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Molecular Identification of ESBL Genes *bla*$_{GES-1}$, *bla*$_{VEB-1}$, *bla*$_{CTX-M}$ *bla*$_{OXA-1}$, *bla*$_{OXA-4}$, *bla*$_{OXA-10}$ and *bla*$_{PER-1}$ in *Pseudomonas aeruginosa* Strains Isolated from Burn Patients by PCR, RFLP and Sequencing Techniques

Fereshteh Shacheraghi, Mohammad Reza Shakibaie, Hanieh Noveiri

Abstract**—**Fourty one strains of ESBL producing *P.aeruginosa* which were previously isolated from burn patients in Kerman University general hospital, Iran were subjected to PCR, RFLP and sequencing in order to determine the type of extended spectrum β-lactamases (ESBL), the restriction digestion pattern and possibility of mutation among detected genes. DNA extraction was carried out by phenol chloroform method. PCR for detection of *bla* genes was performed using specific primer for each gene. Restriction Fragment Length Polymorphism (RFLP) for ESBL genes was carried out using *EcoRI, NheI, PVUII, EcoRV, DdeI,* and *PstI* restriction enzymes. The PCR products were subjected to direct sequencing of both the strands for identification of the ESBL genes.The *bla*$_{CTX-M}$, *bla*$_{VEB-1}$, *bla*$_{PER-1}$, *bla*$_{GES-1}$, *bla*$_{OXA-1}$, *bla*$_{OXA-4}$ and *bla*$_{OXA-10}$ genes were detected in the (n=1) 2.43%, (n=41)100%, (n=28) 68.3%, (n=10) 24.4%, (n=29) 70.7%, (n=7)17.1% and (n=38) 92.7% of the ESBL producing isolates respectively. The RFLP analysis showed that each ESBL gene has identical pattern of digestion among the isolated strains. Sequencing of the ESBL genes confirmed the genuinety of PCR products and revealed no mutation in the restriction sites of the above genes. From results of the present investigation it can be concluded that *bla*$_{VEB-1}$ and *bla*$_{CTX-M}$ were the most and the least frequently isolated ESBL genes among the *P.aeruginosa* strains isolated from burn patients. The RFLP and sequencing analysis revealed that same clone of the *bla* genes were indeed existed among the antibiotic resistant strains.

Keywords**—**ESBL genes, PCR, RFLP, Sequencing, *P.aeruginosa*

I. INTRODUCTION

Pseudomonas aeruginosa is ranking second among gram negative hospital acquiring pathogens and one of leading cause of burn infections reported to the National Nosocomial Infection Surveillance System [1, 2]. The idea of eradication of *P. aeruginosa* from burn patients through intense antimicrobial therapy may lead to significant selection of resistance strains in burn unit of the hospitals [3]. One of the important features of these strains is resistant to multiple clinically important antibiotics like third generation of cephalosporins, imipenem and aztronam [4]. Many *P.aeruginosa* strains produces different class of extended spectrum β-lactamases (ESBLs) that enable bacterium to stand against extended –spectrum cephalosporins, such as cefotaxime, ceftriaxone and ceftazidime and have been reported with increasing frequency [5, 6]. ESBL mediate resistance to cephalosporin antibiotics and were first discovered in Europe in the early 1980s. They have become a widespread problem, particularly in *Klebsiella pneumoniae*, and increasingly in non-typhoid Salmonella species. The OXA-type ESBLs have been found mainly in *P. aeruginosa* isolates from Turkey and France [7].

