Emergence of Storm Resistant Mechanisms in Pseudomonas aeruginosa Isolated from Burn Patients Hospitalized in Ghotbeddin Shirazi Burn Hospital

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Abstract

Introduction: Pseudomonas aeruginosa is a bacterium has capability of presenting different policies against antimicrobial agents. These capacities make this bacterium as one of the most important cause of nosocomial infection especially among burned patients.

Methods: In this cross sectional study, total of 253 hospitalized burned patients (During September 2013 to November 2014) in Ghotbeddin Shirazi were evaluated for P. aeruginosa. Confirmed isolates tested for Metallo-β-lactamase (MBLs) producing and related genes blaIMPI, II, blaSPM, blaKHM, blaVIM, efflux pump activity, and OprD mutations.

Results: From total of 56 (22%) P. aeruginosa isolates, 32 (57.14%) were MBLs while in genetic evaluation, only blaIMPI, blaKHM and blaVIM genes were detected. While 52 (92.8%) isolates had MexAB-OprM gene but 44 (78.6%) isolates had efflux pump activity. In OprD sequencing evaluation it has been proved that the most imipenem resistance isolates have mutations in this related gene. Based on the results it has been proved that the circulating P. aeruginosa isolates in Shiraz burn center are armed with the most potential resistant mechanisms.

Conclusion: According to that the most isolates have the potential of express different mechanisms of resistance, it is highly recommended to evaluate the circulating infectious agents periodically and determine their resistant algorithm to control them before being resistant and being dominant in health centers.

INTRODUCTION

Pseudomonas aeruginosa (PA) is one of the most leading important causes of nosocomial infections which holds the second place after Escherichia coli among gram-negative pathogens reported to the National Nosocomial Infection Surveillance System. An important subject about this organism is its notoriously capability which makes it difficult to control the organism with antibacterial or disinfectants [1].

Beta-lactam antibiotics are a broad spectrum class of antibacterial agents, consisting of all antibiotic agents that contains a β-lactam ring in their molecular structures. Among these groups, carbapenems and meropenems are two potent agents for serious treatment of gram-negative bacterial infections especially P. aeruginosa infections in hospitalized patients. However, resistance to carbapenems has emerged because of production of hydrolyzing enzymes such as metallo-beta-lactamases (MBLs), impermeability to drug due to loss of OprD porin and the up-regulation of an active efflux pump system present in the cytoplasmic membrane of these organisms are another mechanisms of resistant in this bacteria [2].

Nowadays the detection of antimicrobial resistance mechanisms specially in nosocomial infections may help to find appropriate antimicrobial treatment methods[3]. Recent reports on the antibiotic sensitivity patterns of P. aeruginosa in the world have highlighted the problem of antibacterial resistance in cystic fibrosis (CF) and burn isolates in comparison with other hospital isolates[4]. As described before carbapenems are well-suited to use because of their broad spectrum activity and resistance to hydrolysis by most β-lactamases, including the extended-spectrum β-lactamases (ESBLs). These properties have led to an increase in the use of carbapenems,
especially in hospitals in which ESBLs are highly prevalent.
According to increase use of carbapenems in Gram negative infections, other forms of resistant mechanisms like Metallo-β-lactamase (MBLs) is increasingly reported as the main cause of high-level carbapenem resistance among P. aeruginosa isolates [5]. This group of enzymes is resistant to all commercially available β-lactamase inhibitors and only inhibited by metal ion chelators [6]. According to the recent studies in Iran and other parts of the world, there has been a dramatic increase in detection and spread of acquired and transferable families of MBLs (IMP, VIM, SPM, SIM, KHM-1 and AIM enzymes) [6, 7]. Other than hydrolizing enzymes it has been documented that inactivating mutations in OprD can confer resistance to carbapenems either [8]. OprD is an outer membrane protein (OMP) [8], which has a specialized role in the uptake of positively charged amino acids and carbapenems. According to that the Carbapenems are a small hydrophilic compound they can efficiently crosses the outer membrane of the bacterium by passing through the aqueous channels provided by porin proteins which mainly introduced as an OprD [9]. Another remarkable mechanism in bacterium resistance is efflux pump system, which are proteinaceous transporters localized in the cytoplasmic membrane of bacterial cells. Some mutations in efflux pump genes leads to the up-regulation of the MexAB-OprM active efflux system and which may increase the resistance to meropenem and doripenem but with no effect on the susceptibility of P. aeruginosa to imipenem, which is not a substrate for this system [10].

