International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:4, No:11, 2010

A New bla_{VIM} Gene in a *Pseudomonas putida* Isolated from ENT Units in Sulaimani Hospitals

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Abstract**—**A total of twenty tensile biopsies were collected from children undergoing tonsillectomy from teaching hospital ENT department and Kurdistan private hospital in sulaimani city. All biopsies were homogenized and cultured; the obtained bacterial isolates were purified and identified by biochemical tests and VITEK 2 compact system. Among the twenty studied samples, only one *Pseudomonas putida* with probability of 99% was isolated. Antimicrobial susceptibility was carried out by disk diffusion method, *Pseudomonas putida showed re*sistance to all antibiotics used except vancomycin. The isolate further subjected to PCR and DNA sequence analysis of bla_{VIM} gene using different set of primers for different regions of *VIM* gene. The results were found to be PCR positive for the bla_{VIM} gene. To determine the sequence of bla_{VIM} gene, DNA sequencing performed. Sequence alignment of bla_{VIM} gene with previously recorded bla_{VIM} gene in NCBI- database showed that *P. putida* isolate have different bla_{VIM} gene.

Keywords**—**Clinical isolates, Putida, Sulaimani, Vim gene.

I. Introduction

MICROORGANISMS might exhibit resistance to drugs by many different mechanisms. The most important mechanism is β- lctamase enzymes production, which are a group of enzymes capable of hydrolysing the 4-membered β-lactam ring of beta-lactam antibiotics [1], which can be either chromosomally encoded or plasmid mediated [11]. Several novel MBLs were identified, including VIM-1 from *P. aeruginosa* and IMP-2 from *Acinetobacter baumannii* in Italy [15], VIM-2 from *P. putida* in France [119, and IMP-3 from *Shigella flexneri* in Japan. The spread of MBLs in gram-negative rods has been described in several other countries and is becoming an emerging threat [7]. It remains unknown whether these MBLs have appeared in other countries. The aim of the study is to identify the molecular mechanism of the multidrug resistant *P. putida* among the isolates.

II. Methods

A. Isolation and Identification

Samples were collected from Teaching Hospital (ENT Dept.) and Kurdistan Private Hospital. Biopsies were taken after tonsillectomy. Biopsy was transferred to laboratory in a sterile container which contains normal saline. Samples were prepared for bacteriological examination by homogenization and centrifugation. Prepared samples were cultured on nutrient agar, and then single colonies were selected and inoculated on selective media for the purpose of obtaining pure cultures. Isolate identification performed microscopically, biochemical tests, and then the identification confirmed using VITEK 2 compact system.

B. Antimicrobial susceptibility ans Isoelectric focusing of β-lactamase: Antibiotic-containing discs (BBL, Cockeysville, MD, USA) were used for routine antibiograms by disc diffusion assay. MICs of antimicrobial agents were determined by the agar dilution method. *Escherichia coli* ATCC 25922 were used as MIC reference strain. Modified Hodge and EDTA-disc synergy tests were performed for the screening of metallo-β-lactamase-producing strains. The results were compared to CLSI standard 2008. The isoelectric points of β-lactamases were determined by loading cell sonicates to precast pH 3 to 10 gels. The gel was overlaid with a filter paper soaked in 20 mM EDTA for 5 min, before the imipenem (0.5 mg/L)-containing Mueller–Hinton agar was added. In this manner, inhibition of imipenem-hydrolysing activities could be observed.

