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Detection of TEM, SHV and PER Type Extended-Spectrum ß-Lactamase Genes among Clinical Strains of *Pseudomonas aeruginosa* Isolated from Burnt Patients at Shafa-Hospital, Kerman, Iran

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Abstract

Objective(s)
This study was carried out to evaluate the existence of the TEM, SHV and PER ESBL genes in ESBL producing strains of *Pseudomonas aeruginosa* isolated from burnt patients at Shafa-hospital, Kerman, Iran.
Materials and Methods
A total of 120 strains of *P. aeruginosa* were isolated from 245 patients in burn unit of Shafa-hospital during January 2006 to December 2007. MIC of antibiotics was measured using agar dilution test. ESBL producing strains were detected by double-disc synergy method containing amoxicillin and amoxicillin+clavulanic acid and phenotypic confirmatory test. All the clinical isolates resistant to imipenem (IMP) were screened for the production of MBL by E-test with IMP/IMP+EDTA strips. PCR and multiplex-PCR performed for the detection of different types of ESBL producing genes in ESBL positive isolates.
Results
Of 120 the isolates, 3-5% showed MIC greater than 16 µg/ml to IMP and meropenem, 66% showed MIC greater than 32 µg/ml to ceftazidime, 42% to azteronam and 60% of the isolates showed MIC greater than 64 µg/ml to cefotaxime, 41 (34%) confirmed as ESBL producers. Not any isolate could produce MBL ($P \leq 0.05$). The PCR assay of all ESBL producing isolates revealed that 6.6%, 4.1% and 2.5% of them were positive for SHV, PER and TEM genes, respectively.
Conclusion
Many ESBL producing strains of *P. aeruginosa* isolated from patients in burn unit of Shafa-hospital. However, none could produce MBL enzyme. The genes among ESBL producing strains were SHV, PER as well as TEM type of β-lacatamases.

Keywords: Antibiotic resistance, ESBL, MBL, *Pseudomonas aeruginosa*

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Introduction

Pseudomonas aeruginosa is a leading cause of nosocomial infections especially in burn unit of the hospitals, create huge burden in treatment of infections caused by this organism and pose high rate of morbidity and mortality (40-50%) among burn infected patients (1-3). Extended-spectrum β-lactamases (ESBLs) are class of enzymes that mediate resistance to extended-spectrum cephalosporins, such as cefotaxime, ceftriaxone, and ceftazidime and are most commonly found in *Klebsiella pneumoniae*, *Escherichia coli* and have been recently detected in *P. aeruginosa,* at low frequently in burnt patients (1, 2, 4). ESBLs in *P. aeruginosa* are mostly associated with over production of chromosomal Amp-C cephalosporinase, or with non-enzymic mechanisms such as drug efflux or outer membrane impermeability (4). Plasmid acquired Ambler class -A ESBLs such as PER, VEB, GES, IBC, TEM and SHV types were commonly detected in Enterobaceriaceae (5, 6). Class-A ESBLs recently reported in *P. aeruginosa* but they have so far been found in a limited number of geographic areas (8). The PER type of enzyme mostly reported from Turkey. It was found that as many as 11% of *P. aeruginosa* isolated at hospitals in Turkey produce this type of enzyme (9). The VEB-type is integron-associated class- A that was first detected in *P. aeruginosa* by Nordmann *et al* (10, 11) and in south east Asia from a four month old Vietnames child transferred from Vietnam and hospitalized in France (5). TEM and SHV (refer to sulfhydryl variable) are able to hydrolyze ampicillin at a greater degree than carbenicillin, oxacillin, or cephalothin, and have negligible activity against Extended-spectrum cephalosporins (3, 10). TEM –types are mainly reported in *P. aeruginosa* as a rare isolate in France (2). Poirel *et al* (11) detected PER, WER TEM, SHV and VEB-1-like Extended-Spectrum ß-lactamases in *P. aeruginosa* in Kuwait. Lee *et al* (12) reported prevalence of Ambler class-A and -D ß-lactamases among clinical isolates of *P. aeruginosa* in Korea. They found that extended-spectrum derivatives were rare among *P. aeruginosa* in Korea. Similar results were obtained by other authors in Korea (13, 14) and Japan (21).

