappears and vancomycin use should be considered. Unfortunately vancomycin resistant strains from several *Enterococcus* species are reported. The plasmidal nature of vancomycin resistance in some *E. faecium* strains and its conjugal transfer to some other Gram positive bacteria rise the frightening possibility of dissemination of vancomycin resistance (20). For this reason vancomycin resistance was searched in our strains. In disk diffusion tests, 8 strains gave inhibition zones which should be indicative for resistance but would be classified as intermadiate if they were 1 mm wider. Then these strains were subjected to tube dilution tests with vancomycin and MIC values were obtained as 0.5 µg/ml for 3 and 1 µg/ml for 5 strains, and they were not accepted as vancomycin resistant. Up to date no vancomycin resistant *Enterococcus* strain was reported in this country (11,19).

Beta-lactamase producing enterococcal isolates are also increasing in the World but none of our 100 isolates gave positive results by nitrocefin test. Strains isolated in Ankara were also found to be beta-lactamase negative (10).

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