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Frequency of *blaOxa10* Beta-lactamase gene in *Pseudomonas aeruginosa* isolated from different clinical swabs

Dalal S. AL- Rubaye[1*], Wasan W. Albassam[1], Hayder M.al-habobi[2], Ilham Ab.H.K.AL-Rubaye[3]

[1]Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq
[2]Genetic Engineering and Biotechnology, University of Baghdad, Baghdad, Iraq
[3]Al-Razi Center for Research and Diagnostic Kits, Ministry of Industry and Minerals, Baghdad, Iraq

Abstract

Thirty one isolates of *P. aeruginosa* were collected from 100 clinical samples include (pus, burn and wound swabs). All the isolates were identified as *P. aeruginosa* by conventional methods, PCR and DNA sequencing. Antibiotic sensitivity tests were undertaken for 8 groups of antibiotics (Cephalosporins, Rifamycin, fluoroquinolones, Penicillins, Carbapenems, Aminoglycosides, Chloramphenicol and Beta-lactamase inhibitor+ Penicillin). The highest resistance rate was recorded to Rifampin, and Pipracillin with 31(100%), followed by Chloramphenicol ,Cefotaxime and Cefixime with 28 (90.3%), and the lowest resistance rate to Imipenem with 8 (25.8%). Cefpodoxime resistance value was less than values reported in other study. Six strains did not carry the *blaOXA10* gene that is hydrolyze Cloxacillin , Oxacillin and Carbapenem, in spite of their resistance to more than 3 different antibiotic group. According to present study, we concluded that the drug of choice is the Imipenem, the most effective anti-pseudomonal agent, and the *bla OXA-10* gene frequency was 6 (19.4%) among *P. aeruginosa* isolates from patients in different hospitals in Baghdad.

Keywords: ESBLs, *blaOXA10,*, ambler class D , *P. aeroginosa* and B-lactamases

تردد جين بيتا لاكتاميزالاوكساسلين 10 في الزائفة الزنجارية المعزولة من مسحات سريرية مختلفه

دلال صالح الربيعي[1]*، وسن وائل البصام[1]، حيدر مازن الحبوبي[2]، الهام عبد الهادي خلف الربيعي[3]

[1]قسم التقنيات الأحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق
[2]معهد الهندسة الوراثية والتقنيات الأحيائية، جامعة بغداد، بغداد، العراق
[3]مركز الرازي للبحوث والعدد التشخيصية، وزارة الصناعة والمعادن، بغداد، العراق

الخلاصة

عزلت واحد وثلاثون عزله من الزائفه الزنجاريه من مجموع 100 عينه سريريه تضمنت (قيح ، حروق وجروح) جميع العزلات شخصت اعتمادا على الطرق التقليديه تفاعل البلمرة المتسلسل وتسلسل الحمض النووي. اختبرت نتائج فحص الحساسيه للمضادات الحياتيه ل8 مجاميع (Cephalosporins, Rifamycin fluoroquinolones, Penicillins, Carbapenems, Aminoglycosides Chloramphenicol و Penicillin inhibitor –Beta-lactamase) . سجلت اعلى مقاومه لل Rifampin و Pipracillin حوالي31 (100%) وبعدها Chloramphenicol , Cefotaxime و Cefixime حوالي 28 (90 . 3%) واقل معدل مقاومه كانت لImipenem حوالي 8 (25. 8%) . قيمه المقاومه لل Cefpodoxime اقل من معدلات المقاومه المسجله في الدراسات الاخرى . اظهرت ست عزلات بانها غيرحامله للجين *blaOXA10 المسؤول عن المقاومة لل* Oxacillin , Cloxacillin و Carbapenem بالرغم من مقاومه تلك العزلات لاكثر من 3 مجاميع لمضادات حيويه مختلفه . نستنتج من

*Email:dr.tbdalal@gmail.com

هذه الدراسه الحاليه ان ال Imipenem كان الاكثر تاثيرا ضد الزائفه الزنجاريه , وكانت نسبه التردد للجين *blaOXA10* 6 (% 4. 19) *في الزائفه* الزنجارية المعزوله من المرضى في مختلف مستشفيات بغداد.