There is no paper published on prevalence of PER-1, SHV, and TEM genes in Iraq, Syria, Jordan and there are few authors reported the existence of above genes among ESBL producing *P. aeruginosa* in the infected burnt patients, in Iran (15, 20). Therefore, the present study was carried out for the first time to address this issue.

Materials and Method

Bacteria strains

A total of 245 samples collected from the infected burnt patients hospitalized in the burn unit of Shafa- hospital in Kerman, Iran from January 2006 to December 2007. Among them, 120 *P. aeruginosa* were identified and confirmed using standard microbiological and biochemical tests as previously described (7). *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 (ESBL sensitive) obtained from Tarbit Modarrs University, Iran (Bacteriology unit) and used as a quality reference strain for MIC and ESBL tests. *Kelabsiella pneumoniae* ATCC700603 (SHV positive) strain were kindly received from Dr. Mobain (Azad University, Tabriz, Iran).

K. pneumoniae 7881 and *P. aeruginosa* KOAS (TEM and PER positive respectively), provided by Professor Nordmann (Institute Pasteur, France).

Antibiotic susceptibility testing

Minimal inhibitory concentration (MIC) of each antibiotic was determined for all the isolates, using a standard agar dilution method according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (16). About 10^4-10^5 CFU/ml of bacterial cell concentrations were used to spot the Muller-Hinton agar (Hi-media, India), containing different amounts of antibacterial agents. MIC recorded when no visible growth observed on the agar plate, after 24 hrs of incubation at 37 °C. The antimicrobial agents included in this study, such as cefotaxime (CTX), ceftazidime (CAZ), imipenem (IMP), meropenem (MEM) and aztreoname (ATM),

purchased as powder and discs from Glasgow Company Ltd. England with 99.8% purity.

Detection of ESBL and MBL

Production of ESBL was tested for all the isolates by double-disc synergy method containing amoxicillin-clavulanic acid and PCT (phenotypic confirmatory test) containing 30 µg/ml ceftazidime (CAZ), 30 µg/ml cefotaxime (CTX) and 10 µg/ml cefpodoxime (CPD) alone and with CAZ 30 µg + CA 10 µg, CTX 30 µg + CA 10 µg and CPD 10 µ g+ CA 1 µg per disc respectively. The zones of inhibitions for the CTX, CAZ, CPD discs were compared with that of the CAZ 30 µg + (CA) 10 µg, CTX 30 µg + CA 10 µg and CPD 10 µg + CA 1µg discs according to the CLSI guidelines. An increase in zone diameter of ≥5mm in the presence of clavulanic acid indicated the existence of ESBL in the test organism. Furthermore, phenotypic screening activity for MBL production was also carried out by E-test MBL strips (AB BIODISK, Solna, Sweden), under the conditions recommended by the manufacturer.

DNA extraction

1.5 ml of a 24 hrs grown *P. aeruginosa* culture in Tripticase Soy Broth (TSB) medium (Merck, Germany), transferred into Eppendorff microfuge tubes, and centrifuged at 7,000 rpm for 10 mins. The pellets were dissolved in 600 µl of the lysis buffer (NaCl 1 M, Tris -HCl 1 M, EDTA 0.5 M and DD/water). SDS solution 20 µl (25%) and 3 µl of proteinase K (20 mg/ml) were added to the mixture. The microfuge tubes incubated at 60 °C for 1 hr. After the lysis of bacterial cells, 620 µl of phenol, chloroform and isoamylalchol (25:24:1vol/vol) added to the above solutions, carefully mixed and the mixtures centrifuged at 10,000 rpm and 4 °C for 10 mins. The supernatants transferred to other microfuge tubes and 1.5 ml of 95% cold ethanol was added to them for one hr. Then the DNA precipitated at 10,000 rpm centrifugation and room temperature for 10 mins. The supernatant discarded and the precipated DNA dissolved in 50 µl of 10 mM TE-buffer containing 10 µl RNase and was used for further investigation.