C. Molecular methods

Amplification of *VIM* gene of *pseudomonas putida by* direct colony PCR: A single bacterial colony which is previously cultured on nutrient agar was dissolved in 50 µl dd H_2O (MQ). The cells suspension was incubated at 37C° water bath for at least 3 min. The cells were disrupted by heating by the insertion of the PCR tube containing the bacterial suspension into the thermocycler using the following program: 2 cycles for 10 min. at 99 C° heating and 1 cycle for 5 min. at 4 C° cooling. The samples were centrifuged at 13000 rpm for 10 minutes. The pellet was discarded and 5 µl of supernatant were used as template in the PCR reactions. Master mix was prepared by adding 5 µl of tag buffer, 2.5µl of (f and r) primer, 1µl of dNTP(10mM), 5µl of supernatant, 3 µl MgCl2, and 0.3µl tag polymerase to 30.7µl DDH2O (50 µl total volume in a sterile 0.5 ml PCR tube on ice). The PCR reactions were inserted into the PCR programs: A- PCR for the detection of VIM-type metallo-lactamase genes was carried out with primers VIM-DIA/f and VIM-DIA/r in a 50µl volume Reaction parameters were as follows: Annealing at 55°C for 60s extension at 72°C for 90s denaturation at 94°C for 50s for 25 cycles. The samples were analyzed by gel electrophoresis at 80V for 1 hr. The gel running was stopped and the DNA was visualized, and the DNA bands were

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photographed digitally. as was the preparation of recombinant plasmids containing PCR product, and transformation of them into *E. coli* DH5, Plasmids from successful clones were used to determine the sequence of the bla_{VIM} gene by the dideoxynucleotide-chain termination method, with an automatic DNA sequencer (ABI 3700, in Adden institute for molecular biology techniques/ Tehran- Iran The determination of the sequence was repeated with more than two clones from independent amplicons. Both strands were sequenced. Sequence alignment of *VIM* gene: Homology searches were conducted between the sequence of other reported sequences of *VIM* gene for *P. Putida* and other Gram negative bacteria in database of NCBI using BLAST program which is available at the NCBI online at (ww.ncbi.nlm.nih.gov) and the sequence of the same gene of the natural isolates.

III. Results and Discussion

The results showed that out of 20 samples, only one was positive for *Pseudomonas putida*, the identification levels (Confidence and probability) by VITEK 2 compact system was 99%. *Pseudomonas putida*, being that the bacterium rarely colonizes mucosal surfaces but from other previously reported cases, it was determined that risk factors for developing such infections include the insertion of catheters, intubation, and/or intravascular devices [3]. *P. putida* infection was found in contaminated bottle of StaKleer. StaKleer is an anti-fog solution used on mirrors and endoscopes to prevent condensation from occurring, allowing for the proper visualization of ear and nose tissues. Sometimes unopened bottles of the solution at the clinic were found to be contaminated with *Pseudomonas putida* [9]. Disc diffusion testing revealed that *Pseudomonas putida* local isolate was resistant to most β-lactams, including ampicillin, ampicillin–sulbactam, piperacillin, piperacillin–tazobactam, cefalothin, cefoxitin, cefotaxime, ceftazidime and aztreonam. The isolate was also resistant to tobramycin, intermediate to gentamicin, but susceptible to amikacin and ciprofloxacin. MICs of imipenem and meropenem for the isolate were 4 mg/L, and that of aztreonam was 64 mg/L. MICs of ampicillin, ampicillin–sulbactam, piperacillin, piperacillin– tazobactam, cefalothin, cefoxitin, cefotaxime and ceftazidime were >128 mg/L. Isoelectric focusing of extract of the isolate showed two β-lactamase bands of pI ~5.3 and 9.0. The isolate showed positive modified Hodge and EDTA-disc synergy tests, and the only pI 5.3 band was no longer present when the gels were overlaid with EDTA, which are findings suggesting a metallo-β-lactamase. The band of pI ~9.0 was likely to be chromosomal AmpC cephalosporinase. A plasmid harbouring a carbapenem resistance determinant was not detected (data not shown). These results suggest that a metallo-β-lactamase gene may be located on the chromosome. VIM-2 metallo-β-lactamase has no hydrolytic activity against aztreonam, but the MIC of aztreonam for *P.putida* was 64 mg/L, which is higher than the resistant breakpoint. This result was possibly due to production of a chromosomal cephalosporinase (pI ~9.0). The gel electrophoresis analysis showed a band about 800 bp for bla_{VIM} gene in accordance with bla_{VIM} gene sequence. Carbapenem-hydrolyzing metallo-ß-lactamases, especially IMP-type and VIM-type metallo-ß-lactamases, are clinically important, because these enzymes effectively hydrolyze almost all ß-lactam antibiotics except monobactams, conferring resistance to penicillins and cefepime in addition to carbapenems on pathogenic bacteria. Since genes encoding these metallo-ß-lactamases (bla_{IMP} and bla_{VIM}) and their variant genes have become easy to detect using the PCR method, since 1989 the dissemination of these genes in clinical isolates has been widely observed in gram-negative bacteria, especially in *Pseudomonas aeruginosa* and other non-glucose-fermenting bacteria [16]. Multiple-drug resistance *P. putida* isolates producing VIM-type metallo-ß-lactamases were reported in Italy as a causative species of nosocomial infections. [14, 19, 4, 8]. Luzzaro *et al*., 2004 [7] reported that the sizes of the integron carrying the bla_{VIM} varied among the isolates from 3 to 6 kb. Prevalence of metallo-ß-lactamase-producing *P.putida* is an important clinical problem, representing a reservoir of genetic determinants of multi-drug resistance. The *P. putida* isolate PCR product which has been amplified and used as template for sequence reaction (Fig.1). The result of sequence alignment of bla_{vim} sequence from Sulaimani hospitals against bla_{Vim2} gene of . *putida* class 1 integron which published by Lee *et al*. in 2002, in korea, (ACCESSION: AF327064.1) showed that the sequence has a length of about 3057 bp., identities were 792/803 (98%), which indicate that there were 11 mutations in bla_{Vim} of *p. putida* isolated from Sulaimani (Fig. 2).

