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Research article

Detection and sequencing of *bla*$_{VEB-1}$ gene in clinical isolates of Proteus mirabilis Isolates from Baghdad City`s hospitals

Maysoon Munther Al-Hamando[1], Dimah Nazar Faraj[1]*, Lina Arjamand[1]

ABSTRACT

In Present study, 25 clinical isolates of *Proteus spp.* of clinical samples, urine, wounds and burns collected from different hospitals in Baghdad city, all isolates were identified as *Proteus mirabilis* using different bacteriological media, biochemical assays and Vitek-2 system. It was found that 15 (60%) isolates were identifying as *P. mirabilis*. The susceptibility of *P. mirabilis* isolates to cefotaxime was 66.6 %, while to ceftazidime was 20%. Extended spectrum β-lactamses producing *Proteus* was 30.7 %. DNA of 5 isolates of *P. mirabilis* was extracted and detection for *bla*$_{VEB-1}$ gene by using multiplex polymerase chain reaction (PCR). Results showed that the presence of this gene in all tested isolates, as an important indicator for increasing risk of extended spectrum beta lacatmases resistant *P. mirabilis* isolates against antimicrobial agents due to its spread recently in Middle and Far East of Asia. Sequencing of DNA nucleotides was carried out with automated sequencer (Macrogen/ Korea) and confirmed that 3 isolates of *P. mirabilis* have *bla*$_{VEB-1}$ gene, while one isolate has *bla*$_{VEB-3}$ gene.

Keywords: *Proteus mirabilis*, bla$_{VEB-1}$ gene, beta-lactamases, EXBLs enzymes

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INTRODUCTION

Proteus genus members are widely distributed in the natural environment. They can be found in polluted water, soil and manure, where they play an important role in decomposing organic matter of animal origin [1, 2]. *Proteus spp.*, especially *Proteus mirabilis* are common opportunistic pathogens in human. *P. mirabilis* is the third most common (after *Escherichia coli* and *Klebsiella pneumoniae*) cause of complicated urinary tract infection (UTI), it has ability to form stones in the bladder and kidney, as well as its ability to form crystalline biofilms on the outer surface and in the lumen of indwelling urinary catheters in hospital acquired patients.

Furthermore, it consider as infectious agents of the respiratory tract and wounds, burns, skin, eyes, ears, nose, and throat, as well as in gastroenteritis resulting from the consumption of contaminated meat or other kinds of food [3, 4].

Beta-lactam antimicrobial agents contain a β- lactam ring that inhibits cell wall synthesis of bacteria by binding to penicillin binding proteins (PBPs), [5, 6]. β-lactams comprise a very large family, that are distinguished by the structure of the ring attached to the β-lactams ring [7].

β- lactamases enzymes are the main cause of bacterial resistance to β-lactam antimicrobial agents, which are produced

* Correspondence: Faraj DN.dimahpassat2011@gmail.com
Department of Biology, College Science, University of Baghdad, Baghdad, Iraq.
Full list of author information is available at the end of the article.

by some bacterial genus and able to hydrolyze beta-lactam antimicrobial drugs. Widespread use of antimicrobial agents cause increasing the rate of β- lactamases producing bacteria, especially extended spectrum β-lactamases (ESBLs), The genes coding for ESBLs are often located on self-transmissible or mobilizable broad- host range plasmids. Most ESBLs are evolved by genetic mutation from native β-lactamases [8, 9, 10]. Most *P. mirabilis* isolates are susceptible to β-lactams, because they do not express β-lactamases, some isolates produce a broad spectrum β-lactamases (TEM, SHV and CARB types), and extended-spectrum β-lactamases (ESBLs). Recently a high prevalence of ESBLs among *P. mirabilis* caused invasive infections. Clinical occurrences of ESBL-producing *P. mirabilis* strains have increased since ESBL production in *P. mirabilis* was first documented in 1987 [11, 4].

Resistance to extended-spectrum lactams antimicrobial agents is a critical clinical problem. In addition to the large TEM, SHV, and CTX families, several minor extended spectrum-lactamases have been identified, including VEB. All VEB enzymes identified to date are minor variants of VEB-1 depending on amino acid differences between enzymes; including (1,1a, 1b, 2, 3, 4, 5, 6), which confers a high level of resistance to ceftazidime, cefotaxime, and aztreonam. *bla*$_{VEB-1}$ genes have been identified in a variety of species of *Enterobacteriaceae* and in non-fermenting bacilli from Asia, Europe, the Middle East, Africa, and North and South America on both plasmids and the chromosome [12]. The most predominant plasmid-mediated β-lactamases found in the *P. mirabilis* clinical isolates are TEM-derived ESBLs and CMY-3, CMY-4, CEP-1, CTX-M-2 and PER-2, which have also been reported in specific geographical area. While VEB-1, has been described in *Enterobacteriaceae* and *Pseudomonas aerugin-osa* from Southeast Asia, and *Acinetobacter baumannii* from France [11].