Before this study, there wasn’t any information about the prevalence of resistant mechanisms in main infectious agent isolated in Ghotbeddin Shirazi burn center (Affiliated with Shiraz University of Medical Sciences). Based on the results obtained in this study, frequency and the mechanisms of antibiotic resistance will be determined in P. aeruginosa isolates (18hours/37ºC). An increase in zone size of at least 7 mm of inhibition around imipenem disks alone and those with EDTA was recorded and compared after the incubation time (24 hours/37ºC) to the CLSI guidelines (2014). To perform this step, antibiotic disks (Aztreonam (Mast/30μg), Ceftazidime (Mast/30μg) Cefotaxime (Mast/30μg) were placed around a Clavulanic acid (Mast 15μg) disk on 10 cm Mueller-Hinton agar plates inoculated with 0.5 McFarland suspensions of the isolates, with 30 mm distance between each disk [3]. After the incubation time (18hours/37ºC) inhibition zone diameters were measured to the minimum distance. Difference of ≥5mm in the zone between each disk with Clavulanic acid disk compare to another side of the disk showed that the strain is ESBL positive. For ESBL negative control, Pseudomonas aeruginosa; ATCC 27853; was used.

In this study the modified Hodge test was performed for detection of Metallo-β-lactamase producing isolates according to the following [12].

**Disk Potentiation Test**

Ethylenediaminetetraacetic acid (EDTA) solution (pH 8.0) with 0.5 M concentration was prepared and was sterilized by autoclaving. Test P. aeruginosa isolates were inoculated onto plates of Mueller-Hinton agar (Opacity adjusted to 0.5 McFarland opacity standards). Two 10µg imipenem disks were placed on inoculated plates which one were impregnated with Sul of prepared EDTA solution before. The zone of inhibition around imipenem disks alone and those with EDTA was recorded and compared after the incubation time (18hours/37ºC). An increase in zone size of at least 7 mm around the impregnated imipenem disk compare with imipenem singly was recorded positive for Metallo-β-lactamase producing [13].

**Efflux Pump Analysis**

**Phenotypic**

In this study phenotypic activity of efflux pumps in all test P. aeruginosa isolates were performed with Ethidium bromide (Et-Br) agar based technique with Cartwheel method to screen these active isolates for detection of mexA and mexB genes [14]. This method is a simple, instrument-free, agar-based method utilizes Et-Br for the demonstration of efflux pump activity for at least in 12 isolates of bacteria simultaneously in one plate. The methodology used is straight-forward employing the preparation of two sets of Nutrient Agar (NA) plates containing Et-Br concentrations ranging from 0.0 to 2.5 mg/L for determining the optimize MIC for P. aeruginosa isolates, which determined to be 2 mg/L in this study. The NA plates were prepared fresh in the same day of the experiment and kept protected from light. Plates were inoculated with a study bacterial concentration adjusted to 0.5 McFarland standards in the radial lines format as described in cartwheel pattern (Fig 1). After the incubation time (24 hours/37ºC) the plates are evaluated under Ultraviolet trans-illuminator and...
photographed for more interpretations.

**Figure 1:** Flowchart Followed to Test Bacterial Strains Using the EtBr-agar Cartwheel Method. Isolates were Swabbed on EtBr-containing NA Plates, According to the Diagram

**Table 1:** PCR Primers used for Introduced Genes with Their Specifications

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA sequence (5’-3’)</th>
<th>Amplicon Size (bp)</th>
<th>Annealing temperature (˚C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>oprD</td>
<td>ATGAAAGTGATGAAGTGGAGC</td>
<td>1323-9</td>
<td>49</td>
<td>35</td>
</tr>
<tr>
<td>oprD</td>
<td>CAGGATCGACACGGGATG</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>oprD</td>
<td>AACCTAGCGCCCTCCCT</td>
<td>Sequencing primer set</td>
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<tr>
<td>oprD</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>MexA-B</td>
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<tr>
<td>mexA F</td>
<td>CTCGACCCGATCTACGTC</td>
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<td>38</td>
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<tr>
<td>mexB F</td>
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<tr>
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<td>blalMP-II</td>
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<tr>
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<td>blasKHM</td>
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<td>AATGCAGCGACACCAGGATG</td>
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</table>
DNA Extraction and PCR Analysis

DNA Extraction

For extraction of the total DNA, *Pseudomonas aeruginosa* isolates were refreshed in Mueller-Hinton broth for about 4 hours and their DNAs were extracted with Accuprep® Genomic DNA Extraction Kit (Bioneer-Korea) according to the manufacturer’s instructions. Extracts were stored at -20°C until they were used in the following procedures.

