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Detection of ISPa1328 and ISPpu21, Two Novel Insertion Sequences in the OprD Porin and bla_{IMP-1} Gene Among Metallo-Beta-Lactamase-Producing *Pseudomonas aeruginosa* Isolated From Burn Patients

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Abstract

Background: Carbapenemes are a good choice for treatment of infections caused by multidrug resistant *Pseudomonads aeruginosa*. The emergence of carbapenem resistance has become a major problem in treatment of this organism especially among immunocompromised patients including burn patients.
Objectives: The aim of this study was to investigate carbapenem-resistance mechanisms among burn patients in Tehran, Iran, during 2014 - 2015.
Methods: The antibiotic resistance phenotypic test was accomplished by the Kirby Bauer disk diffusion method. The phenotypic investigation of metallo-beta-lactamase (MBL) producers was evaluated by the combined disk diffusion test (CDDT) method. The prevalence of MBL genes, including bla_{IMP-1} and bla_{VIM-1} was evaluated by polymerase chain reaction (PCR) and sequencing methods. Amplification of *opr*D was performed by PCR and the results of sequencing were aligned with wild-type *P. aeruginosa* strain PAO1.
Results: A total of 100 *P. aeruginosa* were investigated, of which, 95 were resistance to imipenem. Out Of 95 imipenem resistant isolates,, 81 (85.2%) were MBL producers. Among all isolates, 13 strains carried the bla_{IMP-1} gene, whereas all of the strains were negative for the bla_{VIM-1} gene. Amplification of OprD porin was performed for all 100 *P. aeruginosa* strains. Two insertion sequences (ISs) including ISPpu21 and ISPa1328 were detected in PCR products of OprD gene, that were larger than expected.
Conclusions: The prevalence of β-lactamase-producing isolates and their isolation from life-threatening infections in burn patients is increasing at an alarming rate worldwide. Also, we have identified two novel IS elements, ISPa1328 and ISPpu21, in *P. aeruginosa* isolates from hospitals in Tehran, Iran. In most of the isolates, insertional inactivation of *opr*D by ISPa1328 and ISPpu21 were associated with carbapenem resistance.

Keywords: *Pseudomonas aeruginosa*, Metallo-Beta-Lactamase, Insertion Sequences

1. Background

Burn patients are at risk of acquiring infection because of their damaged skin and impaired immune system. *Pseudomonas aeruginosa* is a prevalent cause of nosocomial infections around the world and, as an opportunistic pathogen, causes other infections such as septicemia, pneumonia, endocarditis, urinary tract infection, skin, ear and eye infections, as well as being a leading cause of morbidity and mortality among hospitalized burn patients (1, 2). Due to the intrinsic and acquired resistance mechanisms to various antimicrobial agents, there is a serious challenge for choosing the appropriate antibiotic for treating infections by *P. aeruginosa* (1). Currently, carbapenems are used for the treatment of antibiotic resistant *P. aeruginosa* infections by targeting cell wall through binding and inactivating penicillin-binding proteins (PBPs) (3, 4). Unfortunately, the emergence of carbapenem resistance among *P. aeruginosa* isolates has challenged the success of these antibiotics for therapeutic purposes (3). Carbapenem resistance in *P. aeruginosa* is associated with mutation in the *opr*D gene encoding the outer membrane porin (OprD) that leads to repression or inactivation of the *opr*D. The other mechanism of carbapenem resistance is insertional inactivation of oprD by insertion sequence (IS) elements, which subsequently increases the activity of multidrug efflux pumps such as MexAB-OprM. The two mentioned mechanisms are due to mutations in chromosomal

genes. Another known mechanism that may cause resistance are transferable elements which code for carbapenemases, especially IMP and VIM that belong to metallo-β lactamases and are responsible for the emergence of resistance to all β-lactams except for aztreonam (2, 5-7).

