

Investigation of the relationship between class-1 integrons and per-1 enzyme in ceftazidime resistant *Pseudomonas aeruginosa*.

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Abstract

Pseudomonas aeruginosa strains, especially multidrug resistant, have great of importance among nosocomial infection isolates. Production of beta lactamase is an important mechanism in gram-negative bacteria for resistance to beta-lactam antibiotics. PER-1 enzyme is derived from an extended spectrum beta-lactamase that is non-TEM, non-SHV-derived in class A and especially causes ceftazidime resistance. In this study, our aim was to investigate the relationship between CLASS-1 integrons and PER-1 enzyme in ceftazidime-resistance *Pseudomonas aeruginosa*. The PER-1 type beta lactamase enzyme that causes ceftazidime-resistance, determines the frequency, and detects the relationship between the enzyme and CLASS-1 integrons by PCR in 100 (one hundred) ceftazidime-resistant *Pseudomonas aeruginosa* (*P. aeruginosa*) isolated at Ondokuz Mayıs University Hospital (Turkey) between 2007 and 2008. In this study, *blaPER-1* was detected in 40% (40/100) of the isolates. Four principal clones, which were detected in *P. aeruginosa* strains were responsible for high prevalence using Random Amplified Polymorphic DNA-PCR (RAPD-PCR) methods. CLASS-1 integron was detected in 62.5% (25/40) of the PER-1 enzyme bearing strains and association between *blaPER-1* and CLASS-1 integrons were shown in 2 (two) *Pseudomonas aeruginosa* strain. Also resistance rates of PRL antibiotic in *blaPER-1* negative group was found to be significantly higher against *blaPER-1* positive group, resistance rates of other antibiotics were no different between these two groups. We concluded that PER-1 enzyme is common in our hospital and their clonal diversity indicates horizontal dissemination, the association between *bla PER-1* and CLASS-1 integrons can accelerate dissemination of this gene.

Keywords: Beta-lactamase, PER-1, *Pseudomonas aeruginosa*, Class-1 integron.

Accepted on November 09, 2016

Introduction

Pseudomonas aeruginosa is one of the most important nosocomial pathogens that causes nosocomial infections and frequently leads to mortality and morbidity [1,2]. *P. aeruginosa* infections are often difficult or impossible to eradicate. To some extent, this is due to high-level resistance to many antimicrobial and disinfectants as a result of both intrinsic and acquired mechanisms [3,4]. Several mechanisms can contribute to acquire beta-lactam resistance in *P. aeruginosa*, including beta-lactamase production, the upregulation of efflux systems, and decreased outer membrane permeability [5,6]. Beta-lactamases are the most common cause of bacterial resistance to beta-lactam antimicrobial agents [2]. Among the acquired enzymes, PER-1 is class A extended-spectrum beta-lactamase (ESBL) of notable clinical importance due to its high level of activity especially causes ceftazidime and to the broad diffusion achieved by it in some epidemiological settings [5,7-9]. Various classes of ESBLs (A, B and D) have been

found recently in *P. aeruginosa*. Five types of class A ESBLs (PER, VEB, GES, IBC, TEM and SHV) were recently reported in *P. aeruginosa*; however, these were found in limited regions [10]. The PSE, OXA and PER types are the most common ESBLs found in *P. aeruginosa* isolates [10-13]. The PER-1 and VEB-1 types belong to class A enzymes and relate to a high level of resistance to cepheems, monobactams and ceftazidime [12-14].

The resistance genes such as *blaPER-1* can be transferred among bacteria by mobile genetic elements, such as integrons and even ESBL located on integron and facilitate these elements [15-17]. Integrons structure contains essential elements for insertion and mobilization of gene cassettes generally possess antimicrobial resistance genes [18,19]. In particular, much research effort has been devoted to integrons belonging to classes 1, 2 and 3, the classes most commonly associated with the spread of antibiotic resistance in pathogens [20]. Class 1 integron is composed of a 5'- conserved segment (5'CS) including the integrase gene *intI* and *attI* genes and Pant