ATGTTCAAACTTTTGAGTAAGTTATTGGTCTATTTGACCGC
GTCTATCATGGCTATTGCGAGTCCGCTCGCTTTTTCCGTAG
ATTCTAGCGGTGAGTATCCGACAGTCAGCGAAATTCCGGTC
GGGGAGGTCCGGCTTTACCAGATTGCCGATGGTGTTTGGTC
GCATATCGCAACGCGGTCGTTTGATGGCGCAGTCTACCCGT
CCAATGGTCTCATTGTCCGTGATGGTGATGAGTTGCTTTGA
TTGATACAGCGTGGGGTGCGAAAAACACAGCGGCACTTCT
CGCGGAGATTGAGAAGCAAATTGGACTTCCTGTAACGCGT
GCAGTCTCCACGCACTTTCATGACGACCGCGTCGGCGGCGT
TGATGTCCTTCGGGCGGCTGGGGTGGCACGTACGCATCACC
GTCGACACGCCGGCTAGCCGAGGTAGAGGGGAGCGAGATT
CCCACGCACTCTCTAGAAGGACTCTCATCGAGCGGGGACG
CAGTGCGCTTCGGTCCAGTAGAACTCTTCTATCCTGGTGCT
GCGCATTCGACCGACAACTTAGTTGTGTACGTCCCGTCTGC
GAGTGTGCTCTATGGTGGTTGTGCGATTTATGAGTTGT
CACGCACGTC
TGCGGGGAACGTGGCCGATGCCGATCTGGCTGAATGGCCC
ACCTCCCATTGAGCGGATTCAACAACACTACCCGGAAGCA
CAGTTCGTCATTCCGGGGCACGGCTTGCCGGGCGGTCTAGA
CTTGCTCAAGCACACAACGAATGTTGTAAAAGCGCACACA
ACGCTCAGTCGTTGAGTAGCAGGCAGATGCGGCATAACAT
GAAGTT

Fig. 1: Complete sequence of the bla_{VIM} gene in *P. putida* isolate

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Query 1  ATGTTCAAACTTTTGAGTAAGTTATTGGTCTATTTGACCGCGTCTATCATGGCTATTGCG
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Query 61 AGTCCGCTCGCTTTTTCCGTAGATTCTAGCGGTGAGTATCCGACAGTCAGCGAAATTCCG
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Sbjct613 AGTCCGCTCGCTTTTTCCGTAGATTCTAGCGGTGGTATCCGACAGTCAGCGAAATTCCG

