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

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Received 10 Sep 2007; Revised 4 Oct 2008; Accepted 14 Oct 2008

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 ampicillin.

Methods: Initially, the minimum inhibitory concentrations (MICs) of ampicillin 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

Enterococcus 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 enterococci is attributed to either β-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
antimicrobial resistance profiles of these strains have been reported previously (1). The reference strains, E. gallinarum VanC, E. gallinarum ATCC35038, E. hirae B61, E. hirae B378, E. hirae ATCC9790, E. mundtii ATCC43186, E. durans ATCC6056, E. faecalis A256, E. faecalis JH2-2, E. casseliflavus 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 macrobroth 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 culture, incubated at 37ºC and the remaining isolates (n=18; 33%) varied from 16 to 64 µg/ml.

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 possess 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 (Accession number X84861). Mutations were verified by nucleotide sequencing in at least two independent experiments.

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’s 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.
Ampicillin resistant isolates of E. faecium in Iran

Figure 1. Complete sequence of \textit{pbp5} gene in \textit{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 ampicillin.
Table 1. Polymorphisms in the C-terminal region of pbp5 in 15 E. faecium isolates and correlation with their specific ampicillin MIC values.

<table>
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<tr>
<th>Isolates No.</th>
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<th>485</th>
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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.

REFERENCES