promoter and 3'-conserved segment (3'CS) [21,22]. Class-1 integrons, which are most commonly found in antimicrobial-resistance clinical isolates, possess two conserved segments located on either side of the integrated genes [16-23] and have a threatening potential for the development of antimicrobial resistance and the emergence of MDR profiles in clinical bacteria, gram negative bacteria as well as *P. aeruginosa* [18]. To date the first report to analyse *blaPER-1* has been shown to be related with these genetic determinants [6-15]. The objective of this study was to determine the PER-1 type beta-lactamase enzyme that causes ceftazidime-resistance *P. aeruginosa* using molecular methods. Then, we would be able to determine the frequency and detect the relationship between the enzyme and class-1 integrons.

Materials and Methods

Clinical isolates

One hundred randomly selected strains among one hundred seventy-nine (N=179) strains of *P. aeruginosa* were analysed in this study. These strains were nonreplicate clinical isolates from hospitalized patients in Ondokuz Mayıs University Hospital (Turkey) during the period 2007 and 2008. All isolates were resistant to ceftazidime. *P. aeruginosa* ATCC 27853 was used as the reference strain for Kirby-Bauer disk diffusion method.

Susceptibility testing and screening for ESBL-producing strains

In vitro susceptibility was determined by a Kirby-Bauer disk diffusion method on Mueller-Hinton (MH) Agar (Merck, Darmstadt, Germany) plates as recommended by the "Clinical and Laboratory Standards Institute" (CLSI) [24]. The results of susceptibility testing were interpreted according to the criteria of the CLSI. Antimicrobial agents were purchased from Oxoid (Chemical Co, Hampshire, England). The double-disk synergy test (DDST) was performed with cefotaxime, ceftazidime, aztreonam, and amoxicillin/clavulanic acid disks on Mueller-Hilton agar plates, and the results were interpreted as described previously [25].

Determination of ceftazidime MIC values

Ceftazidime MIC values of the *blaPER-1* positive strains were determined by microdilution method according to CLSI criteria.

Detection of blaPER-1

Total DNA was extracted from the *P. aeruginosa* isolates as described previously by phenol chloroform method [26]. Primers (Tib Molbiol, Berlin, Germany) were PERA, 5'-ATG AAT GTC ATT ATA AAAGC-3', and PERD, 5'- AAT TTG GGC TTA GGG CAGAA-3', yielding a 926-bp product. *blaPER-1* was sought among the isolates by PCR assay according to [1]. Amplification reactions were performed in 50 μ L including 2 μ L of total DNA (50 ng) as template, 2.5 mM

of $MgCl_2$, 2 mM dNTP, 5U of Hot Start Taq DNA polymerase (Fermentas, Vilnius, Lithuania), and 10 pmol of primers. PCR conditions were 5 min at 94°C, followed by 35 cycles of 45 sec at 95°C, 45 sec at 56°C and 1 min at 72°C, followed by a final extension for 7 min at 72°C. The MultiBlock PCR System (Thermo, CA, USA) was used for gene amplification. Electrophoresis was carried out in conventional 1X Tris-Borate-EDTA (TBE) buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH: 8.4) with 1% agarose. 1 kb DNA ladder mix (Fermentas, Vilnius, Lithuania) was used to provide molecular size markers. PCR products were loaded onto 2% agarose gel containing 1X TBE and stained with 5 μ g/ml ethidium bromide (Sigma, CA, USA). A 100 bp DNA Ladder Plus (Fermentas, Vilnius, Lithuania) was used to provide molecular size markers, and photographed using a Gel/ChemiDoc XRS system (Bio-Rad Laboratories, CA, USA).

