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RESEARCH ARTICLE

# Evaluation of Phylogenetic Tree of *bla*CTX-M Gene in *Pseudomonas earugenosa* Isolated form Corneal Scraping Specimens in Baghdad

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## Abstract

Nosocomial infections caused by *Pseudomonas earugenosa* (*P. earugenosa*) presenting resistance to beta-lactam drugs are one of the most challenging targets for antimicrobial therapy, leading to substantial increase in mortality rates in hospitals worldwide. In this context, *P. earugenosa* isolates that isolated form Ibn-Al Haitham teaching hospital for eyes diseases had carried *bla*CTX-M gene which have the highest clinical impact. Hence, this study was designed to investigate the presence of genes codifying for *bla* CTX-M among beta-lactam resistant *P. earugenosa* isolated in one of Baghdad hospitals. Antibiotic sensitivity was carried out using Kirby-Bauer method, and polymerase chain reaction was carried out in addition to the gell electrophoresis**,** the genotypic and the sequencing in aadtion th**e** phylogetic tree were done. Thirteen *P. aerogenosa* isolates were isolated from corneal scraping specimens and the results of antibiotic sensitivity showed that the most effective antibiotic on *P. aerogenosa* isolates was impeniem with 13(100%) sensitivity rate while the highest resistance was to cefozlen 13(100%) resistance rate. The investigation of the *bla*CTX-M gene via polymerase chain reaction showed the presence of *bla*CTX gene (544bp) in 3(23.1%) *P. aerogenosa* isolates. The sequencing of *bla*CTX-M amplicons confirmed the correct identification of *bla*CTX-M gene among *P. aerogenosa* isolates and the data obtained from the sequencing of *bla*CTX-M gene was submitted to the GenBank of National Center for Biotechnology Information (NCBI) under the accession number (KY966041). Molecular phylogenetic analyses were based on the sequences of *bla*CTX gene and closest relatives (100% identity) with closely related *bla*CTX-M gene of some bacterial isolates in the GenBank by using MEGA6 software.

**Keywords:** *Pseudomonas aerogenosa*, *bla*CTX M gene and phylogetic tree.

## Introduction

Bacterial resistance to antibiotics is ancient (D'Costa et al., 2011) and pre-dates their clinical use to prevent and cure bacterial infections (Allen et al., 2009; Lang et al., 2010). However, since the beginning of the current "antibiotic era" less than a 100 years ago, human-induced selective pressures against antibiotic-susceptible strains has led to an intractable rise in the frequency of resistant populations (e.g., [1] Falagas et al., 2005; Giske et al., 2008; Strahilevitz et al., 2009; Nordmann et al., 2011).

Global public health is now facing an epidemic of bacterial infections with reduced susceptibility to front-line clinical antibiotics such as cephalosporins and fluoroquinolones (Levy and Marshall, 2004), leading to therapeutic failure, worsening patient outcomes, and increased financial burdens on individuals and the health care system as a whole (Centers for Disease Control and Prevention [CDC], 2013; O'Neill Commission, 2014; World Health Organization [WHO], 2014). Viable solutions are urgently needed to protect the long-term efficacy of the antimicrobial agents that we have come to rely upon so heavily. Plasmid-mediated ESBLs confer high level resistance to cefotaxime, ceftriaxone, aztreonam and have only marginal effects on MIC of ceftazidime [2].

The identification of *Pseudomonas earugenosa* bacteria resist to a wide range of antibiotic The *Pseudomonas earugenosa* resist to board-spectrum antibiotics, they are responsible for hospital outbreaks.

They play a major role in antibiotic resistance because of their influence on microbial activity, Hence some mutation that transfer vertical to the next generation as well as there is mutation transfer via plasmid which is an extra genetic material transfer the resistance other bacterial cells which means spread of the antibiotic resistance gene among the group rapidly. The release of the acquired plasmid gene may pose a real public and environmental health risk. Most acquired resistance genes likely evolved in natural habitats before transferring into human pathogens through various horizontal gene transfer mechanisms (Martínez, 2009).

Different developed resistances genes progressed into the natural habitat prior to transfer to pathogenic bacteria. To understand antibiotic resistance genes spread into clinically relevant bacteria, we have focused our attention on the phylogenetic tree that carry same cluster. The aim of this study was to capture and characterize resistance *P. earugenosa* CTX-M-type ß-lactamases in isolates from In Al-Haitham clinical hospital patients, describe the complete nucleotide sequences of novel gene and figure out the phylogenetic tree.