PCR Analysis

PCR was performed in all of following steps by amplification reactions on 2μL of each extracted DNA templates in a final volume of 50μl reaction mixture containing 1x PCR buffer, 0.4dNTP mix, 1.5U of Taq DNA polymerase, 1.5mM MgCl2, 0.4 pmol/μl of each 10pm primer (primers sequences showed in Table 1). The program was performed in a thermal cycler (Eppendorf; Germany) with the following program: 35 cycles with a 10 min first denaturation at 94°C and continued cycles were programmed 1min denaturation at 95°C, 1min annealing, (temperature showed in Table 1), 1min extension at 72°C, and a final 10 min extension cycle at 72°C, before cooling at 4°C. PCR products were analyzed by using 2% agarose gel electrophoresis, stained with ethidium bromide (0.5μg/ml) and visualized with an Ultra-violet trans-illuminator. A 100 bp DNA ladder (Fermentas) was used as a molecular size marker in all gels.

Evaluation of the oprD Gene

To evaluate the entry porin protein for carbapenems (OprD), simple PCR amplification of this operon with whole-DNA extracts from all isolates with the PCR protocol mentioned above. For this step specific primers which previously reported (Table 1) was used [15]. The PCR products of the test isolates were classified in two sensitive and resistant groups to carbapenem (Imipenem) and sequenced with the second set of primers introduced in Table 1. Sequences of oprD were detected as MBLs positive in phenotypic method. Based on the molecular tests performed on the MBLs positive isolates (32 isolates) to evaluate the presence of blaKHM, blaVIM, and blaGES genes, it has been deduced that the frequency of these genes is 0%, 7 (21.9%), and 0% (0%) respectively. Based on the results of cartwheel method to find isolates actively pump Et-Br to the plate environment total of 44 (78.6%) isolates were detected positive for efflux pump activity, where in the genotypic evaluation the presence of mexA and mexB genes has been used in 52 (92.8%) isolates have this operon. Based on the molecular results for oprD all of 56 *P. aeruginosa* isolates showed positive for this fragment of gene with the ≥1302 bp length. All of the amplicons were send for sequencing and grouped in two sensitive (14 samples) and resistant (42 samples) to Imipenem and compare in sequence with together to find if the mutation in sequence of resistant isolates responsible for resistance or not? Based on the results of sequencings, Imipenem resistant and sensitive isolates were categorized in four (I-IV) oprD mutational groups. In group II, nine Imipenem sensitive isolates have the same sequence and was the same to reference strain sequence (PAO1, GenBank Accession no. CAA78448). Group II, which showed some point mutation in deletion or change platform include the rest of Imipenem sensitive (5 isolates). Group III, grouped according to the same alignment in size with no deletion or addition but change in their bases changes. This group was contains 28 isolates which their sequences have no the same or introduced

Identification of Metallo-β-lactamases Genes

All of the carbapenem-resistant *P. aeruginosa* isolates were screened by simple PCR for blaKHM, blaVIM, and blaGES with the above mentioned protocol with specific primers for each as described in Table 1. Confirmation of the related MBL PCR products was done by sequencing of 3 random PCR products for each product by the same primer for PCR [16].

Evaluation of the Existence of Efflux Pumps Genes (mexA and mexB)

The existence of two genes of efflux pump, mexA and mexB, were detected via PCR procedures introduced before using specific primers for each as described in Table 1 [17].

Statistical Analysis

Data were analyzed using SPSS 21 (SPSS Inc., Chicago, IL, USA) software. Fisher’s exact test was used for Data analysis. A P-value below 0.05 was considered statistically significant.