2. Objectives

The aim of this study was to investigate the mechanisms of carbapenem resistance in *P. aeruginosa* isolated from burn patients hospitalized in Shahid Motahari Hospital, Tehran, Iran, during 2014 - 2015.

3. Methods

3.1. Bacterial Identification

From march 2014 to march 2015, 100 nonduplicate nonconsecutive *P.aeruginosa* strains were isolated from burn patients hospitalized in Shahid Motahari Hospital (Tehran, Iran). Prior to sampling, the wounds were washed with isotonic saline. Samples were placed in Stuart's media, cultured on the blood agar and Mac-onkey agar (Merck Co) and incubated at 37°C for 24 hours. All isolates were identified by conventional biochemical methods (8) including the catalase test, oxidase test, reaction on Tripple Sugar Iron (TSI) medium, oxidation/fermentation of glucose using the oxidative-fermentative (OF) medium and growth ability at 42°C.

3.2. Antimicrobial Susceptibility Testing

An antimicrobial susceptibility test was performed by the Kirby Bauer disk diffusion method on the Muller Hinton agar based on clinical and laboratory standards institute (CLSI, 2013) guidelines. The antibiotic disks (Mast, UK) used during this study were imipenem (IPM: 10 μg), meropenem (MEM: 10 μg), doripenem (DOR: 10 μg), ceftazidime (CAZ: 30 μg), cefotaxime (CTX: 30μg), amikacin (AK: 30μg), ticarcillin (TIC: 75μg) , piperacillin (PRL: 100 μg), piperacillin/tazobactam (PTZ: 100/10 μg), ciprofloxacin (CIP: 5 μg), cefepime (FEP: 30 μg), aztreonam (ATM: 30 μg), gentamicin(GEN: 10 μg) and colistin (Co: 10 μg). *Pseudomonas aeruginosa* ATCC 27853 was used as the control strain.

3.3. Minimum Inhibitory Concentration

Strains resistant to imipenem, meropenem, ceftazidime and ciprofloxacin by the disk diffusion test were rechecked by the broth microdilution method according to the guidelines of the CLSI 2013 (CLSI). *P. aeruginosa* ATCC 27853 was used as the control strain.

3.4. Phenotypic Detection of Metallo-Beta-Lactamase

A combined disk diffusion test (CDDT) was performed for the identification of MBLs by imipenem and meropenem (Mast Group, Merseyside, UK) alone and in combination with EDTA. An inhibition zone diameter difference between the discs and discs + EDTA of $\geq$ 7 mm was interpreted as positive for the presence of an MBL (9). *P. aeruginosa* PA53 (ACCESSION: KM359726) was used as the control strain.

3.5. DNA Extraction

DNA was extracted using the DNA extraction kit (GeNet Bio Company, Korea, Cat. No. K-3000) according to the manufacturer's guidelines.

3.6. Detection of blaIMP-1 and blaVIM-1 Genes by Polymerase Chain Reaction

The $bla_{\text{IMP-1}}$, $bla_{\text{VIM-1}}$ and oprD genes were amplified by PCR, using the primers described in Table 1. Three μL of the extracted DNA (100 ng/μL) was added to a final volume of 25 μL PCR mixture containing 12.5 μL of 2$\times$ Master Mix (Sinaclon- Iran, CAT. NO.:PR901638), including 1$\times$ PCR buffer, 3mmol/L $MgCl_2$, 0.4mmol/L dNTP, and 0.08 IU *Taq* DNA polymerase,1 μL of 10 pmol/L from each primer and 7.5 μL of sterile distilled water.

The amplification program was set at 36 cycles of denaturation at 94°C for 45 seconds, annealing at 47°C to 54°C, according to the primers (Table 1), for 45 seconds and elongation at 72°C for 45 seconds. The amplified products were visualized after electrophoresis in 1% agarose gels at 95 V for 45 minutes in 1X TBE containing ethidium bromide under UV irradiation. *P. aeruginosa* PA53 (ACCESSION: KM359726) for IMP-1, *P.aeruginosa* Psa1 (ACCESSION: KT313641) for VIM-1 gene and *P.aeruginosa* PAO1 for *opr*D gene were used as the control strains.

