AKADÉMIAI KIADÓ

Acta Microbiologica et Immunologica Hungarica

67 (2020) 3, 187–192

DOI:
10.1556/030.2020.01173

ORIGINAL ARTICLE

Upregulation of *efrAB* efflux pump among *Enterococcus faecalis* ST480, ST847 in Iran

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Received: March 5, 2020 • Accepted: April 20, 2020
Published online: September 25, 2020

ABSTRACT

Antibiotic resistance and especially multiresistance in Enterococci, is a serious public health issue especially in infections of immunocompromised patients. EfrAB is a heterodimeric multidrug ATP-binding cassette (ABC) transporter that causes endogenous resistance to antimicrobials including fluoroquinolones in *Enterococcus* spp. The aim of this study was to seek the gene expression rate and role of *efrAB* efflux pump in ciprofloxacin resistant *Enterococcus faecalis* and Multilocus Sequence Typing (MLST) of multiresistant isolates. Phenotypic and genotyping identification of 80 *E. faecalis* isolates were performed. Minimum inhibitory concentrations (MICs) to ciprofloxacin (CIP) were measured with and without carbonylcyanide 3-chlorophenylhydrazone (CCCP) by broth microdilution. After DNA extraction and sequencing for detection of *efrA* and *efrB* genes, the *efrAB* efflux positive isolates that were resistant to ciprofloxacin and showed decrease of ciprofloxacin MIC range were identified. Isolates that exhibited decrease in ciprofloxacin MIC range from two to ten folds were assessed for biofilm formation and finally, the expression levels of *efrB, efrA* genes were measured by quantitative Real-Time PCR (qRT-PCR). High rates of resistance to tetracycline and minocycline and low rates of resistance to the most antibiotics used in this study were detected. The results in this study indicated that the incidence of Multiple drug resistance (MDR) was 23.7% and all isolates that were resistant to ciprofloxacin revealed several degrees of overexpression in *efrA* and *efrB* genes. Our study found two ST480 and one ST847 in *E. faecalis* isolates. In conclusion, despite of low frequency of resistance to the most antibiotics and MDRs in our region, we found one ST480 isolate with resistance to eight antibiotics that also exists in other parts of the world.

KEYWORDS

efrAB, Efflux pump, *Enterococcus faecalis*, MLST, MIC, UTI

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INTRODUCTION

The causative bacteria of urinary tract infections (UTIs) and antibiotic resistance among them have variation partly owing to the antibiotic consumption in countries that leads to difficulties in treatment process [1, 2]. The center for disease control (CDC) announced that UTI causes 30% of acute care in hospitals [3]. The most plentiful gram-positive cocci in humans that are normally inhabit the gastrointestinal tracts of nearly all animals are *Enterococci* [4].

Enterococci are more known as nosocomial pathogens because they cause severe diseases such as surgical wound infections, endocarditis, bacteremia and UTIs. Moreover, they are resistant to numerous antimicrobial agents that are using in hospitals [5]. Antibiotic resistance and especially multi resistance in *Enterococci*, is a theatrical public health dilemma because it cause defeat in treatment, especially in immunocompromised patients [6]. *Enterococcus faecalis* has emerged as the greater number of Enterococcal infections with high levels of multiple antibacterial resistance and one of the most important pathogens in UTI that has increased [7–10]. Generally, first-choice antibiotics for treatment of Enterococcal infection are β-lactams and aminoglycosides. Antibiotics like glycopeptides and linezolid are categorized as second-choice [11]. Additionally, *Enterococci* have intrinsic resistance to cephalosporins, clindamycin, low level aminoglycosides, lincomycin, polymixins and quinolones and also can obtain resistance to trimethoprim/sulfamethoxazole, tetracyclines, macrolides, ampicillin and chloramphenicol [12]. One of the significant mechanisms of tolerance to biocides are efflux pumps that are included in the major facilitator superfamily (MFS), the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family, the ATP-binding cassette (ABC) family and the toxic compound extrusion (MATE) family, with broad substrates and also antibiotics [13]. Members of ABC transporter family comprises both transport and uptake systems with using ATP to transport a great range of ingredients counting amino acids, sugars, drugs, ions, proteins and polysaccharides [14, 15]. Multiple drug resistance (MDR) phenotypes in Gram-positive and Gram-negative bacteria are attributed to resistance to three or more antibiotics and can be related to overexpression in efflux pumps [16–19]. *EfrAB*, a heterodimeric multidrug ABC transporter was found to have a role in endogenous resistance to antimicrobials including fluoroquinolones in *E. faecalis* and *Enterococcus faecium* [20, 21]. Additionally, there are other resistance mechanisms in enterococci comprising mutational alteration of the target, for example the substitution of the second D-Ala residue from peptidoglycan termini by means of a D-lactase or D-serine that is responsible for resistance to vancomycin or the enzymatic inactivation of drugs by modification of penicillin-binding proteins (PBPs) [22]. Ciprofloxacin as a fluoroquinolone is a common and effective antibiotic for the treatment of UTIs and has improved antibacterial activity against wide range of bacteria [23, 24]. The increased usage of fluoroquinolones for treatment of *E. faecalis* infections led to the occurrence of *E. faecalis* strains resistant to fluoroquinolone in more than a few countries [8, 25–27]. The main purpose of the study was to seek the role of *efrAB* efflux pump in resistance to ciprofloxacin. So, we identified the Minimum Inhibitory Concentrations (MICs) of different antimicrobials and the expression of *efrAB* genes in ciprofloxacin resistant *E.faecalis* strains by quantitative Real-Time PCR (qRT-PCR). Finally, we chose more resistant isolates for typing by Multilocus Sequence Typing (MLST).