However, according to researches, the emergence of VEB-1 ESBL has not been reported in the Far East but recently; the *bla*$_{VEB-1}$ gene has been reported in *Klebseilla pneumonia* and *E. coli* in Vietnamese, which was plasmid and integron-located; while it was found in *Pseudomonas aeruginosa* chromosomally and integron- located in Thailand [4, 11].

bla$_{VEB-1}$ was first described in a gene cassette in a class 1 integron, and most other examples of *bla*$_{VEB-1}$ genes where enough sequence data are available are also found in cassette arrays in class 1 integrons. These arrays are mostly related, containing different combinations from a limited set of cassettes in different configurations, suggesting rearrang-ements mediated by both homologous and IntI-catalyzed recombineation [12].

This study is an attempt to investigate the presence of *bla*$_{VEB-1}$ *gene* in *P. mirabilis* as an indicator for increasing the risk of extended spectrum beta lacatmases resistant isolates towards antimicrobial agents; because this gene is constricted in the Far East countries of Asia, but the current study showed that the presence of this gene in local isolates of *P. mirabilis* in Baghdad, Iraq.

MATERIALS and METHODS

Isolation and identification of *Proteus mirabilis*

Twenty five isolates of *Proteus spp.* were isolated from clinical samples (urine, wounds, and burns) of patients attending to several hospitals in Baghdad, Iraq during the period from October 2013 to March 2014. The samples were streaked on blood, MacConkey and Xylose lysine deoxychoclate agar (XLD agar plates). The plates were incubated aerobically at 37^{o} C for 24 h. The isolates were identified bacteriologically, biochemically according to [13, 14]. In addition, the morphological features on culture media such as Swarming on blood agar, Non lactose fermented growth on MacConkey agar and coloured growth on XLD agar were examined, then identification of bacteria was confirmed by using Vitek 2 identification system (Biomerieux/ Frane) [14].

Antibiotic susceptibility test

Antibiotic susceptibility test was performed for cefotaxime and cftazidime by Kiruby-Bauer method [13, 14]. The isolate was detected as susceptible, intermediate or resistant to these antimicrobial agents by comparison with standard inhibition zones according to Clinical Laboratories Standards Institute (CLSI, 2011).

Detection of ESBLs

Double-disk synergy test method was carried out for ESBLs-producing isolates detection process [15], by submerging a sterile cotton swab into bacterial suspension standardized to match the turbidity of the 0.5 McFarland turbidity standard (1.5 x 10^8 CFU/ml). The surface of Mueller Hinton agar plates were spread by the bacterial suspension, the plates were left for 10 min to dry. An amoxicillin/clavulanic acid (30 µg) disc was placed in the middle of Mueller Hinton agar plate, and then the discs of cefotaxime, ceftazidime and aztronam were arranged around the amoxicillin/clavulanic acid (30 µg) disc within 2-3 cm distance. The plates were inverted and incubated at 37^{o}C for 18 -24 h. After incubation, synergism activity between the central disk and any one of surrounding antimicrobial agents` discs was noted for ESBLs producing isolates detection.

Extraction of DNA

DNA was extracted from 5 isolates of *Proteus* by using a commercial purification kit (Presto Mini Genomic DNA Kit, (Geneaid, Thailand)). The Gram negative bacteria extraction protocol was used as manufacture protocol.

The extracted genomic DNA was confirmed by using gel electrophoresis in 0.8% agarose gel after staining with ethidium bromide and by UV transilluminator documentation system and photographed with digital camera for documentation of the results. The Nano- drop system was used to estimate concentration and purity of extracted DNA.