Introduction

Pseudomonas aeruginosa is a gram-negative rod, non glucose fermenter, widespread in natural environments and it is the most common pathogen cause of nosocomial infections such as pneumonia, urinary tract infections and bacteremia [1]. The infections can be particularly severe in patients with impaired immune systems, such neutropenic or cancer patients [2]. Beta-lactam antibiotics like penicillins, broad-spectrum cephalosporins and monobactams used in treatment of *P. aeruginosa* [3], can inactivated by B-lactamase. Extended spectrum B- lactamases (ESBLs) are enzymes that mediate resistance to extended-spectrum (third generation) cephalosporins (e.g., ceftazidime, cefotaxime, and ceftriaxone) and monobactams (e.g., aztreonam) but do not affect cephamycins or carbapenem [4]. There are four classes of ESBLs are recognised (A, B, C and D), correlating with the functional classification [5]. The *blaOXA10* belong to *blaOXA* type extended spectrum β- lactamases (ESBLs)-genes ambler class D [6]. They are frequently found in *P. aeruginosa* and have a hydrolytic activity against oxacillin and methicillin which not affected by clavulanic acid. These enzymes confer resistance to amoxicillin and cephalothin [7]. Genes encoding oxacillinase enzymes are intrinsic in Gram negative bacteria including *P. aeruginosa,* and cause a serious therapeutic problem at care units of the hospitals through intense antimicrobial therapy [8], and have the ability to develop multidrug resistance and mutational acquired resistance to antibiotics through chromosomal mutations forces [9]. The aim of this study was to investigate the frequency of ambler class D, B-lactamases in burn, wound and pus swabs and to determine the Carbapenemase Producing *P. aeruginosa* isolated from patients in Baghdad hospitals.

Materials and Methods:

Bacterial isolation

Out of 100 different clinical samples, thirty one *P. aeruginosa* isolates were collected from different Hospitals in Baghdad during the period (October 2012 to May 2013) and (November 2014 to March 2015). Clinical specimens were include pus, burn and wound swabs. Each swab taken carefully from the site of infection and placed in tubes containing readymade media (Amies transport media, china), to maintain the swab wet during transferring to laboratory. Each specimen was inoculated on *Pseudomonas* isolation agar (Cetramide) (Hi-Media). All plates were incubated aerobically in incubator at 37ºC for 24 hrs. Identification was done by using colony morphology, Gram stain, motility, oxidase, citrate utilization, and pyocyanin production [10].

Antibiotic susceptibility test:

All isolates were analyzed for the resistance against antibiotics according to the method of Kirby & Bauer [11], on Mueller Hinton agar (HiMedia) by using commercial available paper discs. The concentration of the discs were ranged in Table-1, and the results were followed depend on the National Committee for Clinical Laboratory Standards (NCCLS) guidelines. Multidrug-resistant (MDR) isolates were defined as those showed resistant to three or more classes of antipseudomonal agents (carbapenems, fluoroquinolones, penicillins, cephalosporins, and aminoglycosides) [12].

Table 1- Concentration of all antibiotics used in this study

Antibiotics	concentrations
Ceftriaxone	30 µg
Imipenem	10 µg
Cefpodoxime	10 µg
Rifampin	5 µg
Chloramphenicol	30 µg
Amikacin	30 µg
Meropenem	10 µg
Amoxicillin/Clavulanic acid	20 µg / 10 µg
Cefotaxime	10 µg
Piperacillin	100 µg
Ciprofloxacin	5 µg
Cefixime	5 µg

DNA Extraction

Genomic DNA extraction carried out based on kit automated method using ExiPrep 16 Plus (Bioneer, Korea). Fresh bacterial culture (0.2 ml) were centrifuged at 6000 RPM for 10 min., the pellet was resuspended with the lysis buffer and incubated at 37˚C for 30 min then loaded to extraction cartridge, DNA was eluted by 50 µl elution buffer. The DNA sample measured for their concentration and purity using Microvolume UV Spectrophotometer (ACTGene).

