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FIRST DETECTION OF *efrAB*, AN ABC MULTIDRUG EFFLUX PUMP IN *ENTEROCOCCUS FAECALIS* IN TEHRAN, IRAN

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Enterococcus faecalis is one of the most significant pathogen in both nosocomial and community-acquired infections. Reduced susceptibility to antibiotics is in part due to efflux pumps. This study was conducted on 80 isolates of *E. faecalis* isolated from outpatients with urinary tract infection during a period of 1 year from April 2014 to April 2015. The antibiotic susceptibility patterns of isolates were determined by the disk diffusion method and presence of *efrA* and *efrB* genes was detected by PCR and sequencing. Minimum inhibitory concentrations (MICs) to ciprofloxacin (CIP) were measured with and without carbonyl cyanide 3-chlorophenylhydrazone (CCCP) by broth microdilution. The highest resistance rate was observed to erythromycin (83.3%) and the prevalence of *efrA* and *efrB* genes in all *E. faecalis* isolates was 100%. This study showed that 9 out of 13 (69.2%) ciprofloxacin-resistant isolates became less resistant at least fourfolds to CIP in the presence of efflux pump inhibitor. Our result showed that CCCP as an efflux inhibitor can increase effect of CIP as an efficient antibiotic and it is suggested that *efrAB* efflux pumps are involved in resistance to fluoroquinolone.

Keywords: *Enterococcus faecalis*, *efrAB* efflux pumps, antibiotic resistance, CCCP

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Introduction

Gram-positive organisms are responsible for a number of the most severe human infections [1]. One of the most significant of such bacteria is *Enterococcus faecalis* which is a common contributor agent to both nosocomial and community-acquired infections in patients, including bacteremia, surgical site infections, and urinary tract infections (UTIs) due to its multidrug resistance (MDR) and colonizing capability. However, their particular pathogenic mechanisms are not completely clarified [2–4]. *E. faecalis* has been graded among the most prominent causes of UTIs, which are predominantly caused by *E. faecalis* [5, 6]. These acquired mechanisms of resistance consist of spontaneous mutation along with genetic exchange with other bacteria in the environment through horizontal gene transfer. Moreover, *E. faecalis* is inherently more resistant to a number of antimicrobials in comparison with the majority of Gram-positive bacteria [7]. Resistance to the first-line antimicrobial agents makes the treatments difficult. Such drug resistance can arise from the activity of membrane-based efflux proteins, and henceforth referred to as "pumps." Transportation processes irrelevant to drug resistance, including efflux, are used by all bacteria to obtain nutrients. While bacteria normally use efflux as a transportation process to obtain nutrients, they can take advantage of the same mechanism to efflux antibiotics [1]. Ciprofloxacin (CIP), a fluoroquinolone, is a broad-spectrum antimicrobial that is important in the treatment of a wide range of clinical infections [8]. Although principal mechanisms of resistance to β-lactams, fluoroquinolones, aminoglycosides, and vancomycin are well documented, relatively little is known about drug efflux pumps in *E. faecalis* [9]. The most extensively studied efflux pumps in *E. faecalis* are a homolog of *NorA, EmeA*, a member of the major facilitator superfamily (MFS), and *efrAB* belonging to the ATP-binding cassette (ABC) superfamily of multidrug efflux transporters [10]. The significance of *E. faecalis* is partly due to the existence of MDR efflux pumps such as *EmeA* which belongs to the MFS and the ABC-type MDR transporters [11]. A type of ABC multidrug efflux pump named *efrAB* was identified in *E. faecalis* [12]. The heterodimeric ABC transporter *efrAB* has been suggested to be an MDR pump, which transports norfloxacin and acriflavine when overexpressed in *Escherichia coli* [13], but its function in *E. faecalis* has not been studied by a respective gene deletion [9]. In this work, we explored the general role of *efrAB* efflux pump inhibitor, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), in the minimum inhibitory concentration (MIC) of CIP in resistant *E. faecalis* isolated from clinical samples. Inhibition of efflux pump is a valuable alternative to decrease the susceptibility of resistant *E. faecalis* to commonly used clinical antibiotics. Moreover, a detailed

understanding of the antibiotic resistance and the underlying mechanisms in *E. faecalis* is critical to the management of antimicrobial resistance problem in clinical care, by using novel management tactics such as efflux pumps inhibitors to control resistance to several antibiotics.