Query121 GTCGGGGAGGTCCGGCTTTACCAGATTGCCGATGGTGTTTGGTCGCATATCGCAACGCGG
         |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct673 GTCGGGGAGGTCCGGCTTTACCAGATTGCCGATGGTGTTTGGTCGCATATCGCAACGCAG
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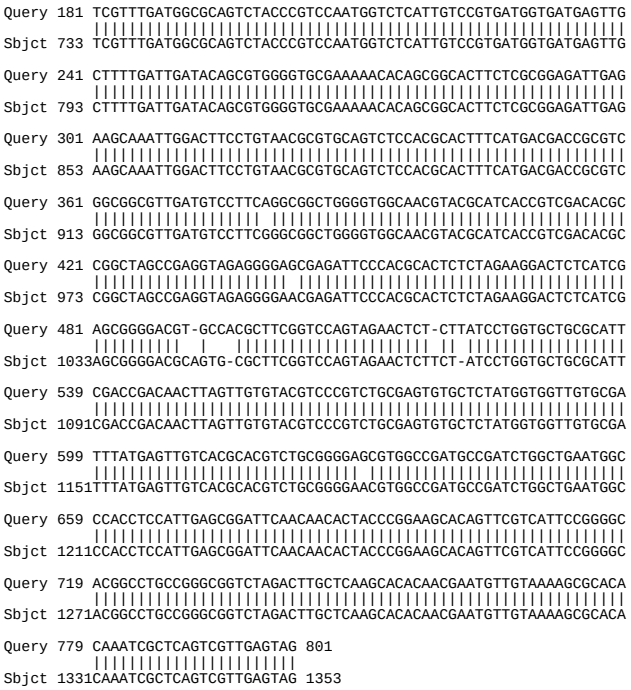


Fig. 2 Sequence alignment of *bla*$_{Vim}$ sequence from Sulaimani hospitals against *Vim2* gene of *p. putida* class 1 integron (*bla* $_{VIM}$-2), (ACCESSION: AF327064.1)

Sequence alignment of *bla*$_{Vim}$ sequence from Sulaimani hospitals against *Vim6* gene of *p. putida* strain DU25165/00 (*bla* $_{VIM-6}$) (ACCESSION: AY165025.1). Sequence has a length of about 828 bp. Identities were 821/830 (98%) (Fig 4). The results showed that there were 9 mutations for the *bla*$_{Vim\ 6}$ of *p. putida* strain DU25165/00 which was first published by Koh *et al*., in (2004)(5) in Singapore (Fig 3).

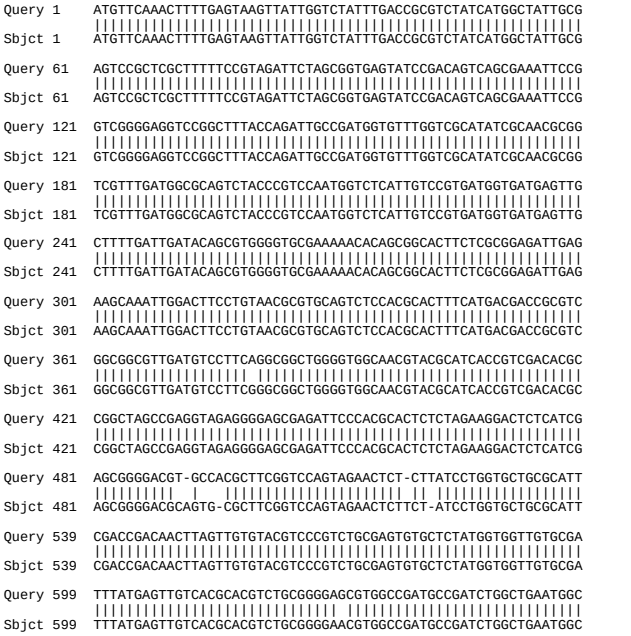


Fig. 3 Sequence alignment of *bla*$_{Vim}$ sequence from Sulaimani hospital against *Vim6* gene of *p. putida* strain DU25165/00 (blaVIM-6) (ACCESSION : AY165025.1)