## Materials and Methods

### Specimens' Collection

In this study, corneal scraping specimens were collected from patients admitted to Ibn-Al haitham teaching clinical hospital for eyes in Baghdad, over a period from April to October, 2016. Isolates were obtained from specimens originated from corneal scraping.

### Identification of Bacterial Strains

Specimens of corneal scraping were cultivated into suitable medium in laboratories, bacterial isolates diagnosed according to standard microbiology method [3]. All bacterial isolates (*Pseudomonas earugenosa)* were diagnosed using Api-20 NE (Analytic Profile Index 20 for non *Enterobacteriaceae*) (Bio-Merieux, France), to identify bacteria belong to non *Enterobacteriaceae* family.

### Antimicrobial Susceptibility Testing

Antimicrobial susceptibility test to 10 different antibiotics was determined by the disk diffusion method, following Clinical and Laboratory Standards Institute recommendations (Tollentino FM, *et al.* 2011). Thirteen *Pseudomonas aerogenosa* isolates were tested for antimicrobial resistant according to Kirby-Bauer (disk diffusion) technique, by using Muller-Hinton agar and different types of antimicrobial discs which supplied commercially (Table-1). Inhibition zones that appeared around antibiotic discs were measured by millimeter (mm) using a metric ruler.

**Table1: Antibiotics discs using in this study Abbreviation and disc potency measured with ((µg/disc)**

| Antibiotic discs | Abb. | Disc potency (µg/disc) | Company/ Origin |
|---|---|---|---|
| Imipenem | IPM | 10 | Bioanalyse/ Turkey |
| Amoxicillin-Clavulanic acid | AMC | 20/10 | Bioanalyse/ Turkey |
| Cefepime | FEP | 30 | Bioanalyse/ Turkey |
| Cefazolin | CZ | 30 | Bioanalyse/ Turkey |
| Cefotaxime | CTX | 30 | Bioanalyse/ Turkey |
| Ceftazidime | CAZ | 30 | Bioanalyse/ Turkey |
| Ceftriaxone | CRO | 30 | Bioanalyse/ Turkey |
| Chloramphinical | C | 30 | Bioanalyse/ Turkey |
| Ciprofloxacin | CIP | 5 | Bioanalyse/ Turkey |
| Gentamicin | CN | 10 | Bioanalyse/ Turkey |

### Investigation of *bla*CTX-M Gene via Polymerase Chain Reaction (PCR)

### DNA Extraction

Few colonies *Pseudomonas aerogenosa* isolates suspend in 500 µl of nuclease-free water (Promega, USA). Heating by using water bath in 90°C for 10 min, then bacterial suspension centrifuged for 10 min at10000 rpm. The DNA material extracted in this procedure employed in different of gene analytical array [4]. The PCR reaction were performed as following Master mix 2X (Kapa, India) (12.5µl), forward primer (1.5µl), reverse primer (1.5µl), nuclease-free water (4.5µl) and DNA sample (5µl). Mixture incubated in PCR and undergoes the cycling conditions which involved at 95°C for 5 min free the strands (denaturation) then followed by 30 cycles of 94°C for 30 sec., to conjugate with the prime (annealing) 55°C for 40 sec. and extension at 72°C for 50 sec.

 

Cycling was followed by a final extension at 72°C for 10 min. nucoltied sequences *bla*CTX-M Forward primer was (5-TTTGCGATGTGCAGCACCA GTAA-3) Reverse primer (5- CGATATCGTTGGTG GTGCCATA-3) (Alpha DNA , Canada) [5].

### Agarose Gel Electrophoresis

PCR product was identifying via Gel electrophoresis analysis using the ethiduim bromide and UV transilluminator documentation system [6].

### Sequencing of PCR Products

The final products of positive PCR results were investigated for further identification. DNA sequencing for amplified fragments of PCR products were employed at Macrogen Company, soul, South Korea were analyzed. Further checks for identification of the local gene sequence were conducted in Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) website online at (http:// www.ncbi.nlm.nih.gov). Aligning of the obtained sequences with those of reference strains in GenBank confirmed the identification of has measured using PCR.

### Phylogenetic Tree Analysis

Phylogenetic data were obtained by the alignment and phylogenetic analysis of the sequences.

By using software MEGA6 software, the phylogenetic relationships were analysed for *bla*CTX-M gene sequences.

## Results and Discussion

### Isolation and Identification of *Pseudomonas aerogenosa*

Thirteen *Pseudomonas aerogenosa* isolates were isolated from corneal scraping specimens and then diagnosed according to standard microbiology method [3]. All bacterial isolates (*Pseudomonas aeruginosa)* were diagnosed using Api 20 and (Analytic Profile Index) (Bio-Merieux, France)), to identify bacteria belong to non *Enterobacteriaceae* family.

