Mutations at C-terminal domain of *pbp5* gene among high level ampicillin resistant isolates of *E. faecium* in Iran

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ABSTRACT

Background and the purpose of the study: The low affinity penicillin-binding protein (PBP) 5 of *Enterococcus faecium* is responsible for intrinsic resistance to beta-lactam antibiotics. This study was conducted to determine the MICs of ampicillin against *E. faecium* strains cultured from Iranian patients (n=54) in Tehran hospitals and to sequence the C-terminal ends of pbp5s from selected strains (n=15) in order to determine possible mechanism of resistance to ampicllin.

Methods: Initially, the minimum inhibitory concentrations (MICs) of ampicllin against 54 isolates of *E. faecium* were determined using broth macro-dilution assay. A PCR was designed to target *pbp5* gene. The PCR products corresponding to the C-terminal ends of PBP5s for each strains (n=15) were sequenced.

Results: Up to 44% of isolates were highly resistant to ampicillin (MIC ≥ 64 µg/ml). Amino acid substitutions were found at position number of 485 (Met 485 to A(T) and also an additional serine residue inserted just after Ser 466 among the high level resistant isolates (MIC ≥ 64 µg/ml). Other substitutions were also found at Q461K and V586L in these strains.

*Conclusion***:** It appears that amino acid alternations near the SDN motif, mainly the amino acid at position 485, were responsible for high-level resistance to ampicillin. Other substitutions outside of this motif (n=7) had no observable effect on resistance.

Keywords: *Enterococcus faecium*. Ampicillin resistance. Penicillin binding protein, Nosocomial infections

INTRODUCTION

Enterococcous faecium is responsible for various opportunistic infections in human and animals. Isolates of *E. faecium* comprise 22.5% of all strains of enterococci cultured from the patients of hospitals in Tehran where they are a common cause of urinary tract infections (UTI) (1, 2).

Since *E. faecium* strains are intrinsically resistant to cephalosporins, semi-synthetic penicillins are being used in combination with aminoglycosides, such as gentamicin for the treatment of infections with these organisms (3). In recent years, due to the increase in the use of beta-lactam antibiotics, resistance has occurred rapidly and up to 76% of Iranian isolates of *E. faecium* have been found to be resistant to ampicillin, and none of them have shown β-lactamase activity (1, 4).

Beta-lactam antibiotics interact with PBPs (transpeptidases) via serine residue of the active-site. PBPs are involved in the late stages of peptidoglycan biosynthesis (transpeptidation) in growing cells. Increased in resistance to ampicillin in entrococci is attributed to either beta-lactamase production, or increase in quantity of PBP5 or point mutations near the three classical conserved motifs, STFK, SDN and KTG (5, 6). It is presumed that these mutations cause lower affinity of the PBP5 molecule for beta-lactam antibiotics and as a result higher MICs values. In this study, the MICs of ampicillin for Iranian clinical strains of *E. faecium* were determined. To identify the role of point mutations in the expression of ampicillin resistance, the C-terminal region of pbp5 for selected strains was sequenced.

MATERIALS AND METHODS

Bacterial isolates and drug susceptibility testing

Fifty four isolates of *E. faecium* recovered from urine specimens of three hospitals in Tehran were examined. The methods for identification and

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antimicrobial resistance profiles of these strains have been reported previously (1). The reference strains, *E. galinarum* VanC, *E. galinarum* ATCC35038, *E. hirae* B61, *E. hirae* B378, *E. hirae* ATCC9790, *E. mundtii* ATCC43186, *E. durans* ATCC6056, *E. faecalis* A256, *E. faecalis* JH2-2, *E. casseliflavis* ATCC25788, *E. faecium* , *E. faecalis* ATCC29212, *E. faecium Tx0016, E. faecalis* ATCC29212 and *E. faecium* TX0016 received from Professor BE Murray of Texas University were used as controls. Two isolates of *E. faecium* expressing the VanA and VanB phenotype which were kindly provided by Frank Aerestup of Danish Veterinary Research Institute were also included in this study. The minimum inhibitory concentrations of ampicillin against the isolates of E. faecium were determined by macro-broth dilution assays (7). Briefly**,** twofold serial dilutions of ampicillin ranging from 512 to 0.25 µg/ml were prepared in Muller-Hinton broth and inoculated with a 100-fold dilution of a 18-24 hrs *E. faecium* culture, incubated at 37ºC and the results were determined after 18 hrs.