3.7. Sequencing

The PCR purification kit (Bioneer Co., Korea) was used to purify PCR products. Purified PCR products were used as templates and sent for sequencing analysis at Bioneer Company, Korea. Sequencing of both strands of the oprD PCR products necessitated two additional internal primers including oprDF2 and oprDR2 (Table 1). The nucleotide sequences were analyzed with the Chromas 1.45 software and BLAST in NCBI.

3.8. Statistical Analysis

The statistical analysis was performed with MINITAB16.

Table 1. Primers Used for Polymerase Chain Reaction and Sequencing

Target Gene	Sequence of Primers (5 to 3)	Product Size (bp)	Annealing Temperature
VIM1-F	GATGGTGTTTGGTCGCATA	390	54°C
VIM1-R	CGAATGCGCAGCACCAG		
IMP1-F	GAAGGCGTTTATGTTCATAC	587	47°C
IMP1-R	GTAAGTTTCAAGAGTGATGC		
OprDF1	ATGAAAGTGATGAAGTGGAG	1329	50°C
OprDR1	CAGGATCGACAGCGGATAGT		
OprDF2	AACCTCAGCGCCTCCCT		Primers for Sequencing
OprDR2	AGGGAGGCGCTGAGGAT		

4. Results

A total of 100 *P. aeruginosa* strains were isolated from burn patients. Twenty-six strains (26%) were isolated from females and 74 (74%) from males.The average age of patients was from 2 to 72 years. (distribution shown in Figure 1).

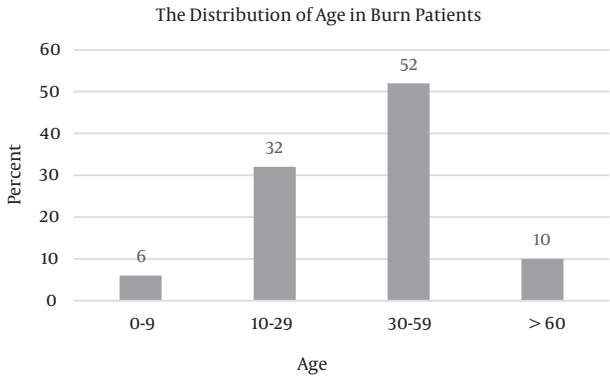


Figure 1. The Distributions of Age in Burn Patients

The resistance rate of the 100 *P. aeruginosa* isolates according to the Kirby-Bauer method was as follows: 95 (95%) to imipenem, 95 (95%) to meropenem, 94 (94%) to doripenem, 75 (75%) to ceftazidime, 93 (93%) to cefepime, 94 (94%) to ciprofloxacin, 91 (91%) to amikacin, 90 (90%) to aztreonam, 98 (98%) to ticarcillin, 90 (90%) to piperacillin, 82 (82%) to piperacillin/tazobactam, 95 (95%) to gentamicin and 0 (0%) to colistin.

The results of the MIC test for imipenem, meropenem, ceftazidime and ciprofloxacin on *P. aeruginosa* isolates are shown in Table 2.

Using the combination disk diffusion test method, it was found that among 95 imipenem nonsusceptible *P.aeruginosa* strains 81 (%85.2) were MBL producers (Figure 2).