Polymerase chain reaction (PCR)

Detection of bla_{TEM}, bla_{SHV} and bla_{PER} type ESBL genes from *P. aeruginosa* strains was carried out by PCR Taq-polymerase amplification technique and confirmed by multiplex- PCR using the PER, SHV and TEM type primers as shown in Table 1, and master-mix preparation Table 2 (the primers and reagents were recommended by Professor Nordmann, Pasteur Institute France and purchased from Fermentas PV. Ltd.). The amplification of respected genes performed in a thermal cycler (Eppendrof, Hamburg, Germany) under the following conditions: 35 cycles, each with one cycle consisting of denaturation at 94 °C for 30 seconds, proper annealing temperature for each primer, at 45 ºC for TEM, 43 ºC for PER and 60 ºC for SHV genes respectively and extension at 72 °C for one minute. Each PCR program preceded by a denaturizing step of 94 °C for 3 mins and a final step of 72 °C for 10 mins.

Multiplex- PCR was performed to confirm the results of PCR. The condition for multiplex-PCR is shown in Table 3. Electrophoresis carried out in the horizontal bed apparatus using 1 mM Tris-EDTA-Borate (TEB) buffer (pH 8.2), either at 60 V for 4 hrs or 90V for 2 hrs and the agarose gel (1%) stained with 0.5 µg/ml ethidium bromide (Sigma, USA). The DNA Bands were then observed using UV gel documentation system (UV DOC, England) and photographed. Simultaneously, a molecular ladder 1000 bp (Fermentas) was run to check the molecular weight of the PCR products.

Table 1. The primers used in PCR for ESBLs producing *P.aeruginosa* isolates.

Primer sequence	Gene encoded	ESBL type	Company
5'-ATGAATGGTCATTATAAAAGC-3'	bla_{PER}	PER-A	Fermentas
5'-AATTTGGGCTTAGGGCAGAA-3'	bla_{PER}	PER-B	Fermentas
5'-AAGATCCACTATCGCCAGCAG-3'	bla_{SHV}	SHV- A	Fermentas
5'-ATTCAGTTCCGTTTCCCAGCGG-3'	bla_{SHV}	SHV- B	Fermentas
5'-GAGTATTCAACATTTCCGTGTC-3'	bla_{TEM}	TEM- A	Fermentas
5'-TAATCAGTGAGGCACCTATCTC-3'	bla_{TEM}	TEM- B	Fermentas

Statistical analysis

All the statistical analyses carried out using SPSS version 7.5 (Norusis,17). Chi-square and fisher tests used for determination of significance of association. The $P \leq 0.05$ was considered significant.

Table 2. Master –Mix preparation for PCR.

Reagent	Quantity	Amount used
Reaction Buffer		2.5 µl
$MgCl_2$	25 mM	0.8 µl
dNTP	10 mM	0.5 µl
Primers	PER10 pmol/µl	0.5 µl
	SHV10 pmol/µl	0.5 µl
	TEM 10 pmol/µl	0.5 µl
Taq Polymerase	5 U/µl	0.1 µl
	PER	0.5 µg
DNA template	SHV	0.5 µg
	TEM	0.5 µg

Table 3. Condition of reaction used for the multiplex-PCR.

Name of Material	Concentration
Reaction Buffer	2.5 µl
$MgCl_2$	2.4 µl
dNTP 10 mM	1.5 µl
Primers PER 10 pmol/µl	1 µl
SHV 10 pmol/µl	0.7 µl
TEM 10 pmol/µl	1 µl
Taq Polymerase 5U/µl	1 µl
DNA Template	2 µg

Results

During one year period, 120 *P. aeruginosa* isolated from 245 patients referred to burn unit of Shafa-hospital in Kerman, Iran. The isolates confirmed as *P. aeruginosa* by the standard microbiological methods as well as PCR technique. Table 4 shows distribution of burnt patients infected with this bacterium according to the age and percentage of burn. Among them 77 (64%) were male and 43 (34%) were female. Most of the burnt patients aged between 11 to 20 years old, however, the most critical ones were in the age range of 21 to 30 years ($P \leq 0.5$).