RESULTS

In this cross sectional study total of 253 patients were evaluated from three main wards of the burn hospital; Males, Females and pediatrics. In sexuality differentiation, 146(57.7%) samples were obtained from males and 107 (42.3%) were obtained from females. In sectional differentiation of samples, 121 (47.8%) samples were related to males ward, 83 (32.8%) were related to females ward and the rest samples, (19.4%), were collected from pediatrics ward. The inclusion criteria for samples were that the burned patients hospitalized at least for 48h in the hospital. According to the microbiological results, from total of 253 samples, 56 (22%) isolates were detected *P. aeruginosa while 8 (3%) isolates were including *Pseudomonas spp.*, 8 (3%) isolates were *Acinetobacter spp.*, 48 (19%) were *Staphylococcus aureus*, 6 (2%) were *Klebsiella sp.*, 3 (1%) were *E. coli*, 9 (4%) isolates not detected according to the used test in this study. About 118 (46%) of samples were not growth in the laboratory condition performed in this study (Fig 2). According to the main goal of the study which only *P. aeruginosa* was the main target for the study other isolates were not consider in the following steps. From total of 56 confirmed *P. aeruginosa* isolates, entire isolates were resistant to almost antibiotics used in this study except colistin where all of the isolates were sensitive to, 20 (35.7%) of isolates were sensitive to meropenem, 14 (25%) of isolates were sensitive to imipenem and 5 (9%) isolates (more sensitive isolates were collected from pediatric ward) were sensitive to chloramphenicol (Fig 3). To introduce isolates for MBLs production, ESBL performed screening test showed that all isolates were ESBL. Out of 56 ESBLs producing isolates, 32 isolates were detected as MBLs positive in phenotypic method. Based on the molecular tests performed on the MBLs positive isolates (32 isolates) to evaluate the presence of blaKHM, blaVIM, and blaGES genes, it has been deduced that the frequency of these genes is 0%, 7 (21.9%), and 0% (0%) respectively. Based on the results of cartwheel method to find isolates actively pump Et-Br to the plate environment total of 44 (78.6%) isolates were detected positive for efflux pump activity, where in the genotypic evaluation the presence of mexA and mexB genes has been used in 52 (92.8%) isolates have this operon. Based on the molecular results for oprD all of 56 *P. aeruginosa* isolates showed positive for this fragment of gene with the ≥1302 bp length. All of the amplicons were send for sequencing and grouped in two sensitive (14 samples) and resistant (42 samples) to Imipenem and compare in sequence with together to find if the mutation in sequence of resistant isolates responsible for resistance or not? Based on the results of sequencings, Imipenem resistant and sensitive isolates were categorized in four (I-IV) oprD mutational groups. In group II, nine Imipenem sensitive isolates have the same sequence and was the same to reference strain sequence (PAO1, GenBank Accession no. CAA78448). Group II, which showed some point mutation in deletion or change platform include the rest of Imipenem sensitive (5 isolates). Group III, grouped according to the same alignment in size with no deletion or addition but change in their bases changes. This group was contains 28 isolates which their sequences have no the same or introduced
algorithm of oprD sequences. The rest sequences of resistant isolates (14 isolates) in last group (IV) showed no homology in rearrangement or alignments especially in size length but in some specifications like deletion and addition in their bases which display a main role in frame shift was detected in their sequences.

DISCUSSION

In comparison between the statistic of prevalent infection agents concluded from this study and the previous studies performed in Ghotbeddin Shirazi burn center, no significant changes were observed in the results [18, 19]. Based on the evaluation of the prevalent infections, it has been showed that the most prevalent infectious agents are *Pseudomonas aeruginosa* and *Staphylococcus aureus* with the relative prevalence rate of 21 percent for both of them. Beside on this, other bacteria are either present but in very low rate, which *Acinetobacter* and other species of *Pseudomonas* are in the prevalence rate of 3% or other like *Klebsiella* sp. And prevalence of *E. coli* is under 2 percent prevalence. This algorithm of bacterial infection compare to the past 3 years results for this center shows that the rate and kind of infections in this center is almost controlled in percent but the treatment algorithm and Antimicrobial agents used is very variable. Based on the different studies results one of the main reasons for incomplete control of infections especially in Gram negative bacteria, is their capability in use several different mechanisms to conquer the antibacterial activity. The results of this study on defining the mechanisms of resistant in main infectious agent in burn center may be helpful to introduce an appropriate therapy.

