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DETECTION OF VANCOMYCIN RESISTANT ENTEROCOCCI (VRE) ISOLATED FROM URINARY TRACT INFECTIONS (UTI) IN TEHRAN, IRAN

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ABSTRACT

This report describes the frequency of *Enteroccoci* phenotypic and genotypic susceptibility patterns of VRE (Vancomycin Resistant Enterococci) from three hospitals in Tehran, Iran. One hundred and twenty enterococcal urine cultures were isolate from patients with urinary tract infection (UTI). After identification of enterococcal species by biochemical tests, glycopeptide susceptibility of each isolate was assessed by disk agar diffusion method according to NCCLS guideline. Glycopeptide minimum inhibitory concentration (MIC) for each VRE isolate was determined by the agar dilution method and the *vanA* gene was detected by PCR. Seven percent (8/120) of the isolates were VRE, including *E. faecalis* 38% (3/8), *E. faecium* 25% (2/8), *E. mundtii* 25% (2/8), and *E. raffinosus* 12% (1/8). All 8 isolates resistant to vancomycin showed vancomycin MIC of >512µg/ml, and teicoplanin MIC's ranging from 8->64µg/ml, and they all possessed the *vanA* gene. Six (75%) of VRE were isolated from a referral tertiary care hospital, i.e. Ahari Children Medical Center (ACMC). Almost 90% of *Enterococci* were *E. faecalis* (57%) and *E. faecium* (30%). The remaining 13% were identified as *E. mundtii* (6%), *E. avium* (3%), *E. durans* (1%), *E. hirea* (2%), and *E. raffinosus* (1%). The diverse VRE species combined with high rate of VRE isolation in Iran, as well as isolation of *E. raffinosus* and *E. mundtii* in the Middle East (ME) region for the first time, suggests a rapid spread of resistance among *Enterococci* along with an emerging shift in VRE distribution in Iran.

Key words: Vancomycin Resistant Enterococci (VRE), Urinary Tract Infection, Disk Agar Diffusion Method.

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INTRODUCTION

Enterococcal species have emerged as important pathogens in Iran as well as throughout the world. *Enterococci* are rated as the second leading cause of urinary tract infections (UTI) and comprise about 10% of nosocomial UTI (1,2,3). Furthermore, the emergence of vancomycin resistant *Enterococci* (VRE) in Iran has presented serious challenges for hospital infection control practitioners as well as clinicians treating patients with enterococcal infections in Iranian hospitals (4). Infections caused by VRE in Iran, like many other countries, have been associated with high morbidity and mortality rates especially in immuno-compromised patients (5, 6). There are several reports on the endemic vancomycin resistance of *Enterococci* in Iran, and also several small short-term VRE prevalence studies from Iranian institutions in International and Iranian medical journals (4, 7).

Despite the sporadic reports of VRE isolation from Iranian medical centers, morbidity and mortality caused by enterococcal infections in Iran, is on the rise (8). This is primarily because appropriate antimicrobial therapy of enterococcal infections has become progressively more difficult for Iranian physicians due to the lack of adequate information regarding the prevalence of VRE and levels of their glycopeptide resistance. In this study the frequency of enterococcal species and VRE isolation in Tehran and VRE glycopeptide susceptibility levels in three different health care settings were investigated.

MATERIALS AND METHODS

Patient specimens and bacterial strains

One hundred and twenty enterococcal isolates were recovered from urine specimens of patients with urinary tract infections (UTI) from three hospitals in Tehran, namely, Ahari Children Medical Center (ACMC), which is a referral tertiary care center and a Tehran University of Medical Sciences teaching hospital, Mehrad Hospital and Pars Hospital. The last two hospitals are tertiary care and secondary care facilities, respectively. Only one Enterococcal isolate was analyzed from each patient.

Enterococcal genus identification was performed based on the following microbiological tests: Gram reaction, catalase reaction, presence of pyrrolidonyl arylamidase (PYR), growth on bile-aesculin agar and 6.5% NaCl media. A previously published scheme (9, 10) was used in this study to identify the enterococcal species. This scheme utilized a motility test, arginine

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decarboxylation in Moeller decarboxylase media, pyruvate utilization, and fermentation of carbohydrates (Arabinose, Raffinose ,Mannitol, Ribose).