Traditionally, ESBL enzymes have been derivatives of TEM and SHV parent enzymes. The last year, however, has seen an explosion of developments in ESBLs of non-TEM, non-SHV lineage in Europe. The CTX-M type ESBLs have become particularly widespread [8]. Bert *et al*., [9] detected *bla*$_{PSE}$ and *bla*$_{OXA}$ gene variants using PCR. The genotypes were distinguished by restriction of PCR products with endonucleases recognizing sites involved in point mutations. Jiang *et al*., [10] studied a total of 75 clinical isolates of *P. aeruginosa.* Thirty-four of 36 multidrug-resistant *P. aeruginosa* clinical isolates were positive for ESBLs and *bla*$_{VEB-3}$ was the most prevalent ESBL gene reported by the authors. Antibiotic susceptibility tests and PCR amplification of genes encoding class A (*bla*$_{PSE-1}$, *bla*$_{PER-1}$, *bla*$_{VEB-1}$, *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{GES-1}$) and class D ß-lactamases (*bla*$_{OXA-groupI}$, *bla*$_{OXA-groupII}$ and *bla*$_{OXA-groupIII}$) in *P.aeruginosa* were carried out by Lee *et al*., (11). In 64 (25.4%) isolates, structural genes for PSE-1 (6.3%), OXA-10 (13.1%), OXA-4 (4.3%), OXA-30 (2.0%), OXA-2 (2.3%) and OXA-17 (0.4%) were found, their distribution varied between provinces. None harboured *bla*$_{PER-1}$, *bla*$_{VEB-1}$, *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{GES-1}$. Similarly, PCR and sequence analysis revealed the presence of the *bla*$_{CTX-M-1}$, *bla*$_{SHV-1}$ and *bla*$_{TEM-116}$ genes in the *P. aeruginosa* and *bla*$_{CTX-M-1}$ and *bla*$_{SHV-1}$ in the *Stenotrophomonas maltophilia* strains [12]. Mirsalehian et al., [13] studied the prevalence of ESBLs and antimicrobial susceptibilities of *P. aeruginosa* isolated from burn patients in Tehran, Iran. It was found that 50 (74.62%), 33 (49.25%) and

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21 (31.34%) strains among 67 ESBL-producing strains amplified *bla*$_{OXA-10}$, *bla*$_{PER-1}$ and *bla*$_{VEB-1}$ respectively. Woodford *et al*., [14] studied *P. aeruginosa* isolates producing VEB-type ESBL in the United Kingdom. In one UK centre, a VEB-1 producing strain was isolated. This strain was resistant to all beta-lactams, aminoglycosides and ciprofloxacin, remaining susceptible only to colistin (MICs </=1 mg/L). Two other *P. aeruginosa* isolates co-producing both VEB and VIM enzymes were received from two other UK hospitals; one isolate represented inter-hospital spread of the O15 strain and the second was distinct. Existence of SHV-type ESBL genes in *P. aeruginosa* by PCR-restriction fragment length polymorphism have been reported by Blagui et al. [15]. Restriction of PCR products by *DdeI* and *BsrI* revealed the same restriction pattern with the *bla* $_{SHV-1}$ positive control.

In pervious study, we isolated 41 strains of ESBL producing *P.aeruginosa* from burn infected patients in Kerman University of Medical Sciences general hospital, Iran [4]. In present study we tried to identify the ESBL genes, the restriction digestion patterns and possibility of mutations in restriction sites among detected *bla* genes by PCR, RFLP and sequencing techniques.

II. MATERIAL AND METHODS

Bacterial sources

120 strains of *P.aeruginosa* were isolated from burn infected patients in burn unit of the Kerman University general hospital, Iran within one year period. Identification of the isolates was done according to standard microbiology procedures [4]. The ESBL production was detected among 41 strains by phenotypic confirmatory test and double disc synergy methods as previously published by Shakibaie et al. [4]. The standard microbial cultures for detection of ESBL genes were including *P.aeruginosa* KOAS containing *bla*$_{PER-1}$ obtained from Prof. Nordman, institutes pasture France. *K. pneumoniae* 7881, *K. pneumoniae* CHU-BICETRE containing *bla*$_{CTX-M}$ and *bla*$_{GES-1}$ respectively provided by Prof. Nordman, France. *P.aeruginosa* ATCC 27853, *E.coli* ATCC25922 ESBL sensitive strains and *P. aeruginosa* strains containing *bla*$_{VEB-1,\ OXA-1\ OXA-4,}$ and *bla* $_{OXA-10}$ were available in the stock collection of Institute pasture of Iran [16].

Antibiotic sensitivity tests

The antibiotic sensitivity of the above strains was carried out by disc diffusion break point assay and MIC was determined by agar dilution method in Muller-Hinton agar (MHA) as described previously [4].

DNA extraction

One ml of 24 hours grown *P.aeruginosa* cultures in Triplicate Soy Broth (TSB) medium (Merck, Germany) were transferred into 1.5ml sterile Eppendorff microfuge tubes and centrifuged at 10.000g for 10 minutes. The pellets were dissolved in 600µl of lysis buffer (NaCl 1M, Tris-HCl 1M, EDTA 0.5M), 20µl SDS (25%), 3µl of proteinase -K (20mg/ml) and incubated at 60ºC for 1 hour. After the lysis, 620µl of phenol/chloroform/isoamylalchol (25:24:1 Volume/Volume) were added to the above solutions, carefully vortexed, and centrifuged at 12.000g for 10 minutes. The supernatants were transferred to other sterile microfuge tubes. 1ml of 95% cold ethanol was added and allowed to stand for 1hour in refrigeration condition (4°C). DNA was then precipitated in each tube by centrifugation at 12.000g for 10 minutes. The precipitated DNA was dissolved in 50µl of 10mM Tris EDTA - buffer (TE) containing 10µl of RNase –A as described by Sambrook *et al.,* (28) and used for further investigation.