The prevalence of the $bla_{\text{IMP-1}}$ gene among MBL-producing *P. aeruginosa* isolates was 13 of 81 (16.04%),

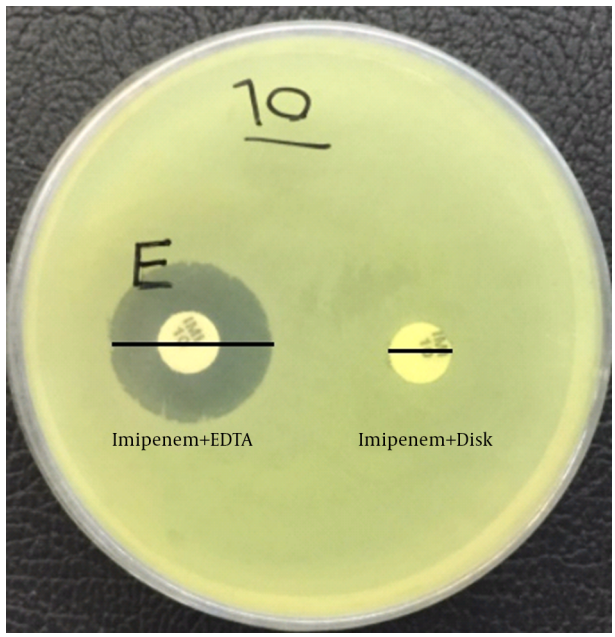


Figure 2. Combined Disk Test Using Imipenem and Imipenem + EDTA. Imipenem + EDTA Disk (on the Left) Produced $\geq$ 7 mm Larger Zone of Inhibition Than the Imipenem Disk (on the Right)

while the $bla_{\text{VIM-1}}$ gene was not detected (Figure 3). The nucleotide sequences data reported in this paper have been submitted to the GenBank sequence database and assigned the accession number KT313640 for the $bla_{\text{IMP-1}}$ gene.

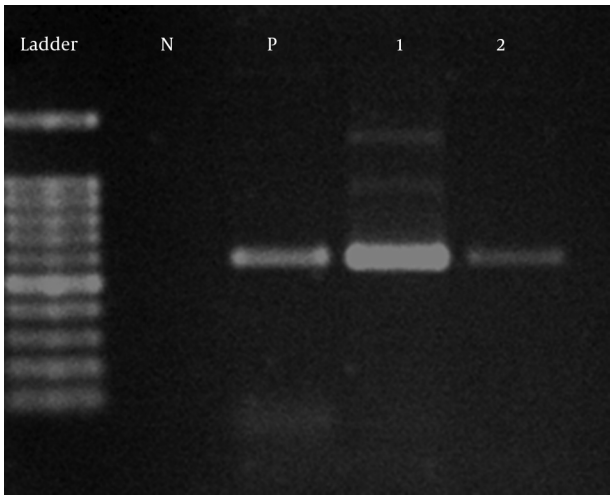
Amplification of the *opr*D porin was performed for all 100 *P. aeruginosa* isolates. Surprisingly, 22 isolates had larger PCR products than expected. The *opr*D gene was not detected in 9 isolates. Sequencing results and alignment of PCR products of *opr*D compared with *Pseudomonas aeruginosa* PAO1 revealed that these large inserts corresponded to ISPpu21 (accession number: KT728193) and ISPa1328 (accession number:KT736319) (Figures 4 and 5).

The nucleotide sequence data reported in this study

Table 2. The Result of MIC Test for Imipenem, Meropenem, Ceftazidime and Ciprofloxacin on *P. aeruginosa* Isolates

Antibiotics	MIC(μg/mL)		
	Resistance	Intermediate	Sensitive
Imipenem	90 (90%)	5 (5%)	5 (5%)
Meropenem	90 (90%)	5 (5%)	5 (5%)
Ceftazidime	72 (72%)	3 (3%)	25 (25%)
Ciprofloxacin	96 (96%)	1 (1%)	3 (3%)

Figure 3. PCR Products of bla_{IMP-1} Producing Strains of *P. aeruginosa* Isolates



N: control negative, P: control positive, 1,2 positive isolates

were submitted to the GenBank sequence database and assigned under the accession number KT313640 for the bla_{IMP-1} gene.