Molecular fingerprinting

The clonal relatedness of the PER-1 positive isolates was investigated by RAPD-PCR analysis with the ERIC-2 primer (Tib Molbiol, Berlin, Germany) 5'-AAG TAA GTG ACT GGG GTG AGC G-3' [3] and an amplification protocol: 1 min at 95°C, followed by 30 cycles of 1 min at 94°C, 1 min at 40°C, and 1 min at 72°C, followed by a final extension for 5 min at 72°C. PCR products were loaded onto 2% agarose gel containing 1X TBE and stained with 5 μ g/ml ethidium bromide (Sigma, CA, USA) observed using a Gel/ChemiDoc XRS system. 1 kb DNA ladder mix (Fermentas, Vilnius, Lithuania) was used to provide molecular size markers. To evaluate similarity between the strains, Jaccard coefficients [$SJ = nAB \times 1 / (nAB + a + b)$] were calculated according to the banding patterns (for symbol explanation and further details see Soll 2000). When the calculated SJ was <0.8, two isolates were accepted as "clonally distinct". Isolates were classified as "clonally related" when the calculated value was 0.8 SJ<1.0; when it was 1.0, the isolates were considered as "identical".

Integron PCR

PCR was performed in a final volume of 50 μ L containing 5 μ L of total DNA (50 ng) as template, 3 mM of $MgCl_2$, 200 μ M dNTP, 5U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania), and 100 pmol of 3' -CS 5'- AAG CAG ACT TGA CCT GA-3' primer and 5'-CS 5'- GGC ATC CAA GCA GCA AG-3' primer (Tib Molbiol, Berlin, Germany). Amplification reactions consisted of denaturation at 95°C for 10 min followed by 30 cycles of denaturation at 94°C/1 min, annealing at 55°C/1 min and elongation at 72°C/5 min, followed by a final extension for 4 min at 72°C. Amplification products were analysed using 1% agarose gel electrophoresis (1X TBE buffer at 900 mA), stained with ethidium bromide solution and visualized using a Gel/ChemiDoc XRS system. 1 kb DNA ladder mix was used to provide molecular size markers.

Investigation of blaPER-1 location in class-1 integrons

Integron location of *blaPER-1* was investigated by PCR. A combination of primer 5'-CS or 3'-CS and a primer (PERA,

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PERD) specific for the *blaPER-1* gene were also used to identify the location of *blaPER-1* in the integrin [19]. The extension step was increased to 6 min, and the amount of Taq DNA polymerase was increased to 6 units per reaction mixture to amplify large DNA fragments [15,27,28].

Statistical analyses

Representatives of each RAPD-PCR pattern were used in integrin-PCR experiments. Dendrograms were formed using Jaccard coefficients and dendrogram's Biomerics version 5.10 with UPGMA (Unweighted pair group method with mathematical averaging) agglomerative hierarchical cluster method for PER-1 producing *P. aeruginosa* strains. Phylogenetic analysis program PAUP (ver. 4.0b10) was also used for implementing the UPGMA method. Pearson chi-square test was used for comparison of PER-1 enzyme including (*blaPER-1*) and non-including groups about in terms of observed resistance rates of aztreonam, cefepime, cefotaxime, piperacillin, piperacillin-tazobactam, imipenem and meropenem. $p < 0.05$ was taken for statistical level of significance and PASW (SPSS, ver. 18) was used for the calculation of descriptive statistics and making chi-square tests.

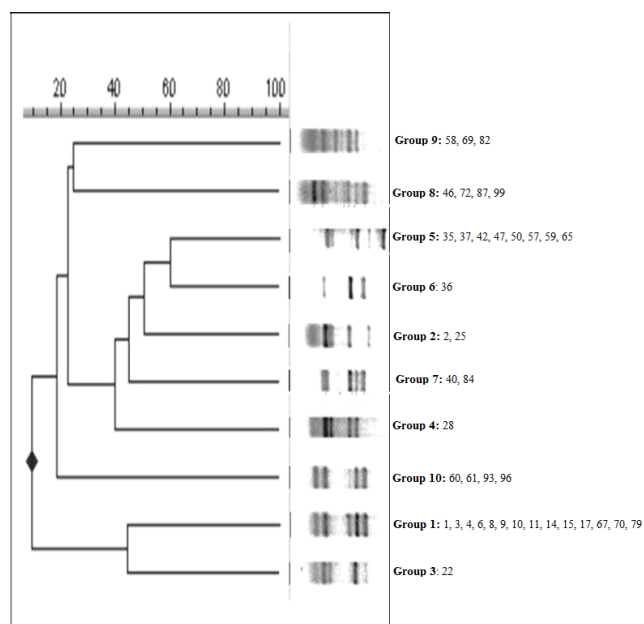


Figure 1. Presentation of clonal relatedness of *P. aeruginosa* isolates by dendrogram of RAPD-PCR.

