A New \textit{bla}_{VIM} Gene in a \textit{Pseudomonas putida} Isolated from ENT Units in Sulaimani Hospitals

Dalanya Asaad Mohammed, and Dara Abdul Razaq

\textbf{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 \textit{Pseudomonas putida} with probability of 99\% was isolated. Antimicrobial susceptibility was carried out by disk diffusion method, \textit{Pseudomonas putida} showed resistance to all antibiotics used except vancomycin. The isolate further subjected to PCR and DNA sequence analysis of \textit{bla}_{VIM} gene using different set of primers for different regions of \textit{VIM} gene. The results were found to be PCR positive for the \textit{bla}_{VIM} gene. To determine the sequence of \textit{bla}_{VIM} gene, DNA sequencing performed. Sequence alignment of \textit{bla}_{VIM} gene with previously recorded \textit{bla}_{VIM} gene in NCBI-database showed that \textit{P. putida} isolate have different \textit{bla}_{VIM} gene.

\textbf{Keywords}—Clinical isolates, Putida, Sulaimani, Vim gene.

\section{I. INTRODUCTION}

\textbf{MICROORGANISMS} might exhibit resistance to drugs by many different mechanisms. The most important mechanism is \(\beta\)-lactamases production, which are a group of enzymes capable of hydrolysing the 4-membered \(\beta\)-lactam ring of beta-lactam antibiotics \cite{1}, which can be either chromosomally encoded or plasmid mediated \cite{11}. Several novel MBLs were identified, including \textit{VIM}-1 from \textit{P. aeruginosa} and \textit{IMP}-2 from \textit{Acinetobacter baumannii} in Italy \cite{15}, \textit{VIM}-2 from \textit{P. putida} in France \cite{119}, and \textit{IMP}-3 from \textit{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 \cite{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 \textit{P. putida} among the isolates.

\section{II. METHODS}

\textbf{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.

\textbf{B. Antimicrobial susceptibility ans Isoelectric focusing of \(\beta\)-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. \textit{Escherichia coli} ATCC 25922 were used as MIC reference strain. Modified Hodge and EDTA-disc synergy tests were performed for the screening of metallo-\(\beta\)-lactamide-producing strains. The results were compared to CLSI standard 2008. The isoelectric points of \(\beta\)-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.

\textbf{C. Molecular methods}

\textbf{Amplification of \textit{VIM} gene of \textit{pseudomonas putida} by} direct colony PCR: A single bacterial colony which is previously cultured on nutrient agar was dissolved in 50 \(\mu\)l dd H\(_2\)O (MQ). The cells suspension was incubated at 37\(^\circ\)C 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\(^\circ\)C heating and 1 cycle for 5 min. at 4\(^\circ\)C cooling. The samples were centrifuged at 13000 rpm for 10 minutes. The pellet was discarded and 5 \(\mu\)l of supernatant were used as template in the PCR reactions. Master mix was prepared by adding 5 \(\mu\)l of tag buffer, 2.5\(\mu\)l of \((f \text{ and } r)\) primer, 1\(\mu\)l of dNTP(10mM), 5\(\mu\)l of supernatant, 3 \(\mu\)l MgCl\(_2\), and 0.3\(\mu\)l tag polymerase to 30.7\(\mu\)l DDH\(_2\)O (50 \(\mu\)l 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-\(\beta\)-lactamase genes was carried out with primers VIM-DIA/\(f\) and VIM-DIA/r in a 50\(\mu\)l volume Reaction parameters were as follows: Annealing at 55\(^\circ\)C for 60s extension at 72\(^\circ\)C for 90s denaturation at 94\(^\circ\)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