Table 4. Distribution of number and percentage of *P. aeruginosa* samples collected from patients hospitalized in burn unit of the Shafa-hospital in Kerman, Iran.

Burns%	Age (year)					Total
	1-10	11-20	21-30	31-40	41>	
1-30	7 (54%)	29 (71%)	21(58%)	4 (25%)	8 (57%)	69 (57%)
31-40	3 (23%)	3 (7%)	5 (14%)	4 (25%)	4 (29%)	19 (16%)
41-50	0 (0.0%)	2 (5%)	1 (3%)	3 (19%)	1 (7%)	7 (6%)
50<	3 (23%)	7 (17%)	9 (25%)	5 (31%)	1 (7%)	25 (21%)
Total	13 (100%)	41 (100%)	36 (100%)	16 (100%)	14 (100%)	12 (100%)

SD= standard deviation

Figures in each column are the number of the isolates.

The MICs of isolates to routinely used antibiotics IMP, MEM, ATM, CAZ and CTX in burn unit of Shafa-hospital are shown in Table 5. As Table 5 indicates, 60% of the isolates showed MIC greater than 64 µg/ml to CTX, 42% were resistant to ATM and 66% to CAZ, while only 3 to 5% of them were resistant to IMP and MEM. This indicates that the patients did not have previous exposure to these antibiotics (since MEM and IMP antibiotics have recently been used in burn unit of Shafa- hospital for the treatment of patients). Phenotypic confirmatory test (PCT) for detection of ESBL by double disk diffusion method, revealed that the isolates were 41 (34%) ESBL producers and 79 (66%) of them negative for ESBL ($P \leq 0.5$), as shown in Figures 1 and 2. However, none of the isolate could produce any MBL enzyme. This was in accordance with to MIC to IMP and MEM.

The ESBL producing strains of *P. aeruginosa* were then subjected to PCR and multiplex-PCR in order to determine which type of ESBL gene they encoded, as shown in Figures 3 and 4. It was found that isolates number 16, 17, 20, 24, 27 and 32 Carried bla $_{SHV\ (200bp)}$, isolates number 12, 14 and 15 carried bla $_{TEM\ (800bp)}$ and isolates 3, 9 and 10 carried bla $_{PER\ (900bp)}$. The above results were further confirmed by multiplex-PCR (data not shown), where only one gene was detected in each positive ESBL isolate. None of the above strains carried two or more genes simultaneously (data not shown), while, no Amp-C gene detected in any isolated strain. Those ESBL producing strains that did not carry the above genes (PCR negative strains) may involve different mechanism of resistance such as efflux process which should further be investigated.

Table 5. MIC (µg/ml) of 120 isolates of *P. aeruginosa* collected from burn patients in Shafa-hospital, Iran.

Anti*	<2	2	4	8	16	32	64	128	≥256
IMP	73 (60.8)	8 (6.6)	15 (12.5)	21(17.5)	1 (0.83)	1 (0.83)	0 (0)	0 (0)	1 (0.83)
MEM	69 (57.5)	4 (3.3)	14 (11.6)	27 (22.5)	2 (1.6)	2 (1.6)	1 (0.83)	0 (0)	1 (0.83)
ATM	38 (31.6)	1 (1.6)	0 (0)	15 (12.5)	16 (13.3)	23 (19.1)	22 (18.3)	3 (2.5)	2 (1.6)
CAZ	5 (4.1)	18 (15)	4 (3.3)	7 (5.8)	7 (5.8)	7 (5.8)	13 (10.8)	41 (34.1)	18 (15)
CTX	3 (2.5)	11 (9.1)	2 (1.6)	4 (3.3)	10 (8.3)	18 (15)	19 (15.8)	46 (38.3)	7 (5.8)

Anti*= Antibiotic; IMP=Imipenem, MEM= Meropenem, ATM= Aztronam, CAZ= Cftazidime, CTX= Cefotaxime
Figures in each column are the number of the isolates, and figures in bracket indicate%