DNA sequencing and in seleco

Primer sets were used to amplify species-specific 23s rDNA gene for *P. aeruginosa* were Forward: TTGAGCCCCGTTACATCTTC and Reverse: GGGAACCCACCTAGGATAA , based on the 23 ribosomal RNA gene sequence in the GenBank (accession no.Y00432). Polymerase chain reaction (PCR) was performed in a 50 µl mixture containing 1× PCR buffer (10 mM Tris–HCl, 1.5 mM MgCl2, 50 mM KCl [pH 9]) (Merck), 100 µM (each) deoxynucleoside triphosphates, 1 U of Taq DNA polymerase (Merck), 10 pM each of forward and reverse primers, and 100 ng of templet DNA. The program for PCR included an initial denaturation 94°C for 5min, 30 cycles of denaturation at 94°C for 60s, annealing at 58°C for 60s, extension at 72°C for 60s and a final extension at 72 °C for 7min. The PCR products were resolved on a 1% agarose gel, stained with ethidium bromide (0.5 µg/ml) and bands observed using a gel documentation system (ATTO). PCR products were sent for sequencing at Bioneer Company/ Korea. The sequences analyzed by NCBI blast to identify *P. aeruginosa.*

PCR Reaction of Oxacillinase encoding gene

PCR primer set of group D ESBLs were used: *blaOxa10* F (5ATTATCGGCCTAGAAACTGG-3 and *blaOxa10* R (5-CTTACTTCGCCAACTTCTCTG-3), to detect OXA10 gene in the multidrug resistant *P. aeruginosa*. PCR was carried out with 2µL of the template DNA, 0.4 pM of each primer, PCR buffer 1x , 200 µM dNTP, 1/5 mM $MgCl_2$ and 1 U of Taq DNA polymerase (Promega/ USA) in a total volume of 50 µl. Amplification was carried out in a thermocycler (Bioneer/ Korea). Agarose gel electrophoresis (1%) of PCR products was carried out using 1 mM Tris-Borate-EDTA (TBE) buffer at 95V for 1 hour, and then the DNA bands were stained with ethidum bromide (Sigma/ USA). DNA ladder 100 bp was used to confirm the size of the specific bla gene. Simultaneously, a negative control was used with no template for blaOXA10 gene. The reaction conditions were as following: pre-denaturation at 94°C for 4 minutes, followed by 35 amplification cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1.5 minutes, with a final extension step of 72°C for 5 minutes.

Results and Discussion

Identification of Isolates

Thirty one isolates of *P. aeruginosa* were collected from pus, burn and wound swabs from patients who were admitted to different hospitals in Baghdad Table-2, by conventional methods on the basis of grow in cetrimide agar, pigment production and oxidase positive. *P. aeruginosa* is one of the important causes of morbidity among hospital patients; it is emerged as an important pathogen and responsible for the nosocomial infection, compared to a study by Qader and Muhamad [13] in sulymania showed that the *P. aeruginosa* isolated from burn swabs (18%),. While another study by Ali and Zahraa [14], showed that the number of *P. aeruginosa* from all swab's types were (41%). This may be due to differences in location and the sources of samples.

Table 2- Total number of isolates in burn, pus and wound swabs

Collected groups	No. of swabs	*P. aeruginosa* isolates
from November 2014 to March 2015	40	15 (37.5%)
from October 2012 to May 2013	60	16 (26.6%)
Total	100	31%

Antibiotic susceptibility test by Disc diffusion

The multidrug-resistant incidence of ESBL-producing *P. aeruginosa* is increasing worldwide [15]. Antibiotic resistance related to plasmid or chromosome occur in *P. aeruginosa* isolates in different hospitals in Baghdad [16]. As shown in Table-3, the highest resistance rate were to Rifampin, and Pipracillin with 31 (100%), followed by Chloramphenicol, Cefotaxime and Cefixime with 28 (90.3%), and the lowest resistance rate to imipenem with 8 (25.8%), the same result reported by Zeynab and Ali [17], the drug of choice is the Imipenem, incontrast to a study by

Mirsalehian *et al*. [18] in Tehran (Iran) showed that, aztreonam, Imipenem and meropenem were the most effective anti-pseudomonal agents.

In this study, Cefpodoxime (an oral third-generation cephalosporin antibiotic) resistance value was less than values reported in study conducted by Oladipo *et al*. [19] in which all isolates of *P. aeruginosa* were absolutely resistant to cefpodoxime, it suggested to repeat the study with large sample size focusing on treatment by Cefpodoxime vs. Cephalosporine to deserves consideration to oral therapy by cefpodoxime in initial treatment because of cost effectiveness in contrast to the parenteral Cephalosporins.