Susceptibility testing
Vancomycin susceptibility testing of enterococcal species was performed by screening of microorganisms on brain heart infusion (BHI) agar (DIFCO, Detroit, Michigan, USA) containing 6 µg/mL vancomycin (Sigma, Steinhelm, GM)). Teicoplanin susceptibility testing was performed by the disk diffusion method on Mueller Hinton agar (DIFCO, Detroit, Michigan, USA) containing 30 µg/mL teicoplanin (MAST Grp. Ltd, Merseyside, UK). Glycopeptide minimum inhibitory concentrations (MIC) were determined by the agar dilution method on BHI agar according to National Committee of Clinical Laboratory Standards guidelines (11).
All susceptibility test results were assessed after 24 h incubation at 35ºC. An enterococcal strain susceptible to vancomycin (ATCC 29212) was used as a negative control and resistant *Enterococcus* strains [*E. faecalis* E206 (*vanA* positive) and *E. faecium* E2781 (*vanB* positive), courtesy of Dr. Edet Udo] were used as the positive control for this study. The MIC break point value considered for resistant isolates using vancomycin and teicoplanin was at ≥ 32µg/mL. For vancomycin, isolates with MIC of ≤ 4µg/mL and for teicoplanin MIC of ≤ 8µg/mL were considered susceptible. All figures including frequency and antimicrobial susceptibility results were rounded down if they were <0.5, and were presented as whole numbers if they were ≥0.5.

Enterococcal DNA extraction
Total DNA was extracted from *Enterococci* as previously described (12). Briefly, enterococcal strains were grown overnight at 35°C on BHI agar. Two or three colonies of each culture were scraped from the surface of the agar plates and resuspended in 200 µL of sterile distilled water. The cell suspension was heated for 15 min at 100°C and then centrifuged at 12,000 *g* for 10 min. The DNA in the supernatant fluid was used as a template for *vanA* gene amplification by polymerase chain reaction (PCR).

Detection of vanA gene by PCR
Genes encoding the vancomycin-resistance determinants *vanA* and *vanB* were investigated by PCR using specific primers (*vanA-1* 5′- GGG AAA ACG ACA ATT GC- 3′ , *vanA-2* 5′- GTA CAA TGC GGC CGT TA - 3′ and *vanB*-1 5′-ATG GGA AGC CGA TAG TC- 3′ , *vanB*-2 5′-GAT TTC GTT CCT CGA CC- 3′) (13). PCR reactions were performed in a 50 µL volume consisting of: 1X PCR buffer, 3.5 mM $MgCl_2$, 0.5µg/mL of each primer, 2.5 U *Taq* DNA polymerase, 0.2 mM dNTP Mix and 3µL of DNA template (10 µg/mL). The PCR conditions consisted of a pre-denaturation step at 94ºC for 5 min, followed by 30 cycles of 45 sec at 94ºC, 45 sec at 54ºC and 45 sec at 72ºC. A final extension step was performed at 72ºC for 5 min. Amplified products were analyzed by electrophoresis on 1.5 % agarose gel. DNA bands were visualized by staining with ethidium bromide and photographed under UV illumination.

RESULTS

In the present study, of 120 Enterococcal isolates 7 % (8/120) were resistant to vancomycin. Various VRE species were isolated, including *E. faecalis* 38% (3/8), *E. faecium* 25% (2/8), *E. mundtii* 25% (2/8), and *E. raffinosus* 13% (1/8). Table 1 depicts the distribution of VRE species according to individual health institution, where each VRE was isolated. In addition to isolation of the majority (75%) of VRE from ACMC, the isolates from this hospital showed the highest diversity (Table 1). Surprisingly, in contrast to the isolated VRE from ACMC, there was no diversity in the few VRE strains isolated from Mehrad hospital and all were identified as *E. faecium*.
Interestingly, although 50% of VRE isolates from ACMC were identified as *E. faecalis;* no *E. faecium* were among VRE isolates recovered from this hospital. Furthermore, all Enterococcal isolates from Pars hospital were sensitive to vancomycin and no VRE were recovered from this hospital. In addition, all isolates of *E. faecalis* isolate in Mehrad and Pars hospitals were susceptible to vancomycin.