Results

A total of 179 nonrepetitive ceftazidime-resistant *P. aeruginosa* isolates were collected from hospitalized patients in Ondokuz Mayıs University Hospital (Turkey) between 2007 and 2008. Ceftazidime resistance accounted for 27% (179/641) of all the *P. aeruginosa* isolates. According to the disk diffusion susceptibility test results, the resistance rates of *P. aeruginosa* isolates to aztreonam, cefepime, cefotaxime, piperacillin, piperacillin-tazobactam, imipenem and meropenem was found 100% (100/100), 88% (88/100), 100% (100/100), 67% (67/100), 43% (43/100), 75% (75/100) and 70% (70/100)

respectively. Multiple antimicrobial resistance phenotypes according to *blaPER-1* occurrences are shown in (Table 1). In this study, the percentage of PER-1 of ceftazidime resistant *P. aeruginosa* strains was found 40% (40/100) similar to other studies. Difference between *blaPER-1* positive and negative strains in terms of resistance ratio of seven antibiotics were compared and only resistance rate of PRL antibiotic significantly higher than any other *blaPER-1* negative group ($p=0.014$). This result was not statistically significant.

Twenty out of the 40 PER-1 producing *P. aeruginosa* isolates were found positive for ESBL by double disk diffusion method. DDST standardize were not done for *Pseudomonas* and ESBL conjectural only the interpretation can be done. The tested isolates were divided into 10 groups according to their banding patterns by RAPD-PCR (Figure 1). Group 1 (n=14), Group 5 (n=8), Group 8 (n=4), Group 10 (n=4) major clones, as well as several strains containing the sub-groups were determined by RAPD-PCR. We showed that four principal clones that were detected in *P. aeruginosa* strains were responsible for high prevalence by RAPD-PCR results. In our data, it has been detected that 22 of *P. aeruginosa* strains (55%) attended two different patterns and these main patterns included strains especially that isolated from urine and endotracheal aspirates from patients admitted in the urology department and adult intensive care unit. Group 1 (n=14) were isolated from urine (n=9), catheter (n=1), wound (n=1), serebro spinal fluid (n=1), tracheal aspirate (n=2), taken from urology and intensive care wards. Group 5 (n=8) were isolated from urine (n=3), tracheal aspirate (n=3), wound (n=1), sputum (n=1) taken from urology and intensive care wards. Group 2, 3, 4, 6, 7 and 9 consisted of 10 isolates were from various wards and specimens. Thirty nine (45.3%) isolates were detected as multi-drug resistant. PCR results showed that 37 (43%) of *P. aeruginosa* isolates harbored the class 1 integron and of these 27 (69.2%) isolates were multidrug-resistant.

CLASS-1 integron was detected in 62.5% (25/40) of the PER-1 enzyme bearing strains. *P. aeruginosa* isolates showed 3-4 banding patterns between 500-4000 bp in length. This result indicated that there were more than one class-1 integron according to number and size. Therefore, we evaluated them in three groups A, B and C in terms of band pattern. PER-1 producing *P. aeruginosa* strain's ceftazidim MIC values were ≥ 64 . Ceftazidim MIC values were different in integron (+) and (-) isolates, MIC values of integron positive isolates were higher (Table 2). The relationship between *blaPER-1* and CLASS-1 integron was also examined with PCR. We obtained PCR product more than 1 kb in three *P. aeruginosa* isolates. These three isolates were used as a template for PCR by using PERA and PERD primers to understand whether they consist of PER-1 gene or not. We obtained PER-1 specific PCR product (900 bp) in only two isolates. We concluded that PER-1 enzyme is common in our hospital and clonal diversity indicates horizontal dissemination, the association between *blaPER-1* and CLASS-1 integrons can accelerate dissemination of this gene. In the past years there were many studies that showed PER-1 enzyme was carried by transposons and plasmids [28,29] but at the present day there was only one

study that demonstrated PER-1 enzyme carried by CLASS-1 integron [15].