Sequence alignment of *bla*$_{Vim}$ sequence from Sulaimani hospitals against *Vim2* gene of *p. putida* strain YMC 98/2/665 class I integron (*bla* $_{VIM-2}$), (ACCESSION: AY907717.1). Sequence has a length of about 5325 bp. Identities were 792/803 (98%).The results showed that there were 11 mutations for the *bla*$_{Vim\ 2}$ of p. *putida* strain YMC 98/2/665 which was first identified in Korea in 2005 by Yan (19) (Fig. 4).

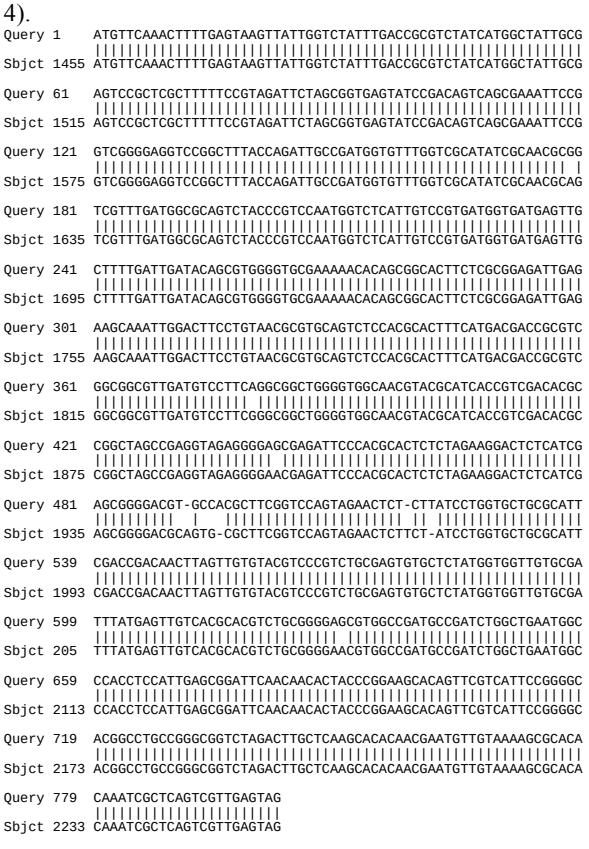


Fig. 4 Sequence alignment of *bla*$_{Vim}$ sequence from Sulaimani hospitals against *Vim2* gene of *p. putida* strain YMC 98/2/665 class I integron (blaVIM-2), (ACCESSION: AY907717.1)

Sequence alignment of *bla*$_{Vim}$ sequence from Sulaimani hospitals against *Vim2* gene of *p. putida* transposon Tn1332 (ACCESSION: DQ174113.1). The sequence has a length of about 11132 bp. Identities were 792/803 (98%) (Fig. 5). The results showed that there were 11 mutations for the *bla*$_{Vim\ of}$ p.

putida transposon Tn1332 which was first published in 2006 by Poirel *et al*., in France[11].