Based on the results of MICs, 12 clinical strains of *E. faecium* and 3 standard isolates including *E. faecium* TX0016, and two Danish isolates were selected for DNA sequencing. These isolates were genetically distinct and belonged to different electrophoretic types (ETs) by multilocus enzyme electrophoresis (MEE) analysis. The methods and loci for MEE analysis have been described previously (4, 8).

Polymerase chain reaction (PCR)

DNAs were prepared from 5 ml of exponential phase culture by boiling the cells for 10 min in cracking buffer (20mM Tris HCl, 2mM EDTA and 1% Triton X-100, pH=8). PCRs were performed in a thermal cycler (Genius thermocycler, Techne, UK) using *Taq* polymerase (Fermentas, Lithuania). Primer sequences used for amplification of the entire 3' terminus of *pbp5* gene are GACAAACGGGATCTCACAA-3' and 5'-CGCTGTACCAGTTTTCGC-3'

corresponding to positions 1155-1173 and 1848-1831, respectively (Figure 1). The primers designed from the *pbp5* sequences of *E. faecium* strains EFM-1 (6). Amplification conditions were pre-denaturation at 94°C for 5 min, 94°C for 1 min, 52°C for 2 min, 72°C for 2 min over 30 cycles followed by a final cycle of 72°C for 5 min. PCR products were analyzed on ethidium bromide-stained agarose gels. MassRuler™ DNA Ladder (Fermentas, Lithuania) was used as molecular size marker.

DNA sequencing

Amplified pbp5 fragments were purified with a PCR purification kit (Roche Diagnostics, Germany) and sequenced by automated DNA sequencer (MWG, Germany). The nucleotide sequences were translated and analyzed using EXPASY molecular biology and aligned with the sequence of *E. faecium* EFM-1 reference strain (Accession number X84861). Mutations were verified by nucleotide sequencing in at least two independent experiments.

RESULTS

Susceptibility testing

The MICs of ampicillin for different clinical isolates ranged from 1-128 µg/ml. A total of 24 isolates (44%) were highly resistant (MIC ≥ 64 µg/ml) and a total of 12 isolates (22%) were susceptible (MIC < 16 µg/ml). The MICs for the remaining isolates (n=18; 33%) varied from 16 to 64 µg/ml.

Amplification of the E. faecium pbp5 gene and sequencing

All *E. faecium* strains in this study were shown to posses the *pbp5* gene following amplification of a 670 bp product using especially designed primers (Figure 2) with the exception of *E. hirae*, which its band was specific to *M. faecium* and absent in *E. faecalis* and other species (Figure 2). A second band (1031bp) was specifically observed for *E. hirae* which differentiated this species from *E. faecium*. The amino acid sequences of PBP5 from Iranian susceptible strains of *E. faecium* (MIC < 16 µg/ml) were very similar to the reported sequence of susceptible strains (6,9). The amino acid sequences of the C-terminal domains of PBP5 from 15 strains of *E. faecium* were compared with that of PBP5 of EFM-1 strain which has a low affinity for ampicillin (6). From the alignment of the primary structures, a consensus sequence was obvious. It seemed that there is a correlation between the MIC level and changes at amino acid residue 485, located three amino acids after the SDN triad. In strains where the MIC was determined to be 32 µg/ml, Thr replaced Met-485, and in strains where the MIC was ≥ 64 µg/ml, Ala replaced Thr-485 (Table 1). However, in 2 resistant strains, there was an additional Ser inserted just after Ser-466, upstream of SDN.