### Antimicrobial Susceptibility Test

The antibiotic susceptibility test of thirteen *Pseudomonas aerogenosa* isolates that isolated from corneal scraping specimens of patient with eye infections to various antimicrobial drugs were shown on table 2. As, the most effective antibiotic on *Pseudomonas aerogenosa* isolates was impeniem with 13(100%) sensitivity followed by Amikacin with 11(90%) sensitivity while the highest resistance was to cefozlen 13(100%) resistance followed by Amoxicillin-Clavulanic acid and Ampicillins 10(88%).

**Table2: The resistance rate of 13 *P. aerogenosa* isolates toward 10 types of antibiotics.**

| Antibiotics | Sensitive % | Resistance % |
|---|---|---|
| Ampicillin | 3 | 10 |
| Amikacin | 11 | 2 |
| Imipenem | 13 | 0 |
| Amoxicillin-Clavulanic acid | 3 | 10 |
| Cefepime | 5 | 8 |
| Cefazolin | 0 | 13 |
| Cefotaxime | 4 | 9 |
| Ceftazidime | 4 | 9 |
| Ceftriaxone | 5 | 8 |
| Chloramphinical | 8 | 5 |
| Ciprofloxacin | 4 | 9 |
| Gentamicin | 5 | 8 |

### Molecular Detection of *bla*CTX-M Gene in *Pseudomonas aerogenosa* by PCR

All thirteen *Pseudomonas aerogenosa* isolates were subjected to PCR assay to detect the presence of *bla*CTX-M gene.

The results in figure-1, showed the presence of *bla*CTX gene (544bp) in 3(23.1%) isolates, out of 13 *Pseudomonas aerogenosa* isolates, while 10(76.9%) isolates not harbored *bla*CTX-M gene.

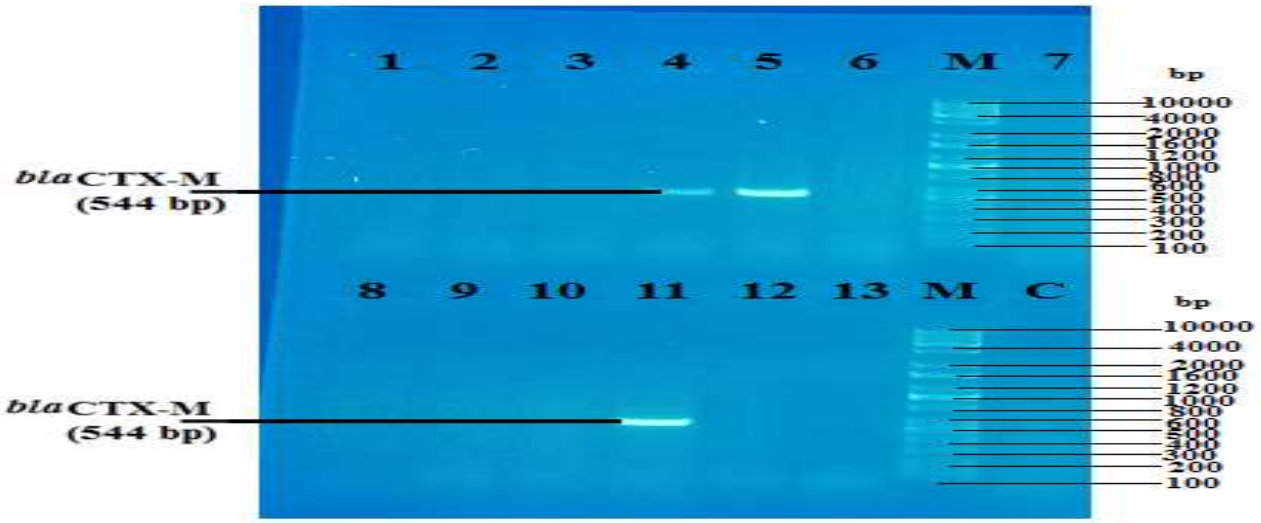


**Figure3: Gel electrophoresis of PCR product of *bla*CTX-M (544 bp) in *Pseudomonas aerogenosa* isolates isolated from corneal infections. Lane M: 100 pb DNA ladder (Kapa , India); lanes 1-13: *Pseudomonas aerogenosa* isolates; lane C: Negative control. Detection was done on agarose gel (1%) at 5 V/cm for one hour, stained with ethidium bromide and visualized on a UV transiluminator documentation system**