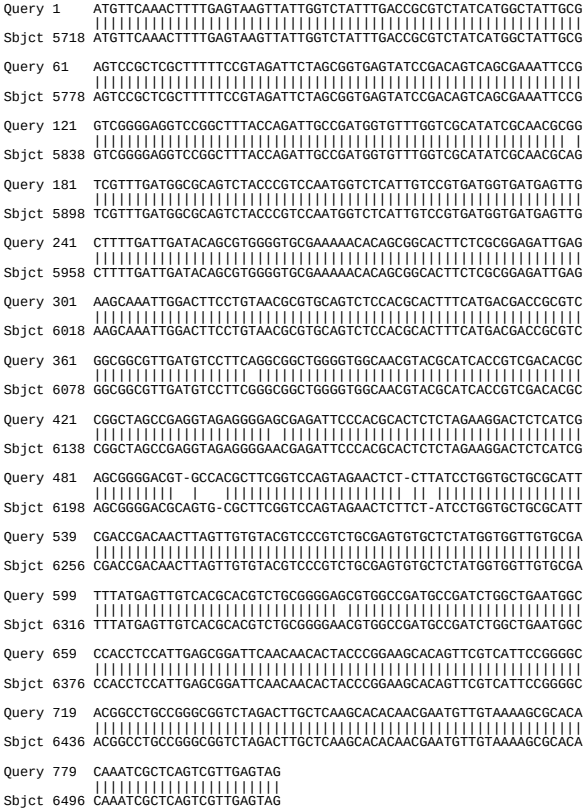
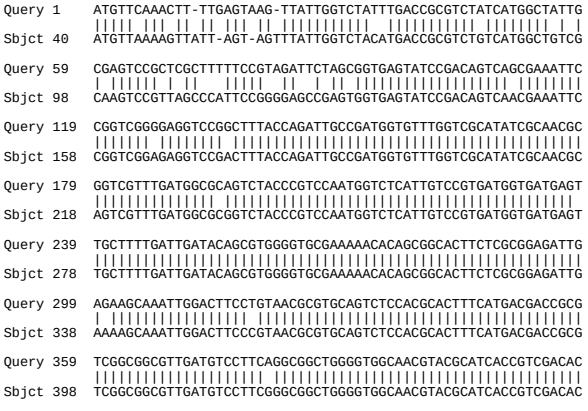


Fig. 5 Sequence alignment of bla_{Vim} sequence from Sulaimani hospitals against *Vim2* gene of *p. putida* transposon Tn1332 (ACCESSION : DQ174113.1)

Sequence alignment of bla_{Vim} sequence from Sulaimani hospitals against *Vim1* gene of *p. putida* strain A2580/277 (VIM-1) gene, (ACCESSION : EU118150.1). The sequence has a length of about 843 bp, Identities were 738/809 (91%) (Fig. 6). The results showed that there were 71 mutations for the $bla_{Vim\ 1}$ of p. *putida* strain A2580/277 which was first identified in Greece by Papadopoulou *et al*. in 2007[12].



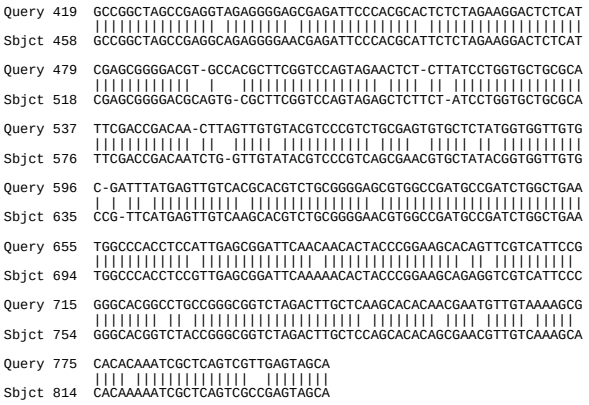


Fig. 6 Sequence alignment of bla_{Vim} sequence from Sulaimani hospitals against *Vim1* gene of *p. putida* strain A2580/277 (VIM-1) gene, (ACCESSION : EU118150.1)

Sequence alignment of bla_{Vim} DNA sequence from Sulaimani hospitals against *Vim* gene of *p. putida* strain PFi class 1 integron (ACCESSION FJ237530). The sequence has a length of about 1904 bp. Identities between the bla_{Vim} DNA sequence from Sulaimani hospitals and $bla_{Vim\ 2}$ of *p. putida* strain PFi isolated in Portugal were 792/803 (98%) (Fig. 7). The results showed there were 10 mutations for the $bla_{Vim\ 2}$ of p. *putida* strain PFi which include transversion, deletion and insertion).The information about this sequence was first submitted by Santos *et al*.(2008) in Portugal [16].