Objectives

There are a number of studies on fluoroquinolones resistance in clinical isolates of *E. faecalis* from Iranian populations, but this is the first study about the role of *efrAB* efflux pump in resistance to fluoroquinolones of *E. faecalis* isolated from clinical samples. So, the aim of this study was to investigate the influence of active efflux system on CIP resistance in clinical isolates of *E. faecalis* using the efflux pump inhibitor, CCCP.

Materials and Methods

Bacterial strains and media

This study was conducted on 80 isolates of *E. faecalis* isolated from outpatients with UTI infection during a period of 1 year from April 2014 to March 2015. Isolated samples were processed immediately after collection for phenotypic detection of *E. faecalis* by standard protocols based on Gram staining, catalase test, bile solubility, growth in sodium chloride, bile esculin test, and sugar fermentation tests [14]. All strains were maintained and stored in brain–heart infusion (BHI) broth (Merck, England) containing 20% glycerol at −80 °C. For routine use, *enterococcal* strains were cultured on BHI broth at 37 °C. The PCR assay was carried out by specific primers to confirm the *E. faecalis* isolates (see the "Molecular examinations" section).

Antibiotic susceptibility testing

The antibiotic susceptibility patterns of *E. faecalis* isolates were determined by the disk diffusion method according to Clinical and Laboratory Standard Institute guidelines (CLSI) [15]. Antimicrobial disks (Mast Group Ltd., United Kingdom) were used to determine the susceptibility of *enterococcal* isolates to penicillin G (10 μg), ampicillin (10 μg), vancomycin (30 μg), tetracycline (30 μg), minocycline (30 μg), CIP (5 μg), levofloxacin (5 μg), gatifloxacin (5 μg),

nitrofurantoin (300 μg), gentamicin (120 μg), and linezolid (30 μg). After incubation at 37 °C for 24 h in Mueller–Hinton agar (Merck, Germany), the diameter of growth inhibition around each disk was measured. *E. faecalis* ATCC 29212 was used as a reference strain.

Molecular examinations

Extraction of the genomic DNA was performed using the High Pure PCR Template Preparation Kit (Roche, Germany), with some modifications. The bacterial pellet was mixed with 200 μL PBS, digested in 5 μL lysozyme, and incubated at 37 °C for 15 min. The mixture was then lysed using a short incubation with a lysis buffer and proteinase K. The solution was then transferred to a spin column to remove any contaminating cellular components. Finally, the DNA was eluted using an elution buffer at 70 °C. PCR was carried out in a total volume of 25 μL Master mix 2× (Sinaclon, Iran; CAT. NO. PR901638) containing 10 pmol of primers, 100 ng of genomic DNA, 0.4 mM of each of four dNTPs, 3 mM $MgCl_2$, and 0.08 U of Taq DNA polymerase. The primer sequences used were ddlE1 (ATCAAGTACAGTTAGTCTTTATTAG) and ddlE2 (ACGATTCAAAGCTAACTGAATCAGT) for *E. faecalis* isolates [16]. PCR was performed in a thermal cycler (Eppendorf Master cycler, Germany) under the following conditions: initial denaturation step at 94 °C for 5 min followed by 36 cycles, including denaturation at 94 °C for 1 min, annealing at 49 °C (for *ddlE*) and 72 °C for 1 min followed by a final extension at 72 °C for 10 min to ensure full extension of the PCR products. The PCR amplification products were identified through electrophoresis in a 1% agarose gel followed by staining with red safe solution and a 100-bp DNA ladder (Fermentas, Germany). The results were visualized under a UV transilluminator. Positive control *efrAB* from *E. faecalis* was isolated from clinical samples (positive control *efrAB* from Bioneer Company, Korea).

PCR amplification of efrA and efrB genes

The presence of *efrA* and *efrB* genes was detected by PCR using the sequence-specific primer sets described in Table I. PCR was carried out in a total volume of 25 μL Master mix 2× (Sinaclon) containing 10 pmol of primers, 100 ng of genomic DNA, 0.4 mM of each of four dNTPs, 3 mM $MgCl_2$, and 0.08 U of Taq DNA polymerase. The cycling program was adjusted as follows: initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 45 s, 57 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 5 min [10].