5. Discussion

Pseudomonas aeruginosa is one of the most prevalent causes of nosocomial infections and its resistance to to antibiotics is increasing among strains isolated from burn patients (10, 11). According to the epidemiological research, which has been accomplished around the world, the prevalence of drug resistance especially MBL-coding genes among *P. aeruginosa* strains has been increased among different countries, regions and even hospitals which are located in various geographical regions. Due to the clinical significance of MBL- producing organisms, isolation of such strains from a patient should be carefully managed (12). The results of this study showed that among all antibiotics, the highest resistance rate was for the following antibiotics: 98% to ticarcillin, 95% to imipenem, meropenem, gentamicin and 94% to doripenem and ciprofloxacin. All the isolates were susceptible to colistin. So, in our study colistin was the most effective antibiotic for treatment of *P. aeruginosa* infections. Two previous studies conducted by Fallah et al. (10) and Shahcheraghi et al. (13) are similar to our study, which showed resistance against various antibiotics such as beta-lactams (including the 3rd generation of cephalosporins and carbapenemas), aminoglycosides and fluoroquinolons. Saffari et al. reported the imipenem resistance rate of 58.7% among *P. aeruginosa* isolates in Ahwaz, which was lower than what we have found in our study (14). However, Radan et al. study, in Isfahan, was in agreement with our results: 96% of *P. aeruginosa* strains which were isolated from hospitalized patients at the burn unit showed resistance to imipenem. Another report, in contrast with our study, found a resistance rate of 21% to imipenem among *P. aeruginosa* isolates from burn patients in Kurdistan (15). Different studies showed increased resistance of *P. aeruginosa* to different antibiotics that are caused by indiscriminate and inappropriate use of antibiotics. This antibiotic resistance can be controlled by appropriate antibiotic prescriptions, including low-resistance-potential antibiotics (14). Production of MBLs is one of the carbapenem resistance mechanisms among *P. aeruginosa* strains (15). In the current study, 85.2% of the isolates were positive for MBL production, which was higher than other studies conducted by Fallah et al. with 58.2% and Hakemi et al. with 17.3% MBL producer isolates (9, 16). In Kurdistan and Ahwaz, 22% and 19.5% of *P. aeruginosa* strains isolated from burn patients were identified as MBL-producing isolates (14). Another study performed by Sadrei et al. indicated a higher incidence of MBLs than our study (17). All phenotypic results were rechecked by molecular methods. According to the PCR results among MBL producing isolates, 13 (16.04%) isolates were positive for the bla_{IMP-1} gene; however, the presence of this gene has been previously proved to be variable in different regions and the bla_{VIM-1} gene has not been detected in *P. aeruginosa*. The result of bla_{VIM-1} in this study is similar to those of two other studies from Iran that were individually performed by Fallah et al. (9) and Hakemi et al. in 2012 (16) and the incidence of bla_{IMP-1} in the mentioned studies was lower than ours and also are in contrast to those of two other stud-