\textbf{Dlnya A. Mohammed} is with Biology department, College of Science, University of Sulaimani, Sulaimani City, Kurdistan Region of Iraq. (phone 009647701414371, 00964533290165; e-mail: dlnya.mohamad@univsul.net.)
photographed digitally, as was the preparation of recombinant plasmids containing PCR product, and transformation of them into *E. coli* DH5<sup>+</sup>. Plasmids from successful clones were used to determine the sequence of the *bla<sub>VIM</sub>* gene by the dideoxynucleotide-chain termination method, with an automatic DNA sequencer (ABI 3700), in Adden institute for molecular biology techniques/Teheran - Iran. The determination of the sequence was repeated with more than two clones from independent ampiclons. 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 StaKleen. StaKleen 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, cefalotin, cefoxitin, cefotaxime, cefazidime 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, cefalotin, cefoxitin, cefotaxime and ceftazidime were >128 mg/L. Isoelectric focusing of extract of the isolate showed two independent amplicons. Both strands were sequenced.

**Fig. 1:** Complete sequence of the *bla<sub>VIM</sub>* gene in *P. putida* isolate

---

IMP-type and VIM-type metallo-β-lactamases, are clinically important, because these enzymes effectively hydrolyze almost all β-lactam antibiotics except monobactams, conferring resistance to penicillins and cephalosporins in addition to carbapenems on pathogenic bacteria. Since genes encoding these metallo-β-lactamases (*bla<sub>VIM</sub>* and *bla<sub>VIM</sub>*<sup>2</sup>) and their variant genotypes have become easy to detect using the PCR method, since 1989 the dissemination of these genes in nosocomial infections, especially in *Pseudomonas aeruginosa* and other non-glucose-fermenting bacteria, especially in *Pseudomonas aeruginosa* and other non-glucose-fermenting bacteria [16]. Multi-drug resistance *P. putida* isolates producing VIM-type metallo-β-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<sub>VIM</sub>* varied among the isolates from 3 to 6 kb. Prevalence of metallo-β-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 genetic sequence reaction (Fig. 1). The result of sequence alignment of *bla<sub>VIM</sub>* sequence from Sulaimani hospitals against *bla<sub>VIM</sub>* gene of *P. 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<sub>VIM</sub>* from *P. putida* isolated from Sulaimani (Fig. 2).
Sequence alignment of blavim sequence from Sulaimani hospitals against Vim2 gene of p. putida strain DU25165/00 (blaVIM-6) (ACCESSION: AY165025.1)

Sequence alignment of blavim sequence from Sulaimani hospitals against Vim2 gene of p. putida strain YMC 98/2/665 class I integron (blaVIM-2), (ACCESSION: AF327064.1)

Sequence alignment of blavim sequence from Sulaimani hospitals against Vim6 gene of p. putida strain DU25165/00 (blaVIM-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 blavim of p. putida strain YMC 98/2/665 which was first published in Korea in 2005 by Yan (19) (Fig. 4).

The results showed that there were 11 mutations for the blavim 2 of p. putida strain YMC 98/2/665 which was first published in 2005 in Korea by Yan (19) (Fig. 4).

Sequence alignment of blavim sequence from Sulaimani hospitals against Vim2 gene of p. putida strain YMC 98/2/665 class I integron (blaVIM-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 blavim of p. putida strain YMC 98/2/665 which was first identified in Korea in 2005 by Yan (19) (Fig. 4).
**putida** transposon Tn1332 which was first published in 2006 by Poirel *et al.*, in France[11].

Sequence alignment of *bla*\textsubscript{Vim} \textsuperscript{1} DNA sequence from Sulaimani hospitals against *Vim*\textsubscript{1} gene of *p. putida* strain A2580/277 (VIM-1) gene, (ACCESSION : EU118150.1).

The results showed there were 10 mutations for the *bla*\textsubscript{Vim} \textsuperscript{1} of *p. putida* strain PFi which include transversion, deletion and insertion.

The information about this sequence was first submitted by Santos *et al.* in Portugal [16].

**Fig. 6** Sequence alignment of *bla*\textsubscript{Vim} \textsuperscript{1} sequence from Sulaimani hospitals against *Vim*\textsubscript{2} gene of *p. putida* strain A2580/277 (VIM-1) gene, (ACCESSION : EU118150.1).