Table 3- The antibiotic susceptibility of thirty one *P. aeruginosa* isolates

Antibiotics	Resistant (%)	Intermediate (%)	Sensitive (%)
Ceftriaxone	24 (77.4)	0 (0.0)	7 (22.6)
Imipenem	8 (25.8)	7 (22.6)	16 (51.6)
Cefpodoxime	24 (77.4)	0 (0.0)	7(22.6)
Rifampin	31 (100)	0 (0.0)	0 (0.0)
Chloramphenicol	28 (90.3)	3 (9.7)	0 (0.0)
Amikacin	20 (64.5)	7 (22.6)	4 (12.9)
Meropenem	24 (77.4)	0 (0.0)	7 (22.6)
Amoxicillin/Clavulanic acid	24 (77.4)	7 (22.6)	0 (0.0)
Cefotaxime	28 (90.3)	3 (9.7)	0 (0.0)
Pipracillin	31 (100)	0 (0.0)	0 (0.0)
Ciprofloxacin	20 (64.5)	11 (35.5)	0 (0.0)
Cefixime	28 (90.3)	3 (9.7)	0 (0.0)

PCR amplification for 23s non–coding RNA gene and sequencing

The genomic DNA of 31 isolates was amplified in PCR with specific primer to investigate partially amplified fragment of 23s non-coding RNA gene, the results showed that the band was approximate 900bp Figure-1. The amplification of the 23 rDNA genes by PCR technique and DNA sequencing of the PCR products was reported by Rantakokko *et al*., [20], to make confirmation to *P. aeruginosa* isolates. PCR product of 11 partial 23r DNA gene were sequenced and analyzed by NCBI-blast, Figure-2A, showed the alignment of the subject with all query, while Figure-2B showed one sample identity (99%).

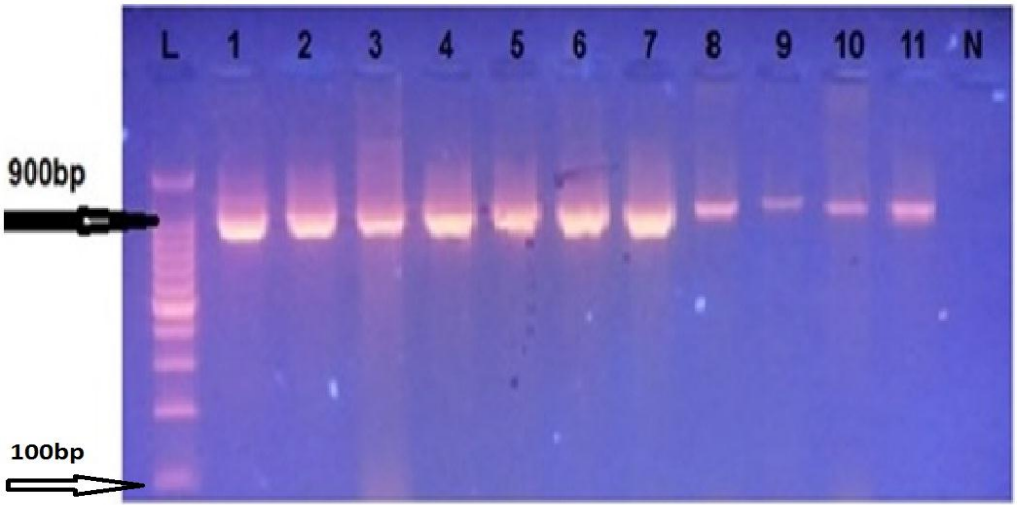

Figure 1- Agarose gel electrophoresis (1%) for 900bp PCR product of 23s RNA, Lane L: 100 bp DNA ladder, lane 1,2,3,4,5,6,7,8,9,10 and 11: 900 bp PCR product of 23r DNA., lane N: negative control.