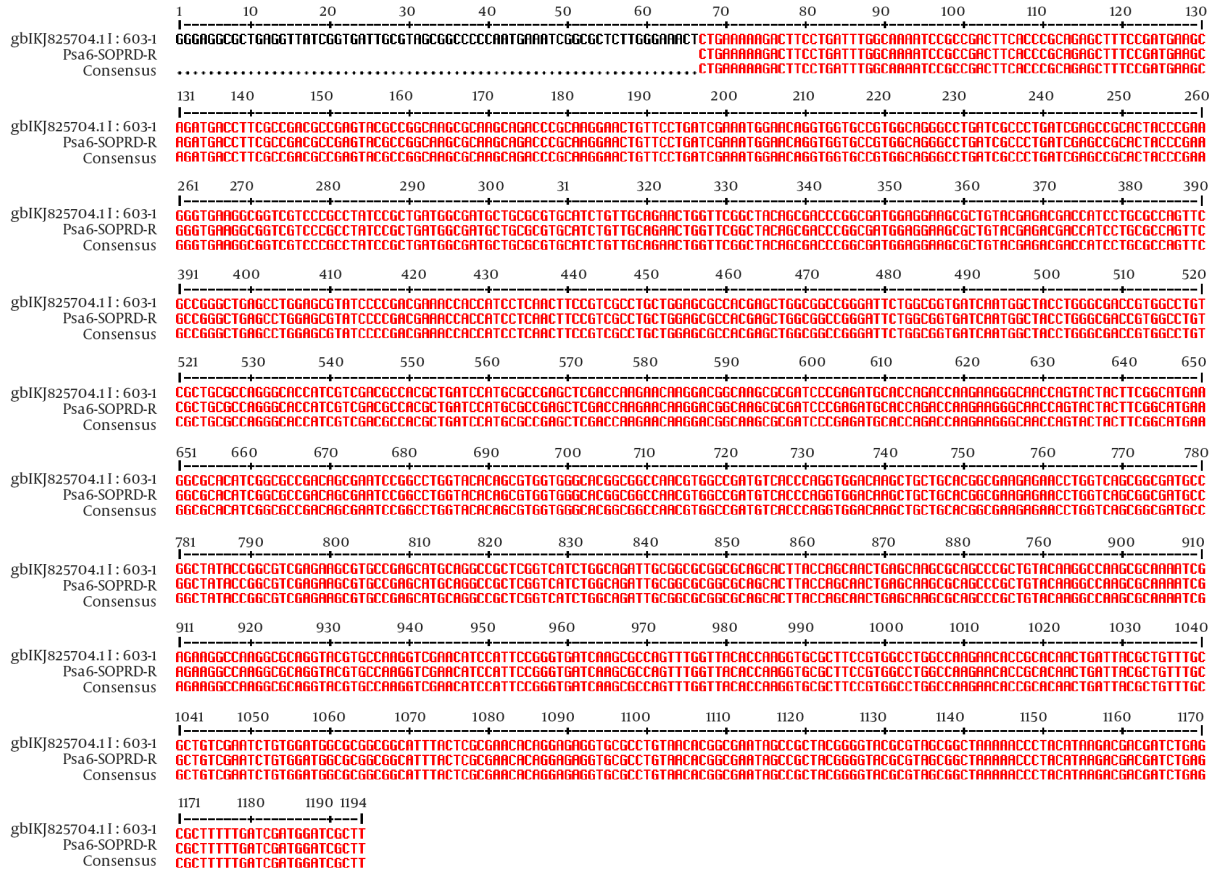


Figure 4. Sequence of the ISPpu21 Located in the *opr*D Gene

ies from Iran: in Tehran 11.43% of the isolated *P.aeruginosa* strains and in Ahwaz 19.51% of *P.aeruginosa* isolates were reported to have the bla_{VIM} gene (16). Also, Saderi et al. reported that 94% of *P.aeruginosa* isolates from Tehran were identified as MBL producers and carried the bla_{VIM-2} gene (17). In another study Ghamgosha et al. detected bla_{VIM-1} gene, while none of them were positive for the bla_{IMP-1} gene (18). Also, Radan et al. reported that all of the imipenem-resistant *P. aeruginosa* isolates were MBL-positive, and 107 out of 144 (74.3%) of the MBL isolates were positive for the bla_{IMP} gene (15). So, these data are in contrast to ours and this may be related to differences in the time of the studies and consequently to changes in antibiotics prescriptions and antibiotic resistance patterns. These results indicated, participation of other factors such as other MBL- encoding genes, lack of *opr*D (which results in membrane permeability change), the over expression of efflux pumps or chromosomal AmpC beta-lactamase. In the current study, amplification of *opr*D showed the presence of the larger fragments of the *opr*D gene in some isolates, which were associated with ISPpu21 and ISPa1328 insertion sequences. In Al-Bayssari et al. (19) study, ISPa1328 and in Estepa et al. study ISPpu21 insertion sequence was detected in the *opr*D gene, which was in accordance with our study (20, 19). Earlier studies conducted by Diene et al. in 2013 have demonstrated the oprD gene disrupted by ISpa46 insertion sequences (21). Other IS elements disrupting the oprD gene have been detected in different regions such as Spain (ISPa133) and the United States (ISPa8) (12, 22). These results demonstrate that insertional inactivation of *opr*D can cause carbapenem resistance among *P.aeruginosa* strains (22). In spite of many unclear reasons about various studies, this discrepancy may be related to differences in time of the studies, geographical regions, kind of infections, antibiotic therapy regimens or the kinds of primers, which have been used in the study.