Burn injury is a major public health problem in many developing and under developing countries in the world [20]. According to the level of injuries in this group of victims it requires immediate specialized care in order to minimize morbidity and mortality. In different studies it has been estimated that as many as 75% of all mortalities following burn injury are related to infections. Burn wounds due to their specification such as moisture and nourishing are a good growth environment for microbes specially environmental bacteria [21]. In this study and others it has been showed that among the infectious agents, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are

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**Figure 2:** The results of Bacterial Culture Results have been Shown in this Figure. In This Study Just Routine Aerobic Bacteria Were Evaluated, Anaerobic Bacteria and Fungi were not Considered and if Presented they have been Proposed as No Growth

**Figure 3:** The Results for Antibiogram Test have been Showed in this Diagram. As Seen in this Figure Most *P. aeruginosa* Isolates are Resistant to the Test Antibacterial Agents
two main infectious agents in burned hospitalized patients according to their condition and exposing to the nosocomial and environmental bacteria. *Pseudomonas aeruginosa* is opportunistic Gram negative bacteria can live and growth in different conditions which the most favorite condition for that is moisture environment. This bacterium as an organism able to accumulate different resistance and virulence factors which is found as major colonizer of the burn wounds surface based on the wounds conditions and either persists well in the hospital environment, once it is established, it can persist for months within a unit [20]; according to the circulating different infectious agents with potential transferable resistant mechanisms, burn hospitals are often harbor multidrug-resistant *P. aeruginosa* that can serve as the source of infection [22].