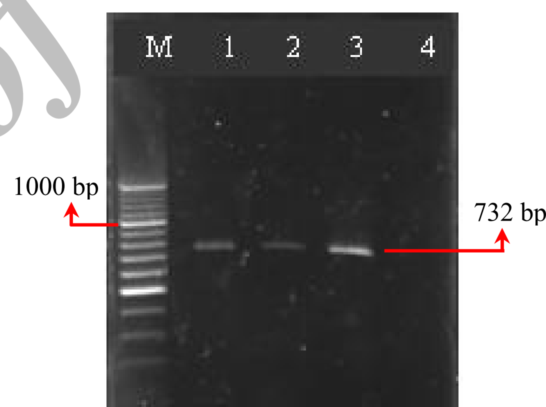

Figure 1. A representative agarose gel electrophoretic analysis of PCR products of *vanA* gene from two Vancomycin Resistant *Enterococci* (VRE), showing the 732 bp amplicon (lane 2 and 3). Positive and negative control samples are shown in lanes 1 and 4, respectively. (M = Molecular weight markers).

The results of teicoplanin and vancomycin MIC levels among various VRE isolates which were examined in this study are shown in Table 2. Overall, the vancomycin MIC levels among isolates of VRE were quite high (i.e. >512 µg/mL) indicating high level of resistance among the ACMC and Mehrad enterococcal isolates. Although, teicoplanin MIC levels of *E. faecalis, E. mundtii* and *E. raffinosus* isolates ranged from 8 µg/mL to 32 µg/mL, the isolates of *E. faecium* displayed the highest teicoplanin resistance levels (i.e. ≥64 µg/mL). Importantly, 100% (8/8) of examined VRE species were identified as having the *vanA* genotype suggesting no diversity in susceptibility genotype among VRE isolates (Figure 1).

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Table 1. Distribution, frequency, and diversity of Vancomycin Resistant Enterococcal species according to the site of isolation in 3 hospitals in Tehran, Iran.

Site	Isolates	VRE(%)[a]	No. (%)[a] of VRE species			
			E. faecalis	*E. faecium*	*E. mundtii*	*E. raffinosus*
ACMC Hospital	87	6 (75)	3 (3)	0	2 (2)	1(1)
Mehrad Hospital	14	2 (25)	0	2 (14)	0	0
Pars Hospital	19	0 (0)	0	0	0	0
Total	120	8 (7)	3 (38)	2 (25)	2 (25)	1(12)

[a] Percentages are calculated based on the total number of VRE isolated.
Note: All percentages are rounded.

Table 2. The minimum glycopeptide inhibitory concentrations for resistant Enterococcal species isolated from 3 hospitals in Tehran, Iran using agar dilution method.

Antibiotic agents	MIC (µg/mL)				
	E. faecalis (ACMC1)*	*E. faecalis* (ACMC2, ACMC3)	*E. faecium* (MR1, MR2)	*E. mundtii* (ACMC4, ACMC5)	*E. raffinosus* (ACMC6)
Vancomycin	>512	>512	>512	>512	>512
Teicoplanin	16	64	>64	64	16

* Acronyms in parenthesis, designate the names assigned to the *Enterococcal Spp.* isolated in this laboratory.

Table 3. Comparison of the frequency of Enterococcal species according to Hospital site of isolation.

Site	Total	No. (%)[a] of *Enterococcal* spp.						
		E. faecalis	*E. faecium*	*E. mundtii*	*E. avium*	*E. hirae*	*E. durans*	*E. raffinosus*
ACMC[b] Hospital	87	50 (58)	26 (30)	8 (9)	1(1)	1(1)	0	1(1)
Mehrad Hospital	14	6 (43)	8 (57)	0	0	0	0	0
Pars Hospital	19	12 (63)	2 (11)	0	3 (16)	1(5)	1(5)	0
Total	120	68 (57)	36 (30)	8 (6)	4 (3)	2 (2)	1(1)	1(1)

[a] All percentages are rounded; [b] Ahari Children Medical Center

As shown in table 3, the frequency of enterococcal species isolation varied among the hospitals examined in this study. Regarding the overall distribution of the enterococcal isolates, by and large, the majority of *Enterococci* were identified as *E. faecalis* and *E. faecium* accounting for 87% of isolates. Other species (*E. mundtii*, *E. avium*, *E. hirae*, *E. durans* and *E. raffinosus*) recovered from the patients with UTI from the hospitals in this study, accounted for the remaining 13% of all Enterococcal isolates. Other enterococcal species consisted of *E. mundtii* (6%); *E. avium* (3%); *E. hirae* (2%); *E. durans* (1%) and *E. raffinosus* (1 %). Again, the highest diversity of enterococcal isolates was shown from ACMC, followed by Pars and Mehrad hospitals. Although Pars hospital provides mostly primary care for patients, this hospital showed a higher diversity of enterococcal isolates than Mehrad hospital. Moreover, despite the absence of any VRE isolates from Pars hospital, low prevalence enterococcal isolates such as *E. avium*, *E. hirea*, and *E. durans* comprised 26% of all *Enterococci* recovered from this hospital.

