

# High Level Resistance of *Enterococcus faecium* and *E. faecalis* Isolates from Municipal Sewage Treatment Plants to Gentamicin

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## Abstract

**Background:** Enterococci are members of the normal gut flora and released into the environment via sewage outlets, where they can survive for long times. Infections with high-level gentamicin resistant (HLGR) enterococci are emerging worldwide. HLGR enterococci have developed a resistance to most antibiotics commonly used for enterococcal infections therefore; treatment of infections caused by HLGR enterococci is difficult. The present study investigated the distribution and antibiotic resistance of HLGR *Enterococcus faecium* and *E. faecalis* isolates from raw wastewater samples in Tehran.

**Methods:** Raw wastewater samples were collected during the period from November 2006 to May 2007 at 3 sewage treatment plants located in different parts of Tehran. All 90 HLGR enterococcal isolates were identified to the species level by biochemical and PCR assays and subjected to antibiotic susceptibility testing.

**Results:** Sixty four percent (58 of 90) of isolates were *E. faecium* and 29% (26 of 90) of them were *E. faecalis*. The highest level of antibiotic resistance was observed with erythromycin (63%), co-trimoxazole (69%) and tetracycline (92%) for *E. faecalis* and with erythromycin (97%), ciprofloxacin (47%), co-trimoxazole (45.5%) and tetracycline (47%) for *E. faecium*. Multiresistance against 3 to 4 antimicrobial was present in 27.5% and 15.5% of the isolates, respectively.

**Conclusion:** HLGR *E. faecium* were more commonly found than *E. faecalis*. Species identification of HLGR enterococci enables us to assess species-specific antibiotic susceptibility patterns in our area. The present study revealed that HLGR *E. faecalis* remained more susceptible than *E. faecium* against the usual first-line and alternative treatments.

**Keywords:** *E. faecalis*, Enterococci, gentamicin resistance, Iran

## Introduction

Enterococci are normal inhabitants of the intestinal floras of human and warm-blooded animals. They are released into the environment through feces (1). Enterococci have the ability to survive adverse environmental conditions (2). The enterococci may be superior indicator organisms than fecal coliforms as a water quality indicator (1). They have emerged as important human pathogens that are responsible for nosocomial infections and with the emergence of aminoglycoside and glycopeptides resistance (3). Of 14 or more, exist enterococcal species, only *E. faecalis* and *E. faecium* commonly colonize and infect humans in detectable numbers (3). They can cause urinary tract and wound infections, septicemia and endocarditis (4). The prevalence, severity and antibiotic resistance

of and the mortality rate from enterococcal infections are often species dependent (5).

The emergence of high-level gentamicin resistance (HLGR) is of serious worldwide due to their multi-resistant (6). According to a study (7), 52% of enterococcal clinical isolates in Tehran hospitals showed HLGR phenotype.

Nevertheless, based on data in the literatures, 90-99% of multiple resistant *E. faecium* and *E. faecalis* found in sewage actually stem from the human population and several cycles of continuation and recontamination with them appears to exist between animals, humans and the environment (8).

This sampling was aimed at obtaining information on prevalence of two most clinically important species *E. faecalis* and *E. faecium* HLGR enterococci in sewage treatment plants in Tehran, Iran.

## Materials and Methods

**Sample collection:** Inflow raw wastewater samples were collected from three sewage treatment plants located in the different parts of Tehran during the period from November 2006 to May 2007. Five hundred milliliter of each sample was collected in a sterile container transported to the laboratory and analyzed within a maximum period of 2 h.

**Isolation of HLGR enterococci:** Samples were subjected to serial 10-fold dilutions with normal saline, before filtration. The membrane filters were put on brain heart infusion agar (Becton Dickinson and Co., Sparks, MD, USA) plates and preincubated for 2 h at 37 °C (9). The membranes were transferred to *m-Enterococcus* agar (Becton Dickinson and Co., Sparks, MD, USA) supplemented with 64 mg of gentamicin per liter. Agar plates were incubated for 48 h at 37 °C. Distinct colony growth was transferred to blood agar plates for following investigations.

**Biochemical identification of species:** All of the enterococcal isolates were tested for phenotypic characteristics by conventional methods, based on the following criteria: growth on Bile Esculin agar and in 6.5% NaCl broth, absence of catalase and presence of pyrrolidonyl arylamidase (PYR test). Species-level identification was performed by biochemical tests including acid fermentation of manitol, sorbitol, sucrose, arabinose and raffinose, motility and arginine hydrolysis (10).

**Detection of genus and species by PCR.** All strains were confirmed for genus and species by PCR with three different primer sets. Amplification of both species specific, and genus (*rrs*) targets produced bands corresponding to their respective molecular size (Table 1). For DNA extraction, one isolated colony was suspended in 200 µl distilled water and boiled at 100 °C for 10 min (11). After centrifugation, 10 µl of supernatant was used as the DNA template. PCR reaction were performed in a total volume of 25 µl containing 0.8 mM dNTP, 0.5 U of Taq DNA polymerase (Roche, Mannheim, Germany), 2.5 pM of each primers, 2.5 µl of 10X PCR buffer, 1.5 mM MgCl<sub>2</sub> and DNA template 5 µl. DNA amplification was carried out with the following thermal cycling profile. Initial denaturation at 94 °C for 5 min, 30 cy-

cles of amplification (denaturation at 94 °C for 1 min ,annealing at 54 °C for 1 min and extension at 72 °C for 1 min ), followed by a final extension at 72 °C for 7 min (12).