Table I. Details of primers used in this study

Genes	Primers (5′–3′)	PCR product size (bp)	Reference
efrA	F: 5′-ACGCCAGTGATGTTTATTGC-3′ R: 5′-ACGAATAGCTGGTTCCATGT-3′	543	This study
efrB	F: 5′-AGTTACTATGTGGTTGCTGG-3′ R: 5′-GGACATCACTACGGTTCATT-3′	439	This study

Sequencing

The PCR purification kit (Bioneer Company) was used to purify PCR products and sequencing was performed by the Bioneer Company. The nucleotide sequences were analyzed with the Chromas 1.45 software and BLAST program from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST).

Treatment of the efflux pump inhibitor

The MICs of CIP were defined in Mueller–Hinton broth according to CLSI [15], using the broth microdilution method in 96-well flat bottom plates. We made two different conditions for each sample. One of the plates contained only CIP and the other plate contained CCCP (Sigma-Aldrich, Shanghai, China) as well as CIP. A set of different concentrations was prepared for each of the two conditions using serial dilution. Similar amounts of bacterial suspensions were further added to each sample. The final concentration of CCCP ranged 25 μg/mL and after incubation for 24 h, the MICs were recorded as the lowest concentration of test compound that was able to inhibit the visual growth [17]. Then, MIC for CIP was determined again for each sample used. A plate containing (25 μg/mL) CCCP without antibiotics was used as control. A reduction of more than fourfolds in the MIC following the addition of CCCP showed that an efflux pump can extrude antibiotics [18].

Statistical analysis

This research was a descriptive-application study. SPSS 16 software was used for statistical analyses. The P value and confidence of intervals were <0.05 and 95%, respectively.

Results

Epidemiological characteristics

Out of 80 *E. faecalis* isolated samples from patients, 41 patients were females (51.2%) and 39 patients were males (48.8%). The mean age of the patients was 21 years (range: 2–84 years old). All the subjects had chief complaints of UTI.

Bacterial isolates and antimicrobial susceptibility test

In a year period, 80 strains of *E. faecalis* were isolated from outpatients with UTI infection. Using biochemical tests and confirmatory PCR assays, we determined that all the strains (100%) were *E. faecalis*. The highest resistance rate was observed in erythromycin (83.3%). The antibiotic resistance profiles of the strains are summarized in Figure 1. The initial results of CIP susceptibility test using the disk agar diffusion method revealed that 13 out of 80 isolates (16.2%) were resistant to CIP. The MIC for CIP in bacterial isolates is summarized in Table II. According to the established breakpoint values suggested by CLSI [19], the *E. faecalis* isolates with MIC ≥4 µg/mL are considered as CIP-resistant. In this study, *E. faecalis* isolates had a CIP MIC range between 4 and 128 µg/mL or greater (Table II), which means they are resistant to CIP.