## Sequencing of PCR Products of *bla*CTX-M

The sequencing of *bla*CTX-M amplicons (544 bp) was carried out and the aligning of the *bla*CTX-M amplicon sequences with the reference strains in the Gen Bank confirmed the correct identification of *bla*CTX-M gene among *Pseudomonas aerogenosa* isolates as shown in figure-2 and Figure-3. The data that obtained from the sequencing of *bla*CTX-M gene was submitted to the GenBank of National Center for Biotechnology Information under the accession number (KY966041)

**Pseudomonas aeruginosa strain t9P1 insertion sequence ISEcp1, partial sequence; and class A extended-spectrum beta-lactamase CTX-M-15 (blaCTX-M) gene, blaCTX-M-15 allele, complete cds Sequence ID: KU926353.1Length: 1001Number of Matches: 1**
**Related Information Range 1: 409 to 857GenBankGraphics**

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 810 bits(898) | 0.0 | 449/449(100%) | 0/449(0%) | Plus/Plus |

Query 1
CAGCGAGTTGAGATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGCGGAAAAGCAC
60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 409
CAGCGAGTTGAGATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGCGGAAAAGCAC
468

Query 61
GTCAATGGGACGATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGATAAC
120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 469
GTCAATGGGACGATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGATAAC
528

Query 121
GTGGCGATGAATAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTTCGCC
180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 529
GTGGCGATGAATAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTTCGCC
588

 

```
Query                                                                 181
CGACAGCTGGGAGACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGTTAAACACCGCC
240
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                 589
CGACAGCTGGGAGACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGTTAAACACCGCC
648

Query                                                                 241
ATTCCGGGCGATCCGCGTGATACCACTTCACCTCGGGCAATGGCGCAAACTCTGCGGAAT
300
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                 649
ATTCCGGGCGATCCGCGTGATACCACTTCACCTCGGGCAATGGCGCAAACTCTGCGGAAT
708

Query                                                                 301
CTGACGCTGGGTAAAGCATTGGGCGACAGCCAACGGGCGCAGCTGGTGACATGGATGAAA
360
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                 709
CTGACGCTGGGTAAAGCATTGGGCGACAGCCAACGGGCGCAGCTGGTGACATGGATGAAA
768

Query                                                                 361
GGCAATACCACCGGTGCAGCGAGCATTCAGGCTGGACTGCCTGCTTCCTGGGTTGTGGGG
420
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                 769
GGCAATACCACCGGTGCAGCGAGCATTCAGGCTGGACTGCCTGCTTCCTGGGTTGTGGGG
828

Query  421  GATAAAACCGGCAGCGGTGGCTATGGCAC  449
       ||||||||||||||||||||||||||||
Sbjct  829  GATAAAACCGGCAGCGGTGGCTATGGCAC  857
```

**Figure 2: Nucleotide sequence of blaCTX-M gene**

**Class A extended-spectrum beta-lactamase CTX-M-15 [Pseudomonas aeruginosa]**
**Sequence ID: AMQ62992.1Length: 291Number of Matches: 2**
**Related Information**
**Gene-associated gene details**
**Identical Proteins-Identical proteins to WP_000239590.1**

| Score | Expect | Method | Identities | Positives | Gaps | Frame |
|---|---|---|---|---|---|---|
| 166 bits(421) | 5e-55 | Compositional matrix adjust. | 80/80(100%) | 80/80(100%) | 0/80(0%) | +1 |

```
Query                                                                   1
YSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDPRDTTSPRAMAQ
180

YSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDPRDTTSPRAMAQ
Sbjct                                                                 132
YSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDPRDTTSPRAMAQ
191
Query  181  TLRNLTLGKALGDSQRAQLV  240
            TLRNLTLGKALGDSQRAQLV
Sbjct  192  TLRNLTLGKALGDSQRAQLV  211
```

**Figure- 3: Amino acid sequence of *bla*CTX-M gene**

 

## Phylogenetic Tree

Phylogenetic tree based on the *bla*CTX-M nucleotide sequences of *Pseudomonas aeruginosa* was shown in Figure-4. The data for the phylogenetic analysis were obtained from sequences in the GenBank nucleotide sequence database. *BlaCTX-M* gene phylogenetic tree of *Pseudomonas aeruginosa* isolate with closely related *bla*CTX-M gene of some bacterial isolates. A phylogenetic tree using sequences of *bla*CTX-M gene from *Pseudomonas aeruginosa* isolate and closest relatives was generated using the software MEGA6 method. Table- 3, show the accession numbers and the percentage of nucleotide identity and similarity of the *bla*CTX-M gene for *Pseudomonas aeruginosa* sequences with other bacteria in the GenBank.