Table 1. Antibiotic resistance phenotypes of *bla*PER-1, positive and negative isolates.

Antimicrobial agent	blaPER (+) resistance rate% (n=40)		blaPER (-) resistance rate% (n=60)		χ^2 with 1 df	P
	Number	%	Number	%		
TZP	17	42.5	27	45	0.061	0.805
FEP	38	95	54	90	0.815	0.367
PRL	16	40	39	65	6.06	0.014
CTX	40	100	60	100	0	1
MEM	24	60	35	58.5	0.028	0.868
ATM	40	100	60	100	0	1
IPM	28	70	41	68.3	0.031	0.86

TZP: piperacillin-tazobactam; FEP: cefepime; PRL: piperacillin; CTX: cefotaxime; MEM: meropenem; ATM: aztreonam; IPM: imipenem

Table 2. RAPD-PCR and integron patterns of *bla*PER-1 (+) *P. aeruginosa* isolates.

Number of isolates	RAPD profile	Service	Specimen	Integron pattern	Ceftazidim MIC values
1	R1	Urology	Urine	A	1024-R
3	R1	Urology	Urine	A	512-R
4	R1	Urology	Wound	A	1024-R
6	R1	Urology	Urine	A	128-R
8	R1	Intensive care	CSF	A	128-R
9	R1	Urology	Urine	A	256-R
10	R1	Intensive care	Catheter	A	512-R
11	R1	Urology	Urine	A	128-R
14	R1	Intensive care	Tracheal aspirate	A	512-R
15	R1	Urology	Urine	A	512-R
17	R1	Intensive care	Urine	A	256-R
67	R1	Urology	Urine	A	64-R
70	R1	Urology	Urine	A	512-R
79	R1	Urology	Urine	A	128-R
25	R2	Intensive care	Pleural fluid	Negative	64-R
2	R2	Internal medicine	Urine	Negative	64-R
22	R3	Intensive care	Tracheal aspirate	Negative	128-R
28	R4	Urology	Urine	Negative	64-R
35	R5	Urology	Urine	B	64-R
42	R5	Intensive care	Tracheal aspirate	B	128-R
37	R5	Cardiovascular surgery	Wound	B	512-R
47	R5	Urology	Urine	B	128-R
50	R5	Neurology	Sputum	B	256-R

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57	R5	Urology	Urine	B	512-R
59	R5	Intensive care	Tracheal aspirate	B	256-R
65	R5	Intensive care	Tracheal aspirate	B	256-R
36	R6	Cardiovascular surgery	Wound	Negative	64-R
40	R7	Pediatrics	Wound	Negative	128-R
84	R7	Neurosurgery	Urine	Negative	128-R
46	R8	Internal medicine	Urine	C	256-R
72	R8	Urology	Urine	C	128-R
87	R8	Pediatrics	Tracheal aspirate	C	256-R
99	R8	Urology	Urine	C	256-R
58	R9	Pediatrics	Tracheal aspirate	Negative	64-R
69	R9	Internal medicine	Sputum	Negative	64-R
82	R9	Pediatrics	Urine	Negative	128-R
60	R10	Neurosurgery	Urine	Negative	128-R
61	R10	Neurosurgery	Urine	Negative	64-R
93	R10	Neurosurgery	Tracheal aspirate	Negative	64-R
96	R10	Neurosurgery	Tracheal aspirate	Negative	256-R

It has been shown that the *P. aeruginosa* strains that have different RAPD-PCR patterns have different integrons. We detected that in the two strains that were group representative *blaPER-1* have relationship with CLASS-1 integrons. It would be necessary to support this relationship with DNA sequence analyses.