DISCUSSION

The spread of antimicrobial resistance among Enterococcal spp. in Iran has presented a serious challenge for the Iranian medical community (4). Unfortunately, treatment failures in enterococcal infections are on the rise because of the lack of adequate information regarding glycopeptide resistance among endemic *Enterococci*. Such information is required for appropriate treatment of patients with enterococcal infections, which rank among the third common cause of bacteremia and the second most frequent cause of UTI. (1, 2). Comprehensive data concerning the endemic prevalence and susceptibility patterns of *Enterococci* in various health institutions is also necessary to prevent spread of antimicrobial resistance in Iran.

This investigation indicates a severe problem of antimicrobial resistance among *Enterococci* in some hospitals in Tehran. The 7 % rate of VRE prevalence in the present study is in agreement with earlier reports of high VRE prevalence (7%) in Tehran (4). In addition, finding of alarmingly high rate of vancomycin resistance in Iran is in sharp contrast with

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studies from other countries in the ME, where low incidence (0-1%) of VRE has been reported (14-17). Moreover, all VRE isolated thus far from ME have been identified as *E. faecium* (4, 18).

Despite the recent isolation of a single *vanB* genotype Enterococcal strain from a Tehran hospital (19), the finding that all VRE's isolated in this investigation were *vanA* genotype illustrates that, *vanA* genotype is the predominant type of Enterococcal vancomycin resistance in Iran, as reported in other countries (2, 16).

The markedly diverse species of VRE and Enterococcal species isolated from ACMC may relate to this hospital's patient population, who are referred from all regions of Iran, representing a wide spectrum of socioeconomic levels. To our knowledge, this is the first report of *E. mundtii* and *E. raffinosus* isolation from enterococcal infections in the ME region. In addition, the high level of Enterococcal glycopeptide resistance in ACMC may in part be explained by antibiotic treatment of most ACMC patients prior to admission. On the other hand, the absence of any VRE from Pars Hospital might be attributed to the primary care patient population visiting this facility, in addition to this hospital's effective enforcement of infection control measures.

The isolation of vancomycin resistant *E. mundtti* and *E. raffinosus* indicates a change in VRE species diversity in some hospitals in Iran. This change might be through mechanisms similar to shifts reported from other countries in which *E. faecium* is gradually emerging as one of the major VRE species (20). This also points out to the increased clinical importance of enterococcal species, other than *E. faecalis* and *E. faecium*, in the ME region.

The wide spectrum of diversity among VRE species of this study is the first report of such diversity from the ME, and is in contrast to earlier reports from this region (4, 16). However, high diversity among our Enterococcal species is consistent with recent reports from other regions of the world, where diverse species of VRE have been isolated (21-24, 18, 25).

In conclusion, the importance of isolating highly resistant VRE from Iranian hospitals raises concern about the use of glycopeptides antibiotic as an appropriate choice for treating life-threatening enterococcal infections in Iran. Additional concern is warranted because like other countries in the region, gentamicin resistance among Enterococcal isolates has been shown to be higher than the *Enterococci* from other geographical areas (26, 8). Our findings also serve to emphasize the value of clinicians' curbing of the inappropriate antibiotic use which is, common in developing countries such as Iran. Additionally, the high level of resistance among VRE species in Iran calls for physicians' vigilance in rapid identification of glycopeptide antibiotic resistance during the treatment of life-threatening enterococcal infections in Iran.

Finally, our results highlight the need for a national Iranian surveillance program, which continuously monitors the changes in bacterial resistance nationwide and will help to set national priorities for local intervention efforts in Iran. Evidence gathered in such programs may help to confirm findings of this investigation with comprehensive endemic surveillance data from all regions of Iran. Reliable bacterial susceptibility surveillance data from Iran and other countries in the ME will further strengthen the reliability of ongoing global surveillance programs in the developed countries, and thus, will enhance attempts to control the spread of VRE worldwide.

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