PCR reaction

The primer sequence for ESBL genes is showed in Table-1. The PER-1 sequence derived from *Pseudomonas aeruginosa* KOAS Producing PER-1 (Pasteur Institute of France), VEB-1 *Pseudomonas aeruginosa* 10.2 (24), GES-1 *K. pneumoniae,* Prf. P. Nordmann CHU Bicetre-France and CTX-M *K.pneumoniae* 7881 kindly provided by P. Nordmann.

A typical 25µl PCR reaction mixture for every primer set was consisted of 1X- PCR reaction buffer (Fermentase, Lithuania), 1.5 µm $MgCl_2$ (25mM), 0.7 µm of each dNTP (10mM), 0.7µl of each primer, 1unit of Taq DNA polymerase 5U/µl (Fermentase, Lithuania) and 0.5 µl of 10µg DNA template. Amplification was carried out in a thermocycler (Eppendorf Mastercycler®, Massachusetts, USA). Agarose gel electrophoresis (1.0%) of PCR products was carried out in horizontal bed apparatus using 1mM Tris-Borate- EDTA (TBE) buffer (pH-7.2) at 90V for 1hour and the DNA bands were then stained with 0.5µg/ml ethidum bromide (Sigma USA) for 10 minutes. The gels were washed twice with D/W and observed under U.V. gel documentation (UV DOC, England) at 280nm. 1000-100bp DNA ladder was used to confirm the size of each specific *bla* gene. Simultaneously, a positive control was run for each ESBL gene.

Restriction Fragment Length Polymorphism (RFLP)

The RFLPs of ESBL genes were carried out using *EcoRI, NheI, PVUII, EcoRV, DdeI,* and *PstI* restriction enzymes respectively. The enzymes were purchased from Fermentas Company Ltd. 1.5µl of each enzyme and DNA mixtures were added to 1µl restriction buffer and kept at 37ºC. Digestions were completed within 3 hours and the mixture then loaded into 1.5% agarose gel concentration. The electrophoresis was conducted for each RFLP set in TBE-buffer at 60V for two hours. The gel was then stained with ethidium bromide solution (0.5µg/ml) and observed under UV light gel documentation system as described above.

Sequencing of the PCR products

DNA sequencing for all 41 strains was performed for identification of detected *bla* genes using primers as shown in Table-1. The PCR products of above genes were further purified with PCR purification Kits (Fermentas) and subjected to direct sequencing of both the strands performed by the Macrogen Company (Seoul, Korea) as described previously [12]. The nucleotide and deduced amino acid sequences were analyzed with CROMASPRO-2 and MEGA- 4 softwares.

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TABLE I
THE PRIMERS AND SEQUENCES USED FOR AMPLIFICATION WITH RESPECT TO ESBL GENES

Primer Name	5' – Sequence - 3'	Detected gene	Molecular Weight	Reference
VEB-1(F)	CGACTTCCATTTCCCGATGC	blaVEB	643bp	21
VEB-1(R)	GGACTCTGCAACAAATACGC			
GES-1(F)	ATGCGCTTCATTCACGCAC	blaGES	643bp	22
GES-1(R)	CTATTTGTCCGTGCTCAGG			
CTX-M(F)	CGCTTTGCGATGTGCAG	blaCTX-M	550bp	23
CTX-M(R)	ACCGCGATATCGTTGGT			
OXA-1(F)	AGCCGTTAAAATTAAGCCC	blaOXA-1	882bp	24
OXA-1(R)	CTTGATTGAAGGGTTGGGCG			
OXA-4(F)	TCAACAGATATCTCTACTGTT	blaOXA-4	216bp	25
OXA-4(R)	TTTATCCCATTTGAATATGGT			
OXA-10(F)	TCAACAAATCGCCAGAGAAG	blaOXA-10	277bp	26
OXA-10(R)	TCCCACACCAGAAAAACCA			
PER-1(F)	AATTTGGGCTTAGGGCAGAA	blaPER	925bp	27
PER-1(R)	ATGAATGTCATTATAAAAGC			