MATERIALS AND METHODS

Isolation and Identification of Isolates

The 80 *E. faecalis* that were isolated from UTI patients during a descriptive study, were grown in bile esculin agar (BEA-Merck, England) and were recognized by biochemical tests like Gram's stain, catalase tests and Growth on 6.5% NaCl. Also, we used Polymerase Chain Reaction (PCR) for final identification [28]. *E. faecalis* ATCC 29212 was performed as a control strain in all of stages of this study.

Antimicrobial susceptibility testing

Determination of the MICs were measured for antibiotics including ampicillin, penicillin G, tetracycline, minocycline, ciprofloxacin, levofloxacin, gatifloxacin, vancomycin, nitrofurantoin and linezolid by E-test method (Liofilchem, Italy). The MIC was found from the scale in terms of µg/mL and where the border of the growth inhibition ellipse crosses with the strip after 24-h incubation period on Mueller-Hinton agar (Merck, Germany) in 37 °C. MICs of isolates were interpreted according to Clinical and Laboratory Standards Institute guidelines (CLSI) [29]. MICs of ciprofloxacin (CIP) were measured with and without carbonylcyanide 3-chlorophenylhydrazone (CCCP) by broth microdilution.

DNA extraction

The DNA extraction of the isolates were performed by the High Pure PCR Template Preparation Kit (GeNet Bio Company, Daejeon, Korea; Cat. No, K-3000), with specific modifications [30].

PCR amplification and sequencing for detection of *efrA* and *efrB* genes

The PCR procedure and the primers for molecular approval of *E. faecalis* and detection of *efrA* and *efrB* genes were performed in circumstances of 94 C for 5 min, then 35 cycles of 94 C for 60 sec, 72 C for 60 sec, and 72 C for 5 min and it was done based on previous study [31]. The isolates which carried these genes and were isolated from clinical samples were used as positive controls and the sequencing process has been done by Bioneer Company (Korea).

Semi quantitative real-time PCR (qRT-PCR) of *efrA* and *efrB* genes:

EfrAB efflux positive isolates of *E. faecalis* that were resistant to ciprofloxacin in our last study were surveyed for the effect of efflux pump inhibitor on reducing MIC range and 11 isolates showed decrease of ciprofloxacin MIC range [31]. Isolates that exhibited decrease in ciprofloxacin MIC range from two to ten folds were assessed for the expression levels of *efrB, efrA* genes by qRT-PCR. RNA extraction was performed (Cat. No., RN7713C; SinaClon) after 24-h cultures grown in Luria-Bertani broth (Merck, Germany) and then

Table 1. Primers for Real-time PCR

Genes	Primers(5′→3′)	PCR products(bp)
efrA	*CGTGAAGAAGAAGGCGTAAC* *ACCTGTGCTCCCAATAAAGG*	159
efrB	*GTGGATCACTTCATTCGGAC* *GGTGGGCAATAACGAAACTC*	238
pheS	*CGTGATACAGATGATGCGAC* *CGCCGCCACATTTAAAACAG*	217

Table 2. Antibiotic susceptibility for *E. faecalis* isolates based on CLSI guideline