Amplification of *bla*$_{VEB-1}$ by Monoplex PCR

Monoplex PCR was performed for detection of whole 1081 bp *bla*$_{VEB-1}$ gene in *P. mirabilis* from 47- 946 bp by using specific primers (**Table 1**). After extraction and detection of genomic DNA samples, PCR reactants and amplification program were prepared in final concentration of 50 µl, template genomic DNA < 250 ng (5 µl), forward and reverse primer 10 pmol/ µl (5 µl), Go Taq green Master mix 2X (25 µl), de-ionized distilled water (10 µl), while the PCR reaction was performed; Initial denaturation at 94 ^{o}C for 10 min., denaturation at 94 ^{o}C for 1 min, annealing at 51 ^{o}C for 1 min, extension at 72 ^{o}C for 3 min. and final extension for 72 ^{o}C for 7 min. repeated for 35 cycles. The PCR product was confirmed by using gel electrophoresis in 1.5 % agarose gel after staining with ethidium bromide and by UV transilluminator documentation system and photographed with digital camera for documentation of the results**.**

Sequencing of monoplex PCR products

Monoplex PCR amlipcons for *bla*$_{VEB-1}$ gene were detected by gel electrophoresis and purification by Gel/ PCR DNA fragment extraction kit (Geneaid, Thailand), and then the sequencing

was carried according to Macrogen Company, Korea. PCR products were sequenced with an automatic sequencer, DNA sequences were analyzed and similarity searches were carried out with the Basic Local Alignment Search tool (BLAST) in the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov).

Table 1. Multiplex specific primers of bla$_{VEB-1}$ (Kim *et al.*, 2004).

Gene	Sequences	Product/ bp
bla$_{VEB-1}$	F 5' CGACTTCCATTTCCCGATGC 3' R 5' GGACTCTGCAACAAATACGC 3'	636

RESULTS and DISCUSSION

Morphological features on blood agar medium, MacConkey agar medium and XLD agar medium in addition to microscopic examination showed that 25 isolates were identified as *P. mirabilis* [13, 4]. Moreover, vitek 2 identification system was used to confirm the identification of these isolates. According to the results, 15 isolates (60 %) were *P. mirabilis*, and 10 isolates (40 %) were *P. vulgaris*.

The susceptibility of *P. mirabilis* isolates to cefotaxime and ceftazidime (The third generation of cephalosporins that have important role as a drug of choice) were investigated which resistant to these antibiotics are increased recently [6, 16]. Out of 15 *P. mirabilis* isolates, 10 isolates (66.6%) were resistant to cefotaxime, while 3 isolates (20%) were resistant to cftazidime (**Fig. 1**).

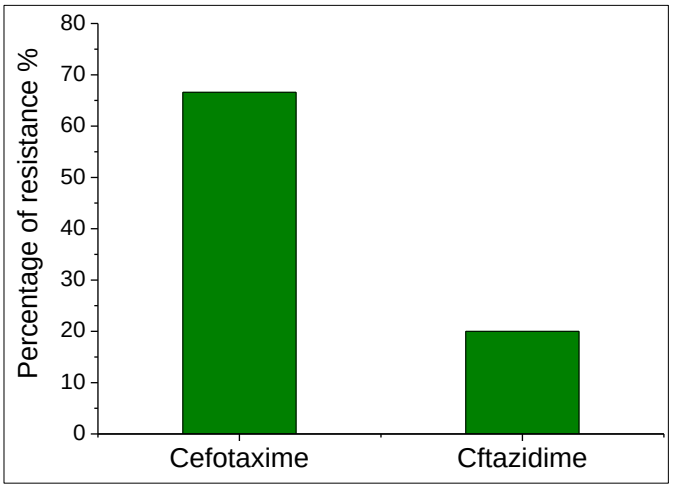

Fig 1. Precentage of resistance of *P. mirabilis* to cefotaxime and cftazidime

Double disk synergy method was carried out to detect the ability of *P. mirabilis* to produce ESBLs by determining the increase of inhibition zone against certain β-lactam antimicrobial agents, such as: amoxicillin/ clavulanic acid, cefotaxime, ceftazidime and aztronam, and compared with same antimicrobial agents lacking clavulanic acid by the same method. The results showed that 6 (40%) isolates of 15 *P. mirabilis* isolates were able to produce EXBLs enzymes **(Fig. 2).**

The percentage of resistance of *P. mirabilis* to antimicrobial agent is due to random use of the antimicrobial agents, mainly and health conditions of population in specific area in addition to ability of genetic material transmission which carried genes responsible of resistant to antimicrobial agents.