F= Forward primer
R= Reverse primer

III. RESULTS

The results of PCR of the *bla* genes of the *P.aeruginosa* strains isolated from one of the Kerman University of Medical Sciences general hospital in Iran are shown in Figure 1. The bla_{VEB-1} (643bp) was the most frequent ESBL gene and isolated from almost all [100% (n=41)] of the ESBL producing strains, while, bla_{OXA-10} (227bp) was detected in 92.7% (n=38) of the isolates. The other ESBL genes were detected among ESBL producing populations including bla_{CTX-M} (550bp) bla_{PER-1} (927bp), bla_{GES-1} (864bp), bla_{OXA-1} (909bp), bla_{OXA-4} (216bp) with frequency 2.43% (n=1), 68.3% (n=28), 24.4% (n=10), 70.7% (n=29) and 17.1% (n=7) respectively as shown in Figure 1. bla_{CTX-M} gene was detected in only one isolate that showed MIC>/=2 to aztronam. Restriction digestion pattern of bla_{VEB-1} PCR product by *EcoR1* enzyme revealed identical digestion pattern among the strains [two bands with 152 and 491bp] while, digestion of bla_{GES-1} gene by *NheI* resulted in two bands [207 and 657bp]. Similarly, digestion of bla_{OXA-1} and bla_{OXA-4} with restriction enzyme *EcoRV* resulted in two bands with 124 and 785 bp and 9 & 207bp respectively. Digestion of bla_{CTX-M} gene with *PVUII* resulted in three bands with 170, 180 and 297bp. The results were further confirmed by sequencing technique (Fig. 2). In order to increase the accuracy of the results, the sequencing of both forward and reverse strands of the ESBL genes were carried out and revealed that the same sequencing pattern with each *bla* positive controls. Figure 2 shows the conserved region for the restriction sequence blaVEB-1gene compared with positive control. This was obtained by comparing with blast data and the chromatograms and predicted amino acid sequences of the above genes. The sequencing of *bla* genes also confirmed the RFLP results and suggested that there were no any mutations in the restriction sites of these genes. It therefore might be suggested that same clone of each ESBL gene has been spread among the *P.aeruginosa* isolated strains by mobile genetic elements like transposons or integrons. This is due to selective pressure (presence of antibiotics) exerted in the hospital environment on the bacteria.

Fig. 1. Agarose gel electrophoresis of PCR products of ESBL genes bla_{CTX-M}, bla_{VEB-1}, bla_{PER-1}, bla_{GES-1}, bla_{OXA-1}, bla_{OXA-4} and bla_{OXA-10} detected among *P.aeruginosa* isolated from burn patients. M= Molecular weight marker (100-1000bp). Positive control was also run alongside the tests as shown in the right side of each gene

5´NAGCNANNGCNNATTGCTTTAGCCGTTTTGTCTGAGATAGATAAAGGGAAT
CTTTCTTTTGAACAAAAAATAGAGATTACCCCTCAAGACCTTTTGCCTAAAAT
GTGGAGTCCGATTAAAGAGGAATTCCCTAATGGAACAACTTTGACGATTGAA
CAAATACTAAATTATACAGTATCAGAGAGCGACAATATTGGTTGTGATATTT
TGCTAAAATTAATCGGAGGAACTGATTCTGTTCAAAAATTCTTGAATGCTAAT
CATTTCACTGATATTTCAATCAAAGCAAACGAAGAACAAATGCACAAGGATT
GGAATACCCAATATCAAAATTGGGCAACCCCAACAGCGATGAACAAACTGTT
AATAGATACTTATAATAATAAGAACCAATTACTTTCTAAAAAAAGTTATGAT
TTTATTTGGAAAATTATGAGAGAAACAACAACAGGAAGTAACCGATTAAAAG
GACAATTACCAAAGAATACAATTGTTGCTCATAAAACAGGGACTTCCGGAAT
AAATAATGGAATTGCAGCAGCCACTAATGATGTTGGGGTAATTACTTTACCG
AATGGACAATTAATTTTTATAAGCGTATTGTGGCGAAAAGATGTCACAAAAA
TCCCATTTTTGTAGATTTGTCCAGGATATGTGCTGTCAGTAGAAAATTTCTGT
AAATGCTCTCTATATTTCCTTCTGACAAAAGGACACTGTTCAACCCTTGGGGA
CATCCATTCCTGGCTATAAGNGNACNGGGGGAAAN 3´-