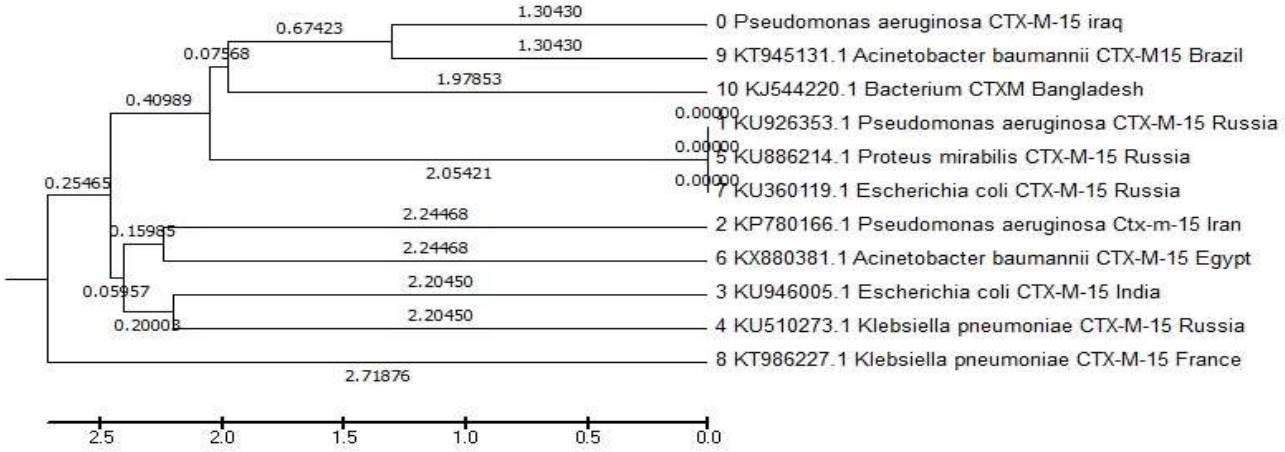


**Figure4: Phylogenetic tree based on the nucleotide sequences of *bla*CTX-M gene**

Thus, molecular phylogenetics is a fundamental aspect of bioinformatics cluster analysis is an approach that finds structure in data by identifying natural groupings (clusters) in the data. A cluster is simply a collection of cases that are more 'similar' to each other than they are to cases in other clusters [3] devise a scheme for grouping the objects into classes so that 'similar' ones are in the same class. Devise a scheme for grouping the objects into classes so that 'similar' ones are in the same class [7, 21].

**Table 3: The accession numbers and the percentage of *bla*CTX-M nucleotide identity**

| | ACCESSION | strain | country | Source | Gene | Identities | expect | score | Range |
|---|---|---|---|---|---|---|---|---|---|
| **1.** | ID: KU926353.1 | t9P1 | Russia | Pseudomonas aeruginosa | CTX-M-15 (blaCTX-M) gene | 100% | 0.0 | 810 | **409 to 857** |
| **2.** | ID: KP780166.1 | 6 | Iran | Pseudomonas aeruginosa | CTX-M-15 (blaCTX-M) gene | ---------- | | | |
| **3.** | ID: KU946005.1 | 862 | India | Escherichia coli | CTX-M-15 (blaCTX-M-15) gene | 100% | 0.0 | 810 | **82 to 530** |
| **4.** | ID: KU510273.1 | I-1719 | Russia | Klebsiella pneumoniae | CTX-M-15 (blaCTX-M-15) gene | 100% | 0.0 | 810 | **413 to 861** |
| **5.** | ID: KU886214.1 | B-1261/15 | Russia | Proteus mirabilis | CTX-M-15 (blaCTX-M-15) gene | 100% | 0.0 | 810 | **409 to 857** |
| **6.** | ID: KX880381.1 | A.b. 140 | Egypt | Acinetobacter baumannii | CTX-M-15 gene | 100% | 0.0 | 810 | **91 to 539** |
| **7.** | ID: KU360119.1 | I-1950 | Russia | Escherichia coli | CTX-M-15 (blaCTX-M-15) gene | 100% | 0.0 | 810 | **409 to 857** |
| **8.** | ID: KT986227.1 | HM | France | Klebsiella pneumoniae | CTX-M-15 (blaCTX-M-15) gene | 100% | 0.0 | 810 | **2120 to 2568** |
| **9.** | ID: KT945131.1 | HUM Ac17 | Brazil | Acinetobacter baumannii | CTX-M-15 gene | 100% | 0.0 | 810 | **6 to 454** |
| **10.** | ID: KJ544220.1 | C47_CLW | Bangladesh | **Bacterium** | CTXM gene | 100% | 0.0 | 810 | **28 to 476** |

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