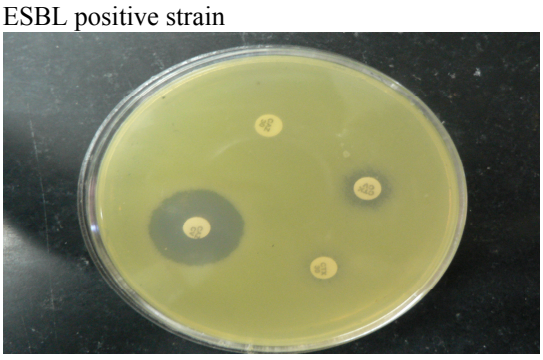

Figure 1. Phenotypic confirmatory test (PCT) for detection of ESBL by disc diffusion method. 10^8 cells/ml in log phase were spread in Muller-Hinton agar and incubated for 15 mins. The CAZ, CTX and CPD with CAZ 30 µg + CA 10 µg, CTX 30 µg +CA 10 µg and CPD 10 µg +CA 10 µg discs were put into the and CPD 10 µg +CA 10 µg discs were put into the plates as described in the text.

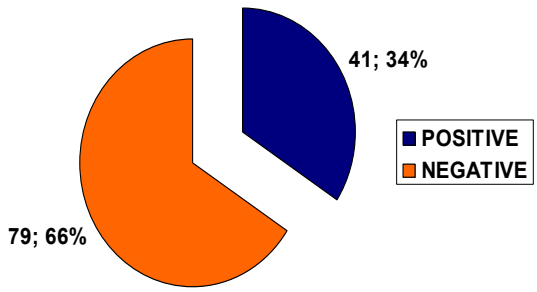

Figure 2. Distribution of ESBL enzymes among 120 strains of *P. aeruginosa* isolated from burn patients.

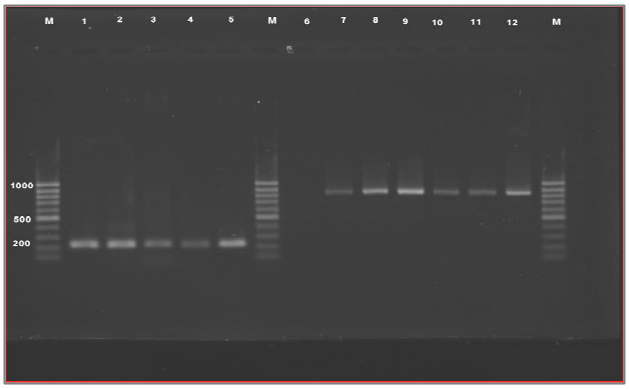

Figure 3. PCR products of ESBL producing strains of *P. aeruginosa* among burn patients.
Lane M: M.Wt. Marker, Lane 2, 3, 4, 5 are those strains which carried bla $_{SHV\ (200\ bp)}$ gene. Lane 7, 8, 9, 10 and 11 are strains contained bla $_{TEM\ (800\ bp)}$ gene. Lane 1 and 12 are positive controls for bla $_{SHV\ (200\ bp)}$ and bla $_{TEM\ (800\ bp)}$ genes in *Klebsiella pneumonia* 7881 received from Prof. Nordmann Pasteur (Institute-France). Lane 6 is negative control containing only buffer.

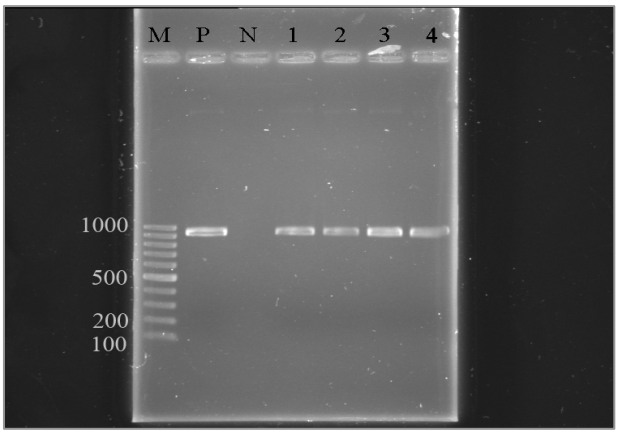

Figure 4. PCR product of bla $_{PER}$ producing strain of *P. aeruginosa* burn isolates.
Lane 1. Marker, Lane 2. Postive control. Lane 3. Negative control. Lanes 1, 2, 3 and 4 are *P.aerugionsa* burn isolates which are positive for bla$_{PER}$ gene.