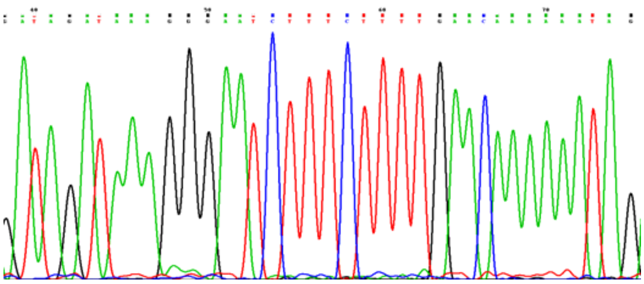

Fig. 2. Sequences and Chromatogram of bla_{VEB-1} of *P.aeruginosa* strains isolated from burn patients in Kerman University general hospital. The nucleotide sequences was analyzed with CROMASPRO-2 and MEGA 4 softwares and confirmed by blast system in internet

IV. DISCUSSION

Chromosomal or plasmid mediated antibiotic resistance is common place in *P.aeruginosa* isolated from burn infected patients in different hospitals in Iran [1]. In this research, the frequency of the ESBL genes among ESBL producing *P.aeruginosa* burn isolates were studied. The *bla*VEB gene was detected in almost all ESBL producing isolate. This was accordance with other results in Middle East and Iran. A retrospective survey was conducted to characterize beta-lactamases in a collection of 43 ceftazidime-resistant *P. aeruginosa* isolates recovered from patients with bloodstream infections hospitalized at a Brazilian teaching hospital between January and December 2005 (17). It was found that nine isolates (20.9%) produced an ESBL, either GES-1 (n = 7, 16.3%) or CTX-M-2 (n = 2, 4.6%).

Drug susceptibility testing and PCR assay were used to determine the antibiotic susceptibility patterns and prevalence of genes encoding five different extended ESBLs (PER, VEB, SHV, GES, and TEM) among 600 isolates of *P.* aeruginosa cultured from patients at two hospitals in Tehran (16**).** The frequency of *bla* $_{VEB}$, *bla* $_{SHV}$, *bla* $_{PER}$, *bla* $_{GES}$, and *bla* $_{TEM}$ among the ESBL isolates (MIC >/=16) were 24%, 22%, 17%, 0%, and 9%, respectively. Isolates containing *bla*$_{VEB}$ were resistant to almost all tested antibiotics except imepenem. However, the frequency of the *bla*OXA group of ESBL in our study was higher as compared with other authors, while only one isolate carried the gene for *bla*CTX-M. 41 *P. aeruginosa* strains were isolated with ESBLs from several wards were collected over 9 months in 2003 and 2004 in a hospital in Warsaw, Poland (18). The isolates were recovered from patients with multiple types of infections, mostly respiratory tract and postoperative wound infections. All 41 isolates produced the PER-1 ESBL, originally observed in Turkey but recently also identified in several countries in Europe and the Far East. The *bla*$_{PER-1}$ gene resided within the Tn1213 composite transposons, which was chromosomally located. The PER, VEB, GES, and IBC beta-lactamases, have been found mainly in *P. aeruginosa* and at a limited number of geographic sites (16, 19). Similarly, in our study, the ESBL gene VEB-1 exhibited similar pattern of digestion in all 41 isolates. Therefore, it might be suggested that the gene was resided on class 1 integron. Prevalence of Class-A ESBL in Clinical Isolates of *Acinetobacter baumannii* and *P.aeruginosa* were studied by *Oh et al* [20]. It was found that the most prevalent class A ESBL genotype in *Acinetobacter baumannii* isolates was *bla*$_{PER-1}$ (n=6), and *bla*$_{SHV-12}$ gene was also found in one *P. aeruginosa* isolate.

V. CONCLUSION

From results of present investigation, it can be concluded that *bla* $_{VEB-1}$ and *bla*$_{CTX-M}$ genes were the most and the least frequently isolated ESBL gene among the *P.aeruginosa* strains detected from burn infected patients while, *bla*$_{OXA-10}$ was the second most frequently isolated gene. They exhibited similar pattern of digestion of PCR products when digested with *EcoR1, DdeI* and *PVUII* restriction enzymes. The sequencing analysis further confirmed the results of PCR and revealed no mutation in the restriction sites of the above genes. The results also confirmed that these ESBL genes evolved from same clone and spread through the *P. aeruginosa* population by selective pressure of antibiotics that prescribed in this region.

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