Isolates of *P. aeruginosa* in this center are often multi-resistant to many classes of potent antimicrobial agents, including fluoroquinolones, aminoglycosides and β-lactams. Carbapenems (Imipenem) are the most introduced potent β-lactams, among which are active even against extended spectrum β-lactamase and AmpC β-lactamase-producing gram-negative bacilli which are used routinely in many centers either in Ghotbeddin Shirazi burn center. Due to acquired metallo-β-lactamase (MBL) ability by Gram negative nosocomial isolates, carbapenem resistance in *P. aeruginosa* isolates has been increasingly reported in different studies. In the first decade of 21st century the imipenem resistance rate of *P. aeruginosa* relatively was high (19%), but the rate rose further to reach more than 50 percent in some circumstances. In following studies performed on clinical isolates of *P. aeruginosa* in recent decades it has been shown that many of the imipenem-resistant isolates armed with genes produce MBL type enzymes [23]. According to the results of antibiogram test in this study it has been shown that 42(75%) of studied isolates were resistant to Imipenem disk and among them, 73 percent were confirmed as MBLs. Among the MBL isolates, *bla IMP-1* and *bla KHM* are the most prevalent (38%) genes where the presence of other introduced genes (*bla SPM, bla VIM, bla IMP-2*) in this study was rare and near negative. Based on the phenotypic results it has been deduced that genes other than introduced could involve and must be evaluated to have a complete algorithm frequency of related genes to MBLs among bacteria circulate in this centers. The important point in hydrolyzing enzymes especially metallo-β-lactamase is their potential to inactive Imipenem as the last assortment choice drugs for *Pseudomonas aeruginosa* infections. Based on increasing active strains in metallo-β-lactamase production in Ghotbeddin Shirazi burn center it can be deduced that treatment of such infected patients is more complicated and is a health treated factor for burned treatment victims. According to the elevation of not responder isolates to routine and choice antimicrobial agents in southwest of Iran burn center, it feels very important to find other mechanisms rather than hydrolyzing enzymes in dominant bacteria to find how they fight with the activity of drugs. Recently increasing in microorganisms resistant to the choice antimicrobial agents, is highlighted and is a big treat for control of infections especially in patients whose health is mainly related to infectious control. Analysis of the data collected by the contributing hospitals in 1998 and 2004 showed an alarming rise in resistant to the Imipenem as the choice drug used in *P. aeruginosa* infection control during recent years leading to 2009 [23]. *P. aeruginosa* is one of the organisms enrich in different mechanism that enable it to conquer the drugs and toxic compounds in their environment which are lethal for most bacteria [24]. According to the most specifications of *P. aeruginosa* in deal with antibacterial agents specially Imipenem the most new and recent introduced resistant mechanisms like metalo-β-lactamase, efflux pumps (exocrine mechanism) and inlet porin structure (inlet duct for drugs) have been evaluated. The results showed that isolates of *P. aeruginosa* in Ghotbeddin Shirazi hospital as the main center for burned victims in southwest of IRAN are armed to the most mechanisms of resistant. It seems likely that most of this multidrug resistance reflects the accumulation of multiple mutations, although this surmise remains to be confirmed by molecular studies, and although reports from other parts of the world document extreme multidrug resistance associated with acquired resistance genes. Previous studies in Iran confirmed resistance to many antibiotics used routinely for treatment of burn wounds infected by *P. aeruginosa*. Hadadi and colleagues showed that *P. aeruginosa* isolates were resistance to Ceftriaxime (99%), Ceftazidime (59.6%), Ticarcillin (50%), Ceftriaxone (44.3%), and Cefoperazone (37.5%) [25]. According to a survey conducted in Ghotbeddin Shirazi Burn hospital in 2006 by Japoni et al. almost all *P. aeruginosa* isolates from burn patients were resistant to all tested anti-Pseudomonal agents except Carbapenems (Meropenem and Imipenem), but in this study resistant to Imipenem and meropenem was increased to 65 percent and 75 percent respectively [26]. According to the different results due to isolates produce hydrolyzing enzymes and the resistant isolates other main mechanisms like efflux pumps and uptake systems were evaluated either. Multidrug efflux pumps particularly the RND-type Mex pumps, have been extensively investigated in *P. aeruginosa*, from the time when they discovered in early 1990s[27]. Studies with clinical isolates support the established role of the drug efflux pumps in multi-drug resistant isolates. Thus, efflux pump mechanisms are considered as a key factor in optimizing the treatment of *P. aeruginosa* infections [28]. In a study in France over expression of MexAB-OprM and MexXY-OprM occurred, respectively, in 11% and 35% isolates of bacteria which suggesting enhanced expression of the efflux systems in bacterial isolates. Efflux pump simultaneously present with other mechanism broaden the resistance profiles of isolates [29]. In this study it has been defined that 78.6% of carbapenem resistance isolates are positive for efflux pump activity where 21.4% of them was due to non-enzymatic mechanisms. Based on the findings of the present study, results clearly showed that carbapenem resistant *P. aeruginosa* isolates are armed with the production of MBLs and efflux pump systems (MexAB-OprM) expressing, simultaneously. In a study performed by Pirnay et al., in 2002, it has been showed that carbapenem resistance in *P. aeruginosa* mainly mediated by chromosomal β-lactamases like metalo-β-lactamases and drug efflux pumps (MexAB-OprM). But in their results another mechanism was introduced which was related to less or loss expression of *OprD* gene a porin protein related [30]. According to the results of recent study it has been found that different changes took place in size of *OprD* gene with deletion or addition or either in alignment of bases, but since these changes were seen in resistant isolates which have other mechanisms of resistant. it has not been deduced that exactly *OprD* changes have either enrolled in isolates resistant. The results of this study highlighted the need for very care-
ful interpretation of results obtained with clinical strains, to introduce new algorithm of drug use for different prevalent resistant mechanisms in isolates to be able conquer the bacterial circulate and control the infections specially in patients whose health is mainly dependent to infectious agents control. One of the most important ways to evaluate and introduce a treatment model in clinical centers is evaluate and predict the frequency and type of resistant that may arise during therapy which will generate more reliable data.

ACKNOWLEDGMENT

This study was financially supported by deputy chancellor of Shiraz University of medical sciences. The authors of this study respectively thanks from the Bacteriology and Virology department of Shiraz medical school, and Ghotbeddin Shirazi burn hospital both affiliated with Shiraz University of medical sciences for their kind's supports.

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest with this study.

REFERENCES

5. Sader HS, Reis AO, Silbert S, Gales AC. IMPs, VIMs and SPMs: the diversity of metallo-beta-lactamases produced by carbapenem-resistant Pseudomonas aeruginosa isolated from burn patients hospitalized in Shiraz Burn Hospital. Iran J Microbiol. 2015;7(1):7-11. PMID: 26644867