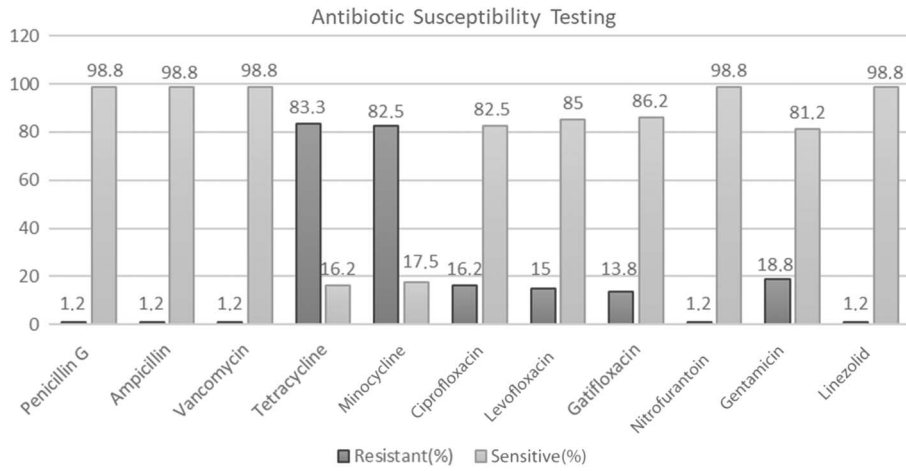

Figure 1. Antimicrobial susceptibility test for *E. faecalis* isolates

Table II. Effects of the efflux pump inhibitor on ciprofloxacin resistance

Isolates, no. (%)	MIC (μg/mL)	MIC (μg/mL) + CCCP	Reduction fold in MIC + CCCP
1 (7.7)	4	1	2
1 (7.7)	8	8	0
4 (30.8)	32	1–8	2–10
4 (30.8)	64	4–32	2–6
1 (7.7)	128	64	2
1 (7.7)	256	256	0

Note: MIC: minimum inhibitory concentration; CCCP: carbonyl cyanide 3-chlorophenylhydrazone.

PCR amplification and sequencing

The results of PCR amplification indicate that prevalence of *efrA* and *efrB* genes in all of isolates of *E. faecalis* is 100% (Figure 2). Sequencing of PCR products displayed conserved regions for the restriction sequence *efrA* and *efrB* genes which were further confirmed by BLAST in NCBI. The nucleotide sequence data described in this paper have been submitted to the GenBank sequence database and have been assigned accession numbers KY131192 and KY131193 for *efrB* gene and accession numbers KY244017 and KY244018 for *efrA* gene (Figures 2 and 3). The sequences of the primers used in this study are listed in Table I.

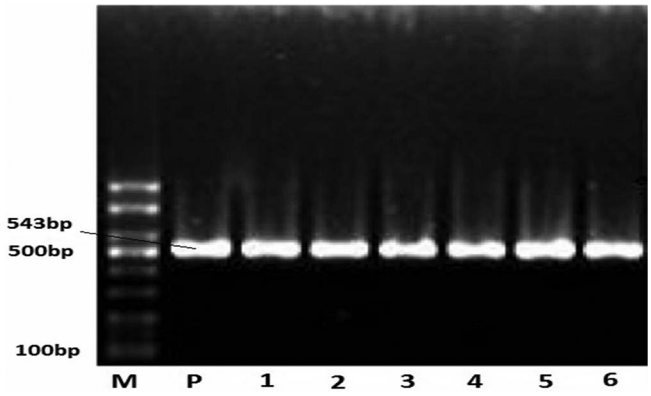

Figure 2. PCR amplification of *efrA* genes. Lanes: M, 100 bp Plus DNA ladder (GeneRuler; Fermentas); P, *E. faecalis* as positive control; 1–6, clinical isolates of *E. faecalis*

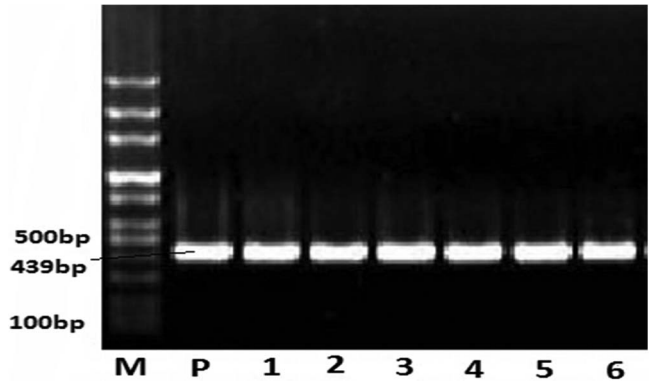

Figure 3. PCR amplification of *efrB* gene. Lanes: M, 100 bp Plus DNA ladder (GeneRuler; Fermentas); P, *E. faecalis* as positive control; 1–6, clinical isolates of *E. faecalis*

Effects of the efflux pump inhibitor on CIP resistance

To determine the role of efflux pump in the CIP-resistant phenotypes in 80 *E. faecalis* isolates, we assessed the MIC of CIP in the presence of 25 μg/mL CCCP and then compared the MICs with and without CCCP. The results showed that 9 out of 13 (69.2%) CIP-resistant isolates became less resistant at least fourfolds to CIP in the presence of efflux pump inhibitor (Table II).