Discussion

Antibiotic resistance is a major factor that derives change in the pattern of antibiotic prescribing and is the most important stimulus to the development of new antibiotics by pharmaceutical industry (2). There is another issue of major importance in relation to epidemiology of resistance, the true incidence is often poorly and sporadically reported (6, 10). In Iran due to inappropriate prescription of antibiotics by some physicians and irresponsible consumption by patients often increases the cost, side effect, treatment failure and antibiotic resistance. This is particularly true in burn unit of the hospitals where antibiotic resistance micro-organisms especially *P. aeruginosa* is dominant (4). Infections due to ESBL-producing *P. aeruginosa* can pose a major threat to life, are often difficult and expensive to treat, and can delay the discharge of patients from hospital (12, 20).

In this study, we tried to identify the prevalence of ESBL producing *P.aeruginosa* in burn unit of the Shafa- hospital in Kerman city, Iran and to detect the type of ESBL genes they encoded. It was found that a considerable number of the isolates showed MIC greater than 64 µg /ml to CAZ, CTX and 42% to ATM. β-lactamase test by PCT method revealed 34% of the isolates could produce ESBL mainly SHV, PER-1 and TEM type β-lactamases respectively.

However, none of the above isolates could produce any MBL enzyme as they exhibited considerable low MIC values to IMP and MEM. This was in accordance with other researches carried out in Iran (20). Serotype's determination, drug resistance patterns and plasmid profiles of *P. aeruginosa* isolated from burnt patients were studied at two hospitals of Tehran (16), and showed a very low rate of resistance to IMP and MEM. Similarly, frequency of *P. aeruginosa* serotypes in burn wound infections and their resistance to antibiotics were studied by Karmi Estahbanati *et al* (7). Occurrence of PER-1 type producing clinical isolates of *P. aeruginosa* were studied in Japan (21). They found that four strains were resistant to ceftazidime, cefepime and aztreonam with MIC of 64≥µg/ml or more, but more susceptible to carbapenem antibiotics. Similar observation was made in our study.

The PCR assay using standard primers for the presence of bla $_{SHV}$, bla $_{PER}$ and bla $_{TEM}$ genes in the ESBL producing strains confirmed that indeed the above types of genes were carried, with different frequency by the isolates. However, no Amp-C gene was detected in any of the above strains. This suggests that they are rare among Pseudomonas strains in Iran, since majority is chromosomally mediated. The results were further confirmed by the multiplex-PCR.

Similarly, of 148 ESBL producing *P. aeruginosa* isolated from different hospital samples in India (1), 30 (27%) were found to be positive for ESBL. Maximum ESBL production was found in sputum and tracheotomy swabs (28.57%) followed by pus (24.13%), urine (19.04%), cerebrospinal fluid (CSF), other sterile body fluids (15.38%) and blood (7.14%). All the ESBL-producing *P. aeruginosa* isolates were multi-drug-resistant. Isolates were also, 100% sensitive to IMP. In one study performed by Jiang *et al (*22) in china, 20% of *P. aeruginosa* carried TEM type ESBL gene.

Conclusion

From the above results, it can be concluded that there is a high prevalence of ESBL

producing *P.* aeruginosa isolates among patients in burn unit of Shafa-hospital in Kerman, Iran. However, none of the above isolates could produce MBL enzyme and most of the isolates showed extremely low MIC values to IMP and MEM antibiotics. This indicates that IMP and MEM are drugs of choice in Shafa-hosapital for the treatment of burn infections and should only be used when the isolated strain was resistant to other type of antibiotics. According to the PCR and multiplex PCR assays, the most prevalent genes among ESBL producers were SHV, PER as well as TEM type of β-lacatamases respectively. While, no Amp-C gene was detected in any of the isolates.

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