**Antimicrobial susceptibility testing:** The susceptibility to antibiotics was tested by the agar disk diffusion method according to CLSI guidelines (13). The antimicrobial disks were used as follows: vancomycin (30µg), ciprofloxacin (5µg), ampicillin (10µg), high content gentamicin (120 µg), trimethoprim/sulfamethoxazole (1.25: 22.75), tetracycline (30µg), erythromycin (30µg) and nitrofurantoin (300µg) were purchased from BBL (BD BBL, Sparks, MD, USA), quinupristin-dalfopristin (15 µg) and linezolid (30 µg) from Mast Diagnostics Ltd. (Bootle, Mersey Side, UK) and teicoplanin (30 µg) from BR (BioRad, Hercules, CA, USA). Isolates with intermediate levels of susceptibility were classified as resistant.

## Results

Ninety HLGR enterococcal isolates were recovered from three sewage treatment plant samples. Among HLGR isolates, the following species were identified; 58 isolates of *E. faecium* (64%), 26 isolates of *E. faecalis* (29%) and six (7%) other enterococcal species. Amplification of genus, *E. faecalis*- specific and *E. faecium*- specific targets produced 320 bp, 941 bp and 658 bp bands, respectively (Fig. 1). Species distribution by PCR method was the same as detected by biochemical tests.

Data obtained from antimicrobial susceptibility testing summarized in Table 2. Among 58 *E. faecium* and 26 *E. faecalis* isolates with HLGR phenotype, resistance percents to erythromycin, ciprofloxacin, co-trimoxazole, tetracycline, ampicillin, nitrofurantoin and synergid were 97% versus 63%, 47% versus 19%, 45.5% versus 69%, 47% versus 92%, 18% versus 7.5%, 17% versus 0% and 3.5% versus 100%, respectively. Simultaneously resistance to ampicillin, ciprofloxacin, tetracycline and co-trimoxazole were 15.5% which was more detected in *E. faecium*.

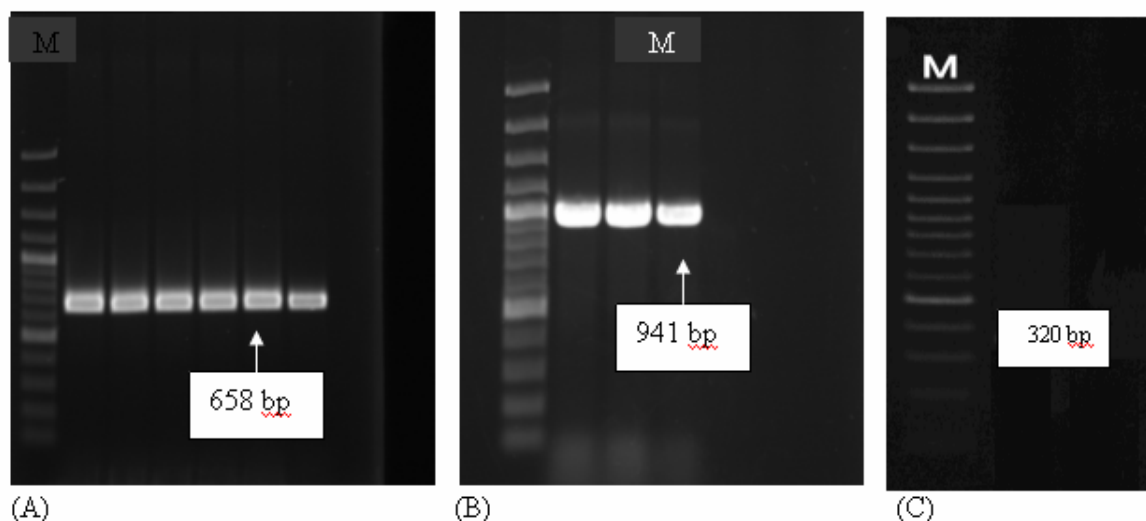
Only 19% of *E. faecalis* isolates were resistant to ciprofloxacin, which differs markedly from 47% obtained by *E. faecium*. No resistance to vancomycin, teicoplanin and linezolid was detected among HLGR isolates.