Discussion

The aim of this study was to determine the PER-1 type beta-lactamase enzyme that causes ceftazidime-resistance *P. aeruginosa* using molecular methods. Then, we would be able to determine the frequency and detect the relationship between the enzyme and class-1 integrons. Ceftazidime-resistance rates of 18-36% have been reported previously in *P. aeruginosa* isolates from hospitalized patients in Turkey [30-32]. Resistance to piperacillin, piperacillin-tazobactam, and other major antibiotics was found to be extremely high, which is similar to previous studies in Turkey [30-32]. Kolaylı et al. [32] reported the PER-1 presence in 55.4% of ceftazidime-resistant *P. aeruginosa* strains, isolated in ICUs of seven university hospitals. Moreover, 86% ceftazidime-resistant ICU (Turkey) isolated strains of *P. aeruginosa* were found to be PER-1 positive [15-32] and found that the percentage of PER-1 of ceftazidime resistant *P. aeruginosa* strains was 46.2%. In addition, [33] also reported that among the 67 ESBL-positive strains, 50 (74.62%), 33 (49.25%) and 21 (31.34%) were positive for OXA-10, PER-1 and VEB-1 genes, respectively, indicating high-level resistance to penicillins, CAZ and CTX.

We also performed double disk synergy test but this test fail to detect strains that produce PER-1 enzymes [30,34]. Therefore,

we also carried out PCR to confirm the detection of *P. aeruginosa* producing PER-1 enzymes. *P. aeruginosa* strains that have different RAPD-PCR patterns have *blaPER-1* and it has been horizontally disseminated. Banding patterns were evaluated in three groups as A, B, C. PER-1 producing *P. aeruginosa* strains ceftazidim MIC values were ≥ 64 . Ceftazidim MIC values were different in integron (+) and (-) isolates. MIC values of integron positive isolates were higher. These results were similar to other studies which investigated presence and frequency of integrons [23,35,36]. In the past years there were many studies that showed PER-1 enzyme was carried by transposons and plasmids [28,29] and one of them demonstrated PER-1 enzyme carried by CLASS-1 integron [15]. A significant correlation was obtained between the presence of integrons and resistance against imipenem, ceftazidime, piperacillin and ciprofloxacin [18]. CLASS-1 integrons were detected in 27.3% of the multi-drug resistant *P. aeruginosa* isolates. Their results suggested the relationship between CLASS-1 integron and antibiotics resistance [37].

In another study by Martinez et al. [20], they studied with environmental and clinical isolates of *P. aeruginosa* to identify presence and spread of class 1 integrons and even characterise their variable regions. For this purpose, they examined a total of 76 isolates and PCR was used for integron detection. PCR detected class 1 integrons in 26 of the 56 clinical isolates. Similar to our study, they also searched 3'CS and 5'CS sequences. Iraj et al. investigated frequency of class 1 integrons between multidrug resistance and non MDR strains from burn wound samples by using PCR [18]. They concluded that 43% had class 1 integrons. The presence of integrons can be effective in antibiotic resistance of *Pseudomonas*

aeruginosa cases. To support this opinion, Kouchaksaraei et al. showed that the resistance of integron positive strains to gentamicin, ticarcillin, ciprofloxacin, imipenem, amikacin, ceftriaxone, ofloxacin, ceftazidime and cefepime antibiotics was 81.9%, 77.3%, 54.5%, 81.1%, 63.6%, 77.3%, 72.7%, 100% and 100% respectively as a result of PCR analyses [38]. In addition, they concluded that all of the integron positive strains were resistant to cefepime and ceftazidime. Budak et al. found that 7 of 14 beta-lactamase- and aminoglycoside resistant *P. aeruginosa* strains were found to be both OXA-2- and OXA-10-positive and PER-1 [39]. It was seen that 2 isolates gave a PCR product with a 5'-3' class I integron PCR.

Integrons are natural expression vectors that permit the insertion of antibiotic resistance genes by a site-specific recombinational mechanism. It has been shown that the *P. aeruginosa* strains that have different RAPD-PCR patterns have different integrons. We detected that in the two strains that were group representative *blaPER-1* have relationship with CLASS-1 integrons. It would be necessary to support this relationship with DNA sequence analyses. We performed that PER-1 enzyme is common in our hospital and their clonal diversity indicates horizontal dissemination, the association between *blaPER-1* and CLASS-1 integrons might accelerate dissemination of this gene.

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