Since various substitutions were found at amino acid residues 461, 496,499, 525 and 586 in the low- and high-level ampicillin-resistant strains, it is likely that they have indirect effects on PBP5 affinity (Table 1). Frame shift mutation causing the insertion of a stop codon near the C-terminal was also found in two isolates of which one belong to the collection of isolates received from Texas (Tx0016) and second one was CH166. The

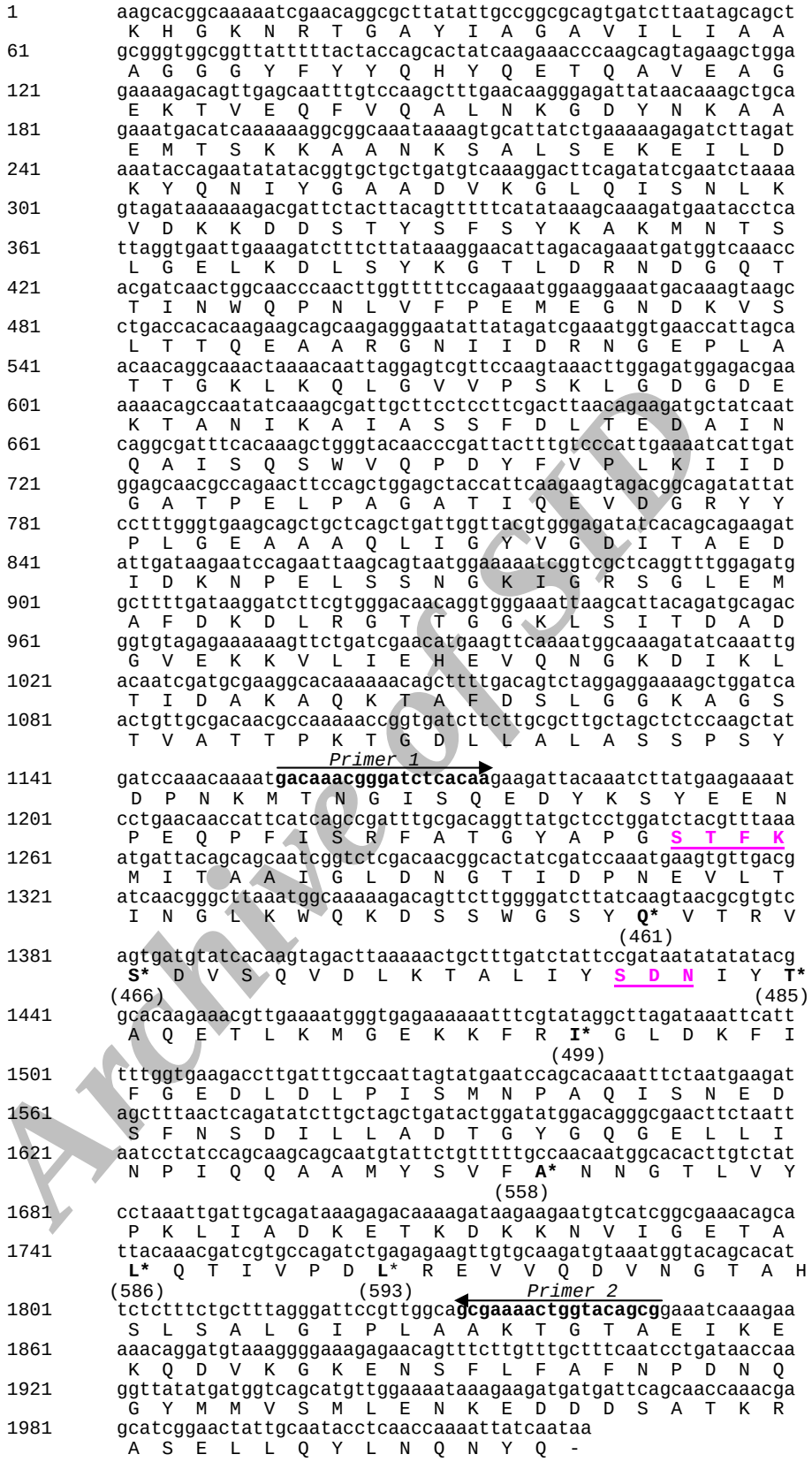

Figure 1. Complete sequence of *pbp5* gene in *E. faecium* EFM-1 strain. The arrows show the of primers used for amplification of C- terminal region. Two important conserved motifs in this region (STFK and SDN) are shown in red color. The asterisks show the amino acids undergo substitutions and responsible for lowering the affinity of PBP5 to ampiciliin..