**Table 1:** Nucleotides sequences of primer sets

Type of primers	Sequence(5'-3')	Reference	Product size(bp)
Genus specific rrs (16S rRNA)	GGATTAGATACCCTGGTAGTCC TCGTTGCGGGACTTAACCCAAC	12	320
<i>E. faecalis</i>	ATCAAGTACAGTTAGTCTTTATTAG ACGATTCAAAGCTAACTGAATCAGT	12	941
<i>E. faecium</i>	TTGAGGCAGACCAGATTGACG TATGACAGCGACTCCGATTCC	12	658

**Table 2:** Antibiotic resistance among HLGR strains isolated from sewage treatment plants in Tehran. The abbreviations are; V: vancomycin, Te: tetracycline, Am: ampicillin, E: erythromycin, Cip: ciprofloxacin, Tei: teicoplanin, Fm: nitrofurantoin, Syn: quinupristin-dalfopristin (Synercid), Lin:linezolid, SXT: co-trimoxazole.

Species	Number of isolates n=90	Number (%) of HLGR isolates resistant to:									
		V	Te	Am	E	Cip	Tei	Fm	Syn	Lin	SXT
<i>E. faecalis</i>	26(29)	(0)	23(92)	2(7.5)	16(63)	5(19)	(0)	(0)	6(100)	(0)	18(69)
<i>E. faecium</i>	58(64)	(0)	27(47)	10(18)	56(97)	27(47)	(0)	10(17)	2(3.5)	(0)	25(43)

**Fig. 1:** A: PCR for *Enterococcus faecium*, B: PCR for *Enterococcus faecalis*, C: PCR for Genus of Enterococci

## Discussion

In the present study HLGR enterococci were found in sewage along with high resistance to other antimicrobial agents especially among *E. faecium* isolates. HLGR multiresistant enterococci have been identified as a major issue of concern, as they have been found associated with nosocomial infections (14). HLGR in *E. faecalis* is now a very common feature worldwide and the reported prevalence exceeds 50% of the *E. faecalis* strains isolated in some hospitals (6). According to an area specific survey in Tehran the prevalence of HLGR *E. fae-*

*calis* isolates reported 30% with high degree of multiresistance (15). This finding was alarming as infection due to HLGR isolates are difficult to treat.

Finding HLGR *E. faecalis* in sewage, which is much more common in human enterococcal infections, suggest a potential high risk for community-acquired HLGR as well as the possibility of transferring antibiotic resistance genes on the other bacteria.

Resistance to multiple classes of antibiotics is common in enterococci as was seen in this study. We found that, resistance patterns differed be-

tween species. Overall, *E. faecium* had a higher prevalence of resistance among the panel antibiotics, while *E. faecalis* isolates had a relatively lower resistance to ampicillin, erythromycin and ciprofloxacin excluding their inherent resistance to quinupristin/dalfopristin (synercid). Resistance rate against synercid among HLGR *E. faecium* isolates was 3.5%. Synercid has a spectrum of activity against multi-resistant enterococci excluding *E. faecalis* and is available for the treatment of multiresistant *E. faecium* infections (16). *E. faecalis* resistance to ampicillin and vancomycin is uncommon (17). However, in this study *E. faecalis* resistance to ampicillin was found in low degree (7.5%) and no resistance to vancomycin was observed.

The resistance rate to co-trimoxazole and tetracycline among *E. faecalis* was more than *E. faecium*. This may be because of widely usage of these two antimicrobials in human and animal infections and selective antibiotic pressure or simply transfer of resistance genes or combination of both.

A high percentage of the *E. faecium* isolates were resistant to multiple drugs, contributes to the challenge of selecting therapeutic measures. Distribution of enterococcal isolates was comparable to other studies. For example Cupakova in Czech Republic during one year (2003) recovered 100 enterococcal isolates from different wastewater samples including 60 isolates as *E. faecalis*, 8 strains were allotted to *E. faecium* and the rest were of other species. The susceptibility to antibiotics showed that the majority of isolates (95%) was resistant to more than one antibiotic tested and no vancomycin resistant isolates was found. Co-resistance to four and five antibiotic simultaneously was reported 37% and 26%, respectively (18).

Similar results were obtained in other study, which showed distribution of enterococci in sewage (18). Out of 45 enterococcal isolates from municipal wastewater, 32 isolates were *E. faecalis*, 10 isolates belonged to *E. faecium* and 3 to *E. hirae* (18).

According to de Costa's report, among 983 enterococci, multidrug resistant isolates were present in 49.4% of the isolates. Only 3.3% and 0.6% of the investigated strains were re-

sistant to ampicillin and vancomycin. Resistance rates to tetracycline, erythromycin, nitrofurantoin and ciprofloxacin were 34.6%, 24.8%, 22.5% and 14% (19).

Ferreira in 2006 in Portugal studied the antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant and demonstrated that the predominant species were *E. faecalis*, *E. faecium* and *E. faecalis*. The percents of resistance observed to erythromycin, ciprofloxacin and tetracycline ranged between 23% and 57%. Only two *E. faecium* of 27 enterococcal isolates were HLGR (20).

All the HLGR *E. faecium* isolates in our study were sensitive to linezolid, which was recently launched for the treatment of Gram-positive infections (16).

Conclusively, our data indicate that the relax usage of antimicrobials had created a large pool of resistance genes which may be disseminate resistant bacteria into the environment and could potentially pose a health treat to human in the future.

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The authors declare that they have no Conflict of Interests.

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