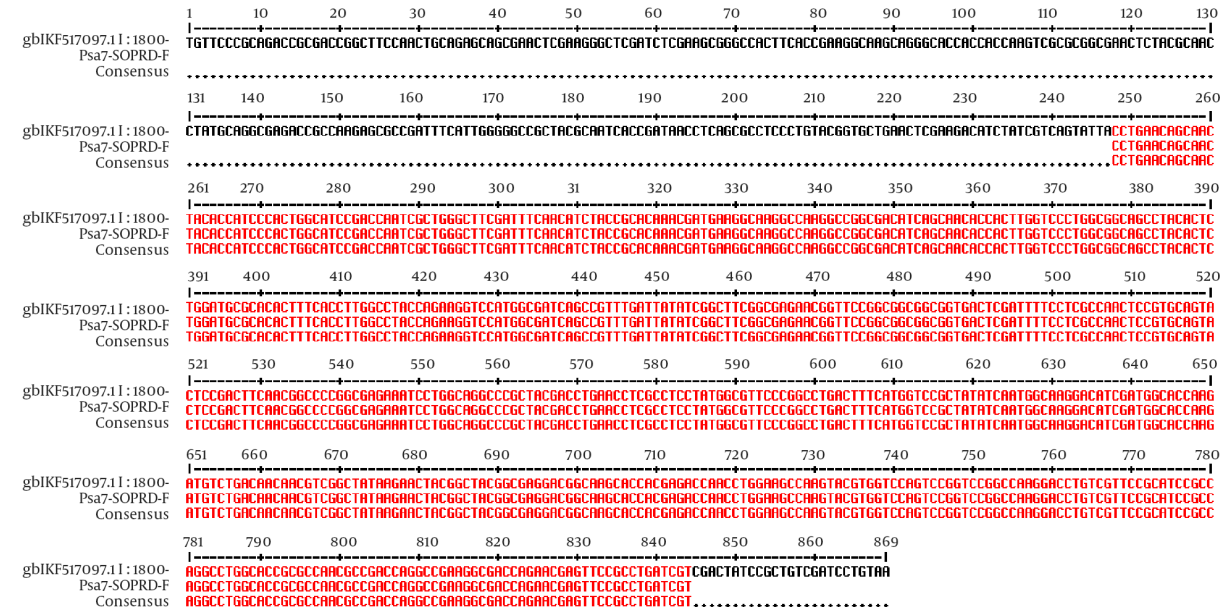


Figure 5. Sequence of the ISPa1328 Located in the *opr*D Gene

5.1. Conclusion

The prevalence of β-lactamase-producing isolates and their isolation from life-threatening infections is increasing at an alarming rate worldwide. Intense pressure for the use of antimicrobial drugs in patients, results in eradication of normal flora and may be a situation of MDR isolates substitution. It was shown in this study that β-lactamase producing *P.aeruginosa* strains are an emerging threat and should be supervised by implementation of timely identification and strict isolation methods that will help to reduce their severe outcomes and mortality rate in these patients. Also, we have identified two novel IS elements, ISPa1328 and ISPpu21, in *P. aeruginosa* isolates from hospitals in Tehran, Iran. In most of the isolates, insertional inactivation of *opr*D by ISPa1328 and ISPpu21 was associated with carbapenem resistance.

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