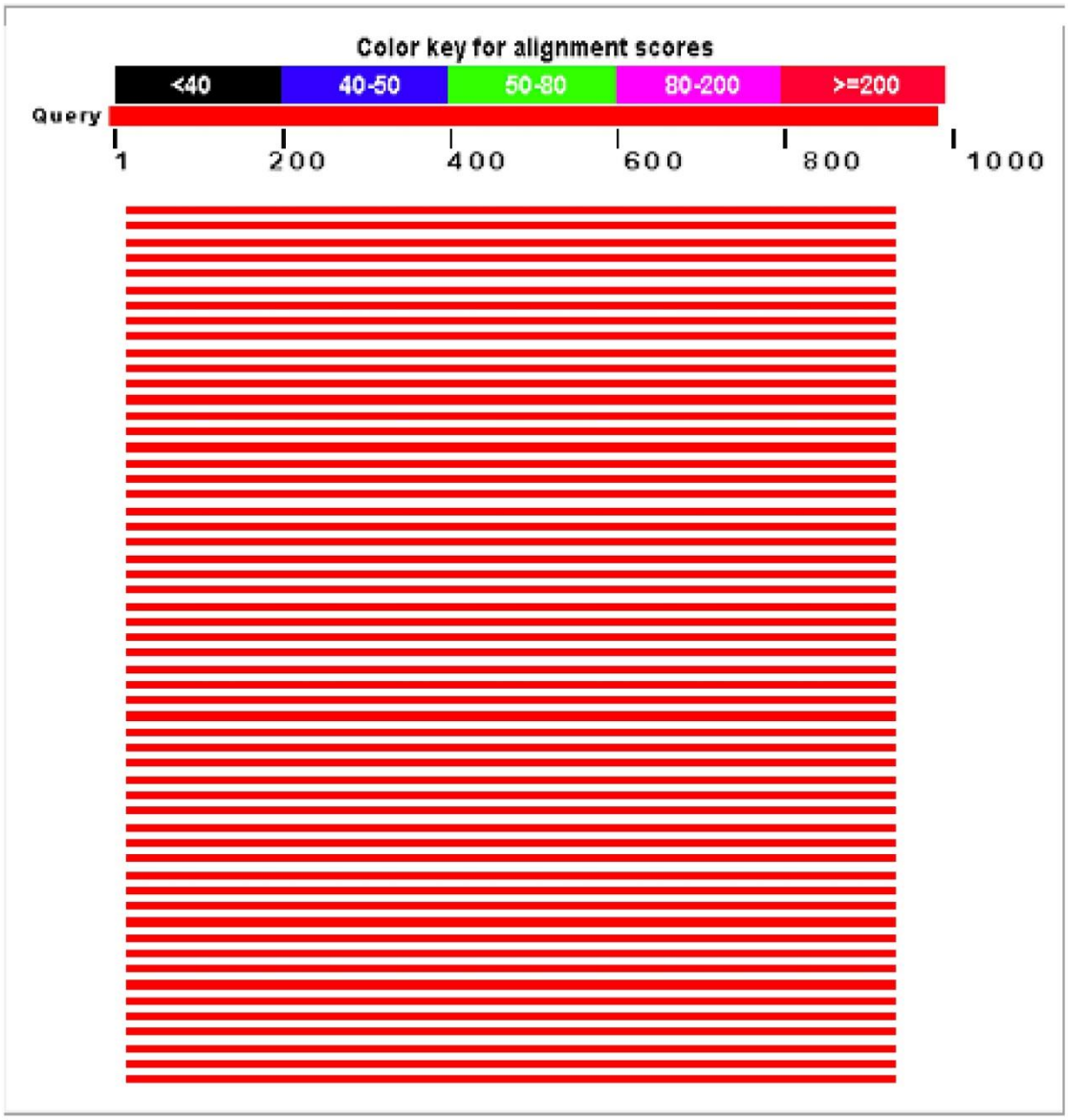

Figure 2A- Graphical output of DNA-level similarities from NCBI BLAST. The color key for alignment score indicates the strength of alignment based on nucleotide matches between the query sequence and the subject sequence (23s non-coding RNA gene)

Pseudomonas aeruginosa strain F9676, complete genome
Sequence ID: gb|CP012066.1|Length: 6368008Number of Matches: 4