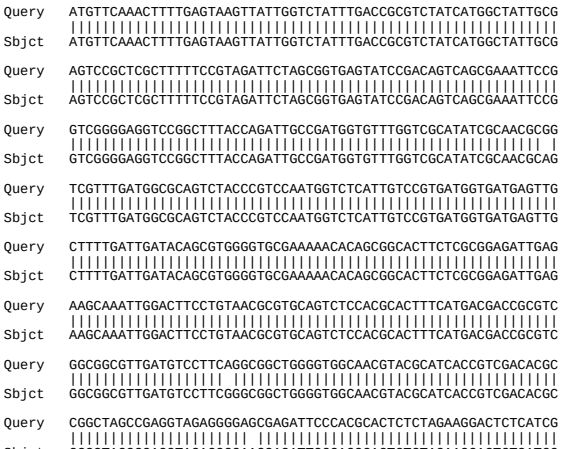


Fig. 7 Sequence alignment of Sequence alignment of *bla*$_{Vim}$ DNA sequence from Sulaimani hospitals against *Vim* gene of *p. putida* strain PFi class 1 integron (ACCESSION FJ237530)

Sequence alignment of bla$_{Vim}$ sequence from Sulaimani hospitals against Vim4 gene of *p. putida* strain 283-02 class 1 integron (ACCESSION : FM179466.1).The sequence has a length of about 3329 bp. Identities between the bla$_{Vim}$ sequence from Sulaimani hospitals and bla$_{Vim\ 4}$ of *p. putida* strain 283-02 isolated in Poland were 736/807 (91%) (Fig. 8). The results showed that there were 71 mutations for the bla$_{Vim}$ 2 of *p. putida* strain 283-02 which was first published in 2009 by Patzar *et al*., in Poland [9].

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Query 1    ATGTTCAAACTT-TTGAGTAAG-TTATTGGTCTATTTGACCGCGTCTATCATGGCTATTG
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Sbjct 274  ATGTTAAAAGTTATT-AGT-AGTTTATTGGTCTACATGACCGCGTCTGTCATGGCTGTCG

Query 59   CGAGTCCGCTCGCTTTTTCCGTAGATTCTAGCGGTGAGTATCCGACAGTCAGCGAAATTC
           | |||||| | ||   |||||  ||  | || ||||||||||||||||||||| ||||||||
Sbjct 332  CAAGTCCGTTAGCCCATTCCGGGGAGCCGAGTGGTGAGTATCCGACAGTCAACGAAATTC

Query 119  CGGTCGGGGAGGTCCGGCTTTACCAGATTGCCGATGGTGTTTGGTCGCATATCGCAACGC
           |||||||| |||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct 392  CGGTCGGAGAGGTCCGACTTTACCAGATTGCCGATGGTGTTTGGTCGCATATCGCAACGC

Query 179  GGTCGTTTGATGGCGCAGTCTACCCGTCCAATGGTCTCATTGTCCGTGATGGTGATGAGT
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Sbjct 452  AGTCGTTTGATGGCGCGGTCTACCCGTCCAATGGTCTCATTGTCCGTGATGGTGATGAGT

Query 239  TGCTTTTGATTGATACAGCGTGGGGTGCGAAAAACACAGCGGCACTTCTCGCGGAGATTG
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query 299  AGAAGCAAATTGGACTTCCTGTAACGCGTGCAGTCTCCACGCACTTTCATGACGACCGCG
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Query 359  TCGGCGGCGTTGATGTCCTTCAGGCGGCTGGGGTGGCAACGTACGCATCACCGTCGACAC
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Sbjct 632  TCGGCGGCGTTGATGTCCTTCGGGCGGCTGGGGTGGCAACGTACGCATCACCGTCGACAC

Query 419  GCCGGCTAGCCGAGGTAGAGGGGAGCGAGATTCCCACGCACTCTCTAGAAGGACTCTCAT
           |||||||||||||| |||||||| |||||||||||||| ||||||||||||||||||||
Sbjct 692  GCCGGCTAGCCGAGGCAGAGGGGAACGAGATTCCCACGCATTCTCTAGAAGGACTCTCAT