Table 1. Polymorphisms in the C-terminal region of pbp5 in 15 *E. faecium* isolates and correlation with their specific ampicillin MIC values.

Isolates No.	MIC (mg/L)	426	461	466	470	485	496	497	499	524	525	558	582	586	593
9SH	128	M	K	S	Q	A	K	F	T	E	D	A	G	V	L
83L	128	M	K	S	Q	A	K	F	T	E	D	A	G	V	L
78L	128	M	K	S	Q	A	K	F	T	E	D	A	G	L	L
44CS	64	M	K	S	Q	A	K	F	T	E	D	T	G	L	L
23SH	128	M	Q	S	Q	A	K	F	T	E	D	A	G	L	L
437L	64	M	Q	S	Q	A	K	F	T	E	D	A	G	L	L
vanB	8	M	Q	S	Q	M	K	F	I	E	D	A	G	V	L
263	4	M	Q	S	Q	M	K	F	I	E	D	A	G	V	L
427L	16	M	Q	S	Q	M	K	F	T	E	D	A	G	V	L
403SH	8	M	Q	S	Q	M	K	F	T	E	D	A	G	V	L
TX0016	8	M	Q	S	Q	M	K	F	T	E	D	A	G	V	L
vanA	4	M	Q	S	Q	M	K	F	T	E	D	A	G	V	L
400CH	1	M	Q	S	Q	M	K	F	T	E	D	A	G	V	L
45CS	32	M	Q	S	Q	T	K	F	T	E	D	A	G	V	M
166CH	2	M	Q	S	Q	M	K	F	T	E	D	A	S	V	L

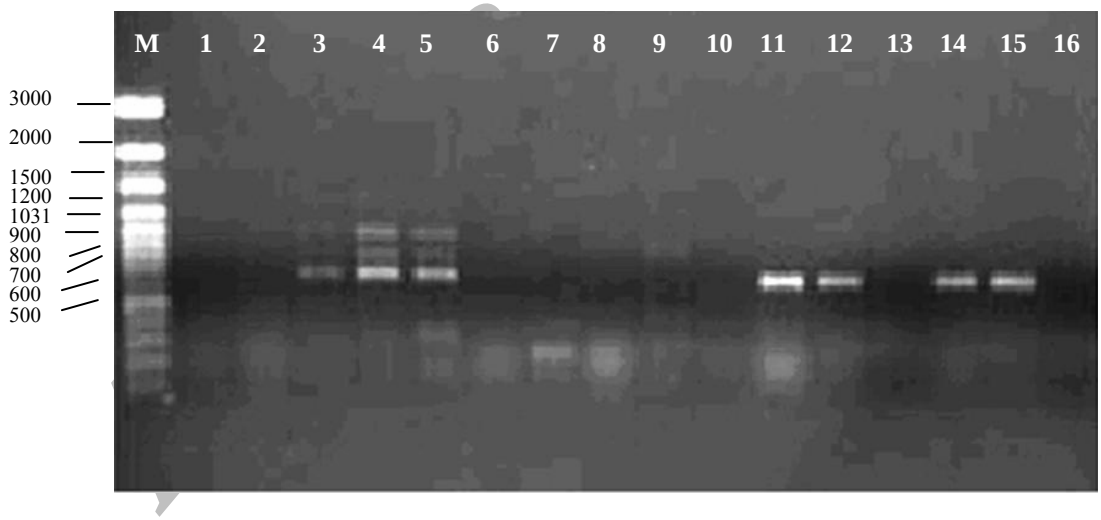

Figure 2. Agarose gel electrophoresis of amplified penicillin binding protein 5 obtained for different species of Enterococci. genes amplicons obtained PCR products . M; molecular weight marker, 1-16:
E. galinarum VanC, *E. galinarum* ATCC35038, *E. hirae* B61, *E. hirae* B378, *E. hirae* ATCC9790, *E. mundtii* ATCC43186, *E. durans* ATCC6056, *E. faecalis* A256, Negative control, *E. faecalis* JH2-2, *E. casseliflavis* ATCC25788, *E. faecium* VanA, *E. faecium* VanB, *E. faecalis* ATCC29212, *E. faecium Tx0016* and *E. faecium* 9SH. M: MassRuler™ DNA Ladder (Fermentas)

latter isolate is unique since it contains frequent mutations. Despite such changes in nucleotide sequences and amino acid substitutions, no changes were identified in the motif regions.