Discussion

Bacteria have evolved sophisticated mechanisms of resistance comprising drug efflux pumps that is adapted for a wide range of substrates, especially antimicrobial drugs. Efflux-mediated resistance can be clinically relevant and causes antibacterial therapy to be useless. It also provides starting point resistance that leads to the emergence of additional resistance mechanisms, such as drug inactivation or drug target modification [20, 21]. The selection of efflux pump-overproducing strains is influenced by bacterial exposure to antibacterials, and limiting such exposure, including minimizing the antibacterial usage, would limit the occurrence of efflux-mediated drug resistance [22, 23].

In this study, we investigated the prevalence of antibiotic resistance especially to CIP among *E. faecalis* isolated from patients with UTIs.

Lee [24] showed that *E. faecalis* isolated from UTI subjects was 46% and 47% resistant to levofloxacin and CIP, respectively [24]. However, the results of this study revealed lower resistance to levofloxacin and CIP (15% and 16.2%, respectively). This differentiation can be attributed to the source of isolated

specimens. In this study, the strains were isolated from UTI in outpatients, but in the Korean study, the strains had been isolated from patients who usually had multiple combined diseases for several years, according to the authors [24]. The results of another study that was conducted by Seo and Lee [25] on *E. faecalis* isolated from patients with chronic prostatitis showed 9.7% resistance to penicillin [25], while the results of this study revealed 1.2% penicillin resistant. However, the Seo and Lee's study did not report any resistance to ampicillin, vancomycin, or nitrofurantoin. These results are similar to this study and showed low prevalence of resistance to ampicillin, vancomycin, or nitrofurantoin.

The analyzed results of antibiotic susceptibility test showed that *E. faecalis* was highly resistant to tetracycline and minocycline. These are the effective antibiotics against a wide range of microorganisms, which are cost-effective, and therefore are frequently used as dominant antibiotics in poultry industry [26, 27]. Antibiotic consumption is one of the most important risk factors for distribution of multidrug-resistant bacteria [28]. Broad-spectrum antimicrobial therapy is associated with increase of resistant bowel flora during or after therapy [29]. Their frequent use in the poultry industry leads us to the hypothesis that the increased antibiotic resistance of the bacteria of normal intestinal flora arises from poultry treatment with tetracycline and minocycline. The fact that in this study, *E. faecalis* isolated from patients with UTIs showed the most noticeable resistance to this group of antibiotics and is consistent with Ayeni et al.'s [30] study in which poultries were used as the samples.

Enterococcus is resistant to numerous antibiotics using efflux pump as a key mechanism of resistance [7]. ABC transporters have important role in MDR *E. faecalis*. *efrAB* is a heterodimeric ABC transporter, which can cause drug resistance when *efrA* and *efrB* genes were expressed together [31]. We also surveyed the existence of *efrA* and *efrB* genes in extracted DNA by PCR. The results were higher than in the results of Valenzuela et al.'s [12] and Kang et al.'s [32] studies. With the aim to explain that involvement of *efrAB* in CIP resistance, we investigated that effect of CCCP as an efflux inhibitor on decrease of CIP MIC. Our results showed that CCCP as an efflux inhibitor can increase the effect of CIP as an efficient antibiotic. In this study, the results of efflux pump inhibitor showed decrease of CIP MIC from two- to tenfolds. The results of this study also suggest that antibiotic efflux pumps are involved in resistance to fluoroquinolone in clinical isolates of *E. faecalis*. ABC transporter is an efflux pump described in *E. faecalis* and its overexpression can confers resistance to fluoroquinolones. This is the first report showing the presence of efflux pump genes *efrAB* in *Enterococcus* spp. isolated from clinical samples in Iran. The use of fluoroquinolones as a feed additive in food animals might contribute to the increase and distribution of *efrAB* to human. So, we suggest that the use of antibiotics should be

carefully evaluated in poultry to avoid the emergence of bacterial resistance to clinically relevant antibiotics.

Acknowledgements

SMJS carried out all experiments and data analysis. FF, AH, and SMJS participated in the design of the study and in the manuscript writing. LA, MR, and PL collaborated in *in vitro* and *in vivo* studies and manuscript writing. All authors have read and approved the final manuscript.

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Conflict of Interest

The authors declare no conflict of interest regarding the publication of this paper.

Availability of Data and Materials

The data sets during this study are available from the corresponding author on reasonable request.

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