The sequence alignment of *bla*\textsubscript{Vim} \textsuperscript{1} DNA sequence from Sulaimani hospitals against *Vim*\textsubscript{2} gene of *p. putida* strain PFi class 1 integron (ACCESSION FJ237530). The sequence has a length of about 1904 bp. Identities between the *bla*\textsubscript{Vim} \textsuperscript{1} DNA sequence from Sulaimani hospitals and *bla*\textsubscript{Vim} \textsuperscript{1} of *p. putida* strain PFi isolated in Portugal were 792/803 (98%) (Fig. 7). The results showed there were 10 mutations for the *bla*\textsubscript{Vim} \textsuperscript{1} 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].
Sequence alignment of \(bla_{\text{vim}}\) sequence from Sulaimani hospitals against \(Vim^4\) gene of \(p.\ putida\) strain 283-02 class 1 integron (ACCESSION : FM179466.1).

The sequence showed a length of about 3329 bp. Identities between the \(bla_{\text{vim}}\) sequence from Sulaimani hospitals and \(bla_{\text{vim}}^4\) 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 \(bla_{\text{vim}}\) 2 of \(p.\ putida\) strain 283-02 which was first published in 2009 by Patzar et al., in Poland [9].

Fig. 8 Sequence alignment of \(bla_{\text{vim}}\) sequence from Sulaimani hospitals against \(Vim^4\) gene of \(p.\ putida\) strain 283-02 class 1 integron (ACCESSION : FM179466.1).

REFERENCES


\beta\)-lactamase, VIM-6, in fluorescent pseudomonads isolated in Singapore.


[6] Lee K., Jong Back Lim, Jong Hwa Yun, 2002 *blaVim* Cassette-Containing Novel Integrons in Metallo-\(
\beta\)-Lactamase-Producing *Pseudomonas aeruginosa* and *Pseudomonas putida* Isolates Disseminated in a Korean Hospital, Antimicrob Agents Chemother. April; 46(4): 1053–1058.


\beta\)-lactamase gene in *Pseudomonas putida* clinical isolates. *J of microbiology and infectious disease*, vol. 4, 118.

\beta\)-lactamase, a class A carbapenem-hydrolyzing enzyme from Enterobacter cloacae. *Antimicrobial Agents and Chemotherapy* 40, 2080–6.

\beta\)-lactamase determinant of *Acinetobacter baumannii* AC-5497 reveals the existence of \(bla_{\text{vim}}\) allelic variants carried by gene cassettes of different phylogeny. Antimicrob Agents Chemother.44:1229–1235

\beta\)-Lactamase Gene *blaIMP* in a Hospital in Japan. *Journal Of Clinical Microbiology*, p. 4246–4251


[17] Siarkou, V., Papadopoulou, C., Thisiadou, K. and Sofianou, 2007. *JOURNAL* Submitted Laboratory of Microbiology and Infectious Diseases, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, University Campus, Thessaloniki 54124, Greece.


Dnya A. Mohamed (M’09) From Iraq-Kurdistan region- Sulaimani in 1970.

Educational Qualifications: (beginning with most recent)

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 Science- Univ. of Sulaimani
3. B.Sc. in Microbiology, Joined in June 1992- College of Science, Univ. of Mostenseryah
4. High school Sulaimani preparatory for girls, Joined in 1988
She was working in bacteriology department, central lab. In Sulaimani city, Ministry of Health from 1993 to 1999, then asst. Lecturer in Biology dept/ College of Science/ Univ. of Sulaimani. (2003-2008), finally she is now lecturer in Biology dept/ College of Science/ Univ. of Sulaimani. (2008-).
1. Member of Scientific committee in biology dept.
2. Member of Biology Syndicate of Kurdistan.
3. Member of Kurdistan universities association
4. Member of Iraqi DNA based diagnosis Research center colaborated with Jordan Institute of DNA based diagnosis.

Published Research:
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 Conference on applied science that held in Kirkoke University in 24-26/3/2009.
3- Participated in (2nd power-lab workshop and new technique in bi-science). Eqlem Danesh Co. on des.29-30, 2008 in shahid Behashi 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.