Query 479  CGAGCGGGGACGT-GCCACGCTTCGGTCCAGTAGAACTCT-CTTATCCTGGTGCTGCGCA
           |||||||||||| |  ||||||| ||||||||| |||| || ||||||||||||||||||
Sbjct 752  CGAGCGGGGACGCAGTG-CGCTTCGTTCCAGTAGAGCTCTTCT-ATCCTGGTGCTGCGCA

Query 537  TTCGACCGACAA-CTTAGTTGTGTACGTCCCGTCTGCGAGTGTGCTCTATGGTGGTTGTG
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Query 596  C-GATTTATGAGTTGTCACGCACGTCTGCGGGGAGCGTGGCCGATGCCGATCTGGCTGAA
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Sbjct 869  CCG-TTCATGAGTTGTCACGCACGTCTGCGGGGAACGTGGCCGATGCCGATCTGGCTGAA

Query 655  TGGCCCACCTCCATTGAGCGGATTCAACAACACTACCCGGAAGCACAGTTCGTCATTCCG
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Sbjct 928  TGGCCCACCTCCGTTGAGCGGATTCAAAAACACTACCCGGAAGCAGAGGTCGTCATTCCC

Query 715  GGGCACGGCCTGCCGGGCGGTCTAGACTTGCTCAAGCACACAACGAATGTTGTAAAAGCG
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Sbjct 988  GGGCACGGTCTACCGGGCGGTCTAGACTTGCTCCAGCACACAGCGAACGTTGTCAAAGCA

Query 775  CACACAAATCGCTCAGTCGTTGAGTAG
           |||| ||||||||||||||  |||||||
Sbjct 1048 CACAAAAATCGCTCAGTCGCCGAGTAG
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Fig. 8 Sequence alignment of *bla*$_{Vim}$ sequence from Sulaimani hospitals against *Vim4* gene of *p. putida* strain 283-02 class 1 integron (ACCESSION : FM179466.1)

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1. Ph.D in Microbiology/ Molecular biology . Joined in June 2008 from College of Science - Univ. of Sulaimani

2. M.Sc. in Microbiology, Joined in November, 2002- College of of Science- Univ. of Sulaimani

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1. Member of Scientific commiti in biology dept.
2. Member of Biology Syndicate of Kurdistan.
3. Member of Kurdistan universities association
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Published Research :

1- The use of Bacillus cereus phospholipase C in prophylaxis and treatment of thromboplastin induced thrombosis in mice. Kurdistan Academic J. 2004.
2- Emergency of vancomycin resistant Staphylococcus aureus burned patients in Emergency hospital in Sulaimani city, Kurdistan region, Iraq. Journal of Karkok University.
3- Comparison of Tn1546 element of vancomycin resistant Staphylococcus aureus isolated from burned patients in Sulaimani hospital. Published in International conference proceeding on bioinformatics and biomedical technology -April 2010.
4- Comparative analysis of the Tn1546 element from newly isolated and identified vancomycin resistant Staphylococcus aureus strain isolated from burn suffering human patients hospitalized at intensified care unit Sulaimani Central Hospital, Iraq. FEBS – June 2010.

Conference Attended:

1- The 2nd Kurdistan Conference on Biological Science that was held in Dohuk university in 6-8/4/2008.
2- The First science Confereance on applied science that held in Kirkoke University in 24-26/3/2009.
3- Participated in (2nd power-lab workshop and new technique in bio-science). Eqlem Danesh Co. on des.29-30, 2008 in shahid Behashti medical science, university, Tehran, Iran
4-Participated in “The 2010 International Conference on Bioinformatics and Biomedical Science“ in 16-18 April 2010, Chengdu, China.
5- Participated in “International Conference on Biological Science and Engineering” in 24-26 Nov. 2010 , Venice, Italy.