A partial gene sequence obtained for the *pbp5* genes of isolates Sh9, Sh23, Ir44, Ir45, Ln403, Ln437, and 263 were submitted to GenBank under AY626970-AY626976 accession numbers respectively. Related sequences from isolates CH400, Ln78, Ln83, CH166, TX0016, VanA and VanB are deposited in GenBank under AY968693 to AY968699 accession numbers respectively.

DISCUSSION

The rate of resistance to ampicillin in Iran is quite high, with the majority of isolates (44%) being highly resistant to ampicillin (MIC > 64 µg/ml) and only 22% of isolates susceptible to this antibiotic (MIC<16 µg/ml). Importantly, as 60% of collected isolates were also highly resistant to gentamicin (1). Therefore other antimicrobial drugs should be considered for the treatment of such infections in Iran. The rates of resistance to both antibiotics are much higher than reported by other investigations (10). In the previous report it was shown that isolates of enterococci cultured from nosocomial infections in Tehran hospitals belong to a genetically diverse population with panmictic structure. Therefore, selection of isolates in this study was based on the results of their susceptibilities to different antimicrobials as well as their genotypes determined by MEE (1, 8). In Norway, the majority of ampicillin resistant isolates were found in a distinct lineage of closely related genotype (5). In contrast, a report from the USA states that ampicillin resistant strains of *E. faecium* collected from diverse geographic areas are also genetically diverse (11). Both groups of susceptible and resistant isolates of *E. faecium* in Tehran hospitals was previously proved to belong to different genetic lineages (8). This suggests that multiple clones of isolates with the same phenotype of resistance have arisen within Tehran hospitals. Such diversity was also obvious in PBP5 coding regions since amino acid substitutions creating 8 different alleles were found at the C-terminal end of the *pbp5* gene (Table 1).

A change from Met-485 to Ala in strains with MICs ≥ 64 µg/ml was found to be the main cause of resistance to ampicillin among the Iranian strains. However, various substitutions were found at amino acid residues 499, 525 and 586, which were far from the active sitewhich also could play a role in observed higher MIC values. The most important amino acid residue substitution associated with high level resistance to ampicillin was amino acid residue 485, located three amino acids after the SDN triad. A change from Met-485 to Thr was observed in a strain (Ir45) with MICs of ampicillin of 32 µg/ml. This isolate was also resistant to vancomycin. Insertion of additional serin (466'S) has been found among all highly resistant strains of isolates (MIC≥128 µg/ml) in the Spain (9). In this study, 2 isolates (Sh9 and Ln83, MICs =128 µg/ml) contained this additional serine just after Ser466 and both of them had amino acid Ala at position 485. There are two additional isolates with this range of MIC (isolates Sh 23 and Ln 78) lacking the above insertion. Therefore insertion of additional serine at position 466' may not have changed the MIC significantly by itself. In contrast to the findings of Ligozzi et al for *E. faecium* 9439 (12), in which changes at amino acid residues 426, 562 and 574 have been reported, in this study it was not found any substitution at these positions of resistant strains. Also, in contrast to the findings of Rybkine et al (13), a similar Leu- for-Val substitution at amino acid residue 586 in some of most resistant strains of this study, no substitution at amino acid residue 558 (Val for Ala) and 574 (Ile for Thr) was determined. From the results it may be concluded that decrease in affinity of PBP5 for ampicillin is the main mechanism of resistance to high level resistance to this drug among Iranian strains of *E. faecium*. This resistance correlates to the presence of different amino acid substitutions, in particular at amino acid residue 485 near the conserved SDN triad. Various changes which were found in the sequences of strain CH166 prove this hypothesis, and since none of the mutations (n=22) occurred around these conserved motifs, isolates remained susceptible to ampicillin. However, since the complete PBP5 genome of these isolates has not been sequenced, other mutations present in the N-terminal, non–penicillin-binding domains could also contribute to the high level of resistance observed in these strains.

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