Antibiotics	MIC interpretive criteria (μg/mL) (%)		
	S (%)	I (%)	R (%)
Gatifloxacin	69(86.2)	1(1.2)	10(12.5)
Levofloxacin	69(86.2)	1(1.2)	10(12.5)
Ciprofloxacin	66(81.2)	0(0)	14(18.8)
Minocycline	18(22.5)	16(20)	46(57.5)
Tetracycline	13(16.2)	1(1.2)	66(82.5)

Residual DNA was taken by DNase I (Fermentas, Lithuania). The quantitation of RNA was accessed with Nanodrop at 260 and 280 nm (A260/280). The product of RNA was reverse transcribed into cDNA via Intron kit (Korea). RNA were suspended in 50 μL DEPC water (0.1% v/v). Real-time RT-PCR assay were accomplished by the Power SYBR Green PCR Master Mix (Bioneer, Korea) and Corbett Rotor-Gene 6,000 Real-Time rotary analyser (Corbett Life Science, Australia). Each run was included triplicate of samples and a negative control without cDNA. *PheS* genes as a housekeeping gene was used for normalization and all of gene expressions were evaluated with pheS gene expression [30]. The $2^{-\Delta\Delta Ct}$ method was used to get the expression fold change and Ct (Cycle of threshold) was measured as the average threshold cycle number. Primers for qRT-PCR were designed in this study and are displayed in Table 1.

Multilocus sequence typing (MLST) of resistant isolates

MLST was done pursuant to the *E. faecalis* MLST scheme via amplifying seven housekeeping genes including *gdh, pstS, gyd, gki, xpt, aroE* and *yqiL* in the reference method [32]. In a word, PCR amplification and sequencing of seven housekeeping genes in the strains of *E. faecalis* with more resistance traits were accomplished (Bioneer, Korea). Set of *E. faecalis* MLST primers for seven housekeeping genes and STs were generated based on MLST website (http://efaecium.mlst.net). Then, characterization of the allelic profiles and STs were obtained.

Biofilm assay

Detection of biofilm formation were done according to microtiter plate test as revealed previously. In short, after cultivation of isolates in LB broth (Merck) overnight at 37 °C, the plates were washed with phosphate-buffered saline (PBS). Then, methanol (Sigma-Aldrich, 99.8%) were added for fixation of the adherent cells and each wells were stained with crystal violet (Sigma-Aldrich, 1%). The optical density ($OD_{570\,nm}$) of the stained adherent cells were determined. Biofilm assay tests were performed in triplicate for all of isolates [33].

Statistical analysis

Statistical analysis was performed by SPSS 18 (SPSS Inc., Chicago, IL, USA). Differences in the mean expression level were evaluated by *t*-test and the relationship between variables was evaluated by calculating Spearman's rho (Correlation Coefficient). P values ≤0.05 were considered significant.

RESULTS

Resistance to tetracycline and minocycline were in high rates; 82.5 and 57.5%, respectively. Moreover, resistance to fluoroquinolones were in lower rates. These findings are listed in Table 2. Survey of resistance to other antibiotics exhibited sensitivity in most of isolates, except in one isolate that showed resistance to ampicillin, penicillin G, ciprofloxacin, levofloxacin, gatifloxacin, vancomycin, nitrofurantoin and linezolid [34].

We screened 19 MDR isolates (resistant to three antibiotics at least) from the 80 isolates of *E. faecalis* from patients with UTIs. The antimicrobial resistance in these 19 (MDR) *Enterococcal* strains is defined in Fig. 1.

Table 3 describes the characteristics of ciprofloxacin resistant strains including folds of expression of *efrA* and *efrB* genes, biofilm formation and also, antibiotic susceptibility (MIC determination) to ciprofloxacin, levofloxacin, gatifloxacin, tetracycline and minocycline by E-test method.

The MLST results in this study showed that ST847 was identified in strains 79 and ST480 were found in strain 20 and 30 (Table 4).

DISCUSSION

Enterococci with a wide range of mechanisms for antibiotic resistance and the capability for occurrence of MDR are the

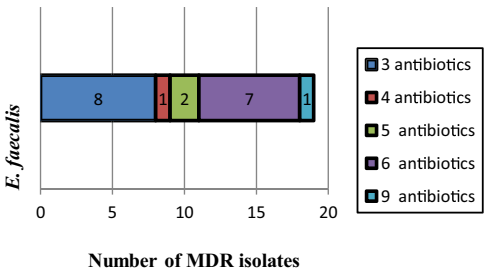

Fig. 1. Antimicrobial resistance in 19 MDR *E. faecalis* isolates

Table 3. Characteristics of ciprofloxacin resistant strains of *E. faecalis*; CIP: ciprofloxacin, LEV: levofloxacin, GAT: gatifloxacin, TET: tetracycline, MIN: minocycline; S: sensitive, R: resistant