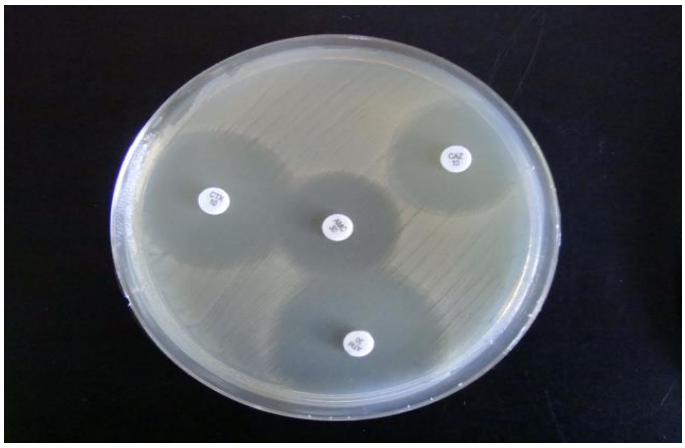

Fig 2. Production of ESBLs by *P. mirabilis* isolate using double disk synergy method (Cefotaxime, Ceftazidime, Aztronam, Amoxicillin/ Clavulanic acid).

In order to detect one of important genes that responsible of *P. mirabilis* resistance to antimicrobial agents by producing EXBLs enzyme; 5 isolates of *P. mirabilis* were selected for detection of *bla*$_{VEB-1}$ gene by extraction DNA from these isolates by using commercial DNA purification kit, then DNA concentration and purity were determined by using nano- drop system. The results showed that the concentrations of DNA were ranged from 53.1 ng/µl to 106.4 ng/µl, while purity was ranged from 1.2 to 2.3, the DNA purity was confirmed by using gel electrophoresis technique.

Detection of *bla*$_{VEB-1}$ gene in 5 isolates of *P. mirabilis* susceptible to cefotaxime was done by monoplex PCR technique. The *bla*$_{VEB-1}$ gene with 636 bp fragment size of the gene was found in all five isolates of *P. mirabilis* after confirmed by gel electrophoresis technique with molecular weight of *bla*$_{VEB-1}$ according to Kim *et al.,* (2004) by using 100bp DNA ladder (**Fig. 3**).

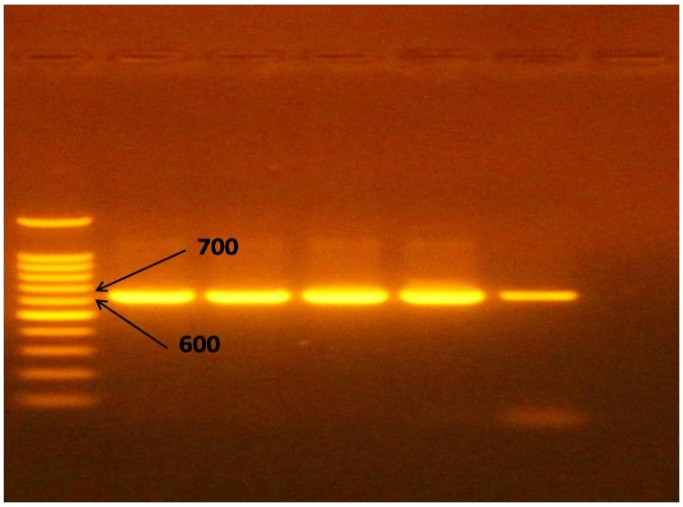

Fig 3. PCR reaction with monoplex set of primers for *P. mirabilis* isolates that contain *bla*$_{VEB-1}$ gene with DNA marker 1000 bp ladder (M), in 1.5 % agarose gel at 5 volt/cm for 1-2 h, visualized by E-graph gel documentation equipped by UV light source

bla$_{VEB-1}$ gene is one of genes that harbor EXBLs enzymes which is concerned as a main cause of *P. mirabilis* resistant towards antimicrobial agents specially the third generation of cephlocporines. This gene is recently detected in *P. mirabilis* in Middle East after detection of this gene first in Far East in Korea according to Kim *et al.* (2004). Attention of of *bla*$_{VEB-1}$ gene is increased because this gene distribution was limited to Europe, Africa, North and South of America but recently it is distribute in Asia specially Middle and Far east.

Sequencing of this gene was done in 5 isolates of *P. mirabilis* in order to confirm presence of *bla*$_{VEB-1}$ gene in *P. mirabilis* local isolates by automated sequencer according to Macrogen company/ Korea The results showed that *bla*$_{VEB-1}$ gene was found in 3 *P. mirabilis* isolates, while one isolates harbor *bla*$_{VEB-3}$, this agree with concept of *bla*$_{VEB}$ genes prevalence as a risk factor of increasing bacterial resistant towards antimicrobial agents in new geographic area. This is due to ability of transmission of genetic material, such as: plasmids carrying these genes between bacterial species, in addition to mutants that cause differences in amino acids sequencing and numbers.

Conflict of interest

The authors declare that they have no conflict of interests.

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Author affiliation

1. *Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.*