Strain numbers	Overexpression rates of *efrA*	Overexpression rates of *efrB*	Antibiotic susceptibility based on MIC					Biofilm formation
			CIP	LEV	GAT	TET	MIN	
2	5 folds	2 folds	32	8	12	96	24	Negative
7	5 folds	2 folds	32	≥128	6	64	16	Negative
24	2 folds	2 folds	32	16	48	≥128	16	Negative
30	2 folds	1 folds	128≤	64	128	96	16	2+(Positive)
40	10 folds	2 folds	4	S	S	64	16	Negative
53	2 folds	2 folds	32	12	64	32	S	1+(Positive)
57	2 folds	1 folds	32	32	64	64	16	1+(Positive)
65	8 folds	3 folds	64	S	S	S	S	Negative
72	1 folds	6 folds	32	S	8	64	12	Negative
75	1 folds	6 folds	64	32	16	≥128	16	1+(Positive)
79	6 folds	4 folds	64	12	128≤	48	S	4+(Positive)

Table 4. Results of Sequence types (STs) for housekeeping gene alleles among highly resistant and biofilm producing isolates

	gdh	*gyd*	*pstS*	*gki*	*aroE*	*yiqL*	*xpt*	STs
Strain 30	1	1	22	22	7	6	17	480
Strain 75	1	1	22	22	7	69	17	480
Strain 79	12	4	46	3	8	47	31	847

third agents for UTIs that have variety from cystitis to perinephric, pyelonephritis and prostatitis [34, 35]. The ABC multidrug transporters as multidrug efflux pumps in *E. faecalis*, are a main problem in forming MDR because multidrug efflux pumps facilitate expulsion of various antibiotics that are structurally different [4, 17, 19, 36]. *EfrAB* as an ABC transporter efflux pump identified in 100% of MDR *E. faecalis* strains [30]. The aim of this study was inquiry of the frequency of antibiotic resistance and the survey of MDR isolates among *E. faecalis* isolated from UTIs for the best results in clinical therapies. A high prevalence of resistance to tetracycline and minocycline were found in this study, while low frequency of resistance to the most antibiotics used in this study were detected. The results in this study indicated that the incidence of MDR was 23.7% that was less than previous studies [37–41]. We would say that the results of some of the studies were related to all species of *Enterococci* or all of the clinical samples were investigated in these studies. Moreover, the purpose was the investigation of expression levels of *efrAB* in ciprofloxacin resistant isolates and then, survey of the correlation of expression level with other antibiotic MIC range of resistance and biofilm formation. In this study, we indicated that all isolates that were resistant to ciprofloxacin revealed several degrees of overexpression in *efrA* and *efrB* genes. This genes co-expression seems reasonable in heterodimeric ABC transporters like *efrAB* and it results increasing drug resistance [42]. Our findings in overexpression of *efrAB* in ciprofloxacin resistant *E. faecalis* showed more proof for the previous study [20]. Additionally, another study found the overexpression in *efrAB* efflux pump, work as an MDR transporter in *Escherichia coli* [4]. The other studies has been investigated in fermented or dairy foods [30, 43, 44]. Our study found one ST847 and two ST480 in *E. faecalis* isolates. Resistance to ampicillin, penicillin G, vancomycin, linezolid, nitrofurantoin, ciprofloxacin, levofloxacin and gatifloxacin were seen in one of the ST480 isolated in this study. Resistance to ciprofloxacin and linezolid in the isolated ST480 in this study were similar to antibiotic resistance patterns of isolated ST480 from Tunisia, Mexico, China and France [45–49]. One limitation to this study was impossiblity of typing all of *E. faecalis* isolates due to the high cost of MLST methods. We recommend further studies defining of the typing of all *E. faecalis* strains by MLST method and prepare the philogenetic diagram of isoated strains.

CONCLUSION

In conclusion, significant overexpression in *efrAB* positive *E. faecalis* that were ciprofloxacin resistant can pose a hypothesis that *efrAB* might have a key function in resistance in *Enterococci*. Moreover, despite of good news of low frequency of resistance to the most antibiotics and MDRs in our region, we found the ST480 isolate with resistance to eight antibiotics that also exists in other parts of the world. It can pose the start of life threatening condition especial for the people at risk.

Competing interests: The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

This study was financially supported by research department of the School of Medicine, Shahid Beheshti University of Medical Sciences (grant no. 6965).

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