Range 1: 1074310 to 1075257 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
1733 bits(938)	0.0	945/948(99%)	2/948(0%)	Plus/Minus

```
Query  12       CTAATGAGCTATTACGCTTTCTTTAA-GGGTGGCTGCTTCTAAGCCAACCTCCTAGCTGT  70
               ||| |||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  1075257  CTAGTGAGCTATTACGCTTTCTTTAAAGGGTGGCTGCTTCTAAGCCAACCTCCTAGCTGT  1075198
Query  71       CTAAGCCTTCCCACATCGTTTACCACTTAACCACAACTTTGGGACCTTAGCTGGCGGTCT  130
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1075197  CTAAGCCTTCCCACATCGTTTACCACTTAACCACAACTTTGGGACCTTAGCTGGCGGTCT  1075138
Query  131      GGGTTGTTTCCCTTTTCACGACGGACGTTAGCACCCGCCGTGTGTCTCCCATGCTCGGCA  190
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1075137  GGGTTGTTTCCCTTTTCACGACGGACGTTAGCACCCGCCGTGTGTCTCCCATGCTCGGCA  1075078
Query  191      CTTCTGGGTATTCGGAGTTTGCATCGGTTTGGTAAGTCGGGATGACCCCCTAGCCGAAAC  250
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1075077  CTTCTGGGTATTCGGAGTTTGCATCGGTTTGGTAAGTCGGGATGACCCCCTAGCCGAAAC  1075018
Query  251      AGTGCTCTACCCCCCAGAGTGATACATGAGGCGCTACCTAAATAGCTTTCGAGGAGAACC  310
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1075017  AGTGCTCTACCCCCCAGAGTGATACATGAGGCGCTACCTAAATAGCTTTCGAGGAGAACC  1074958
Query  311      AGCTATCTCCGAGCTTGATTAGCCTTTCACTCCGATCCACAAGTCATCCCCTACCTTTTC  370
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1074957  AGCTATCTCCGAGCTTGATTAGCCTTTCACTCCGATCCACAAGTCATCCCCTACCTTTTC  1074898
Query  371      AACGGGAGTGGGTTCGGTCCTCCAGTCAGTGTTACCTAACCTTCAACCTGCTCATGGATA  430
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1074897  AACGGGAGTGGGTTCGGTCCTCCAGTCAGTGTTACCTAACCTTCAACCTGCTCATGGATA  1074838
Query  431      GATCGCCCCGGTTTCGGGTCTATACCCAGCGACTAAACGCCCTATTAAGACTCGCTTTCGC  490
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1074837  GATCGCCCCGGTTTCGGGTCTATACCCAGCGACTAAACGCCCTATTAAGACTCGCTTTCGC  1074778
Query  491      TACGCCTACCCTATACGGTTAAGCTTGCCACTGAATATAAGTCGCTGACCCATTATACAA  550
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1074777  TACGCCTACCCTATACGGTTAAGCTTGCCACTGAATATAAGTCGCTGACCCATTATACAA  1074718
Query  551      AAGGTACGCAGTCACCTAACAAGTAGGCTCCCACTGCTTGTACGCATACGGTTTCAGGTT  610
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1074717  AAGGTACGCAGTCACCTAACAAGTAGGCTCCCACTGCTTGTACGCATACGGTTTCAGGTT  1074658
Query  611      CTATTTCACTCCCCTCTCCGGGGTTCTTTTCGCCTTTCCCTCACGGTACTGGTTCACTAT  670
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Figure 2B- Sequences producing significant alignments.

Detection of *blaOXA10* gene by PCR technique

PCR was performed for 31 isolates, *blaOXA10* gene was detected in 6 (19.4%) among them Figure-3, this in agreement with the antibiotic susceptibility test section which shows that the Carbapenem is the lowest resistant drug. The prevalence of *P. aeruginosa* encoding *blaOxa10* in Baghdad was higher compared with Korea (13%) and lesser from France (26%) and from Iran (64%)

[17] while another study by Tamer *et al*., [21] in Palestine showed that the frequency was (41%), which refers to the emergence of ESBLs group D among *P. aeruginosa* isolates must be taken seriously in these countries, while in Baghdad hospitals we need to better treatment to decrease *blaOXA10* gene frequency. Six isolates did not carry the *blaOXA10* in spite of their resistance to more than 3 different antibiotic group, this may be due to that the MDR isolates have another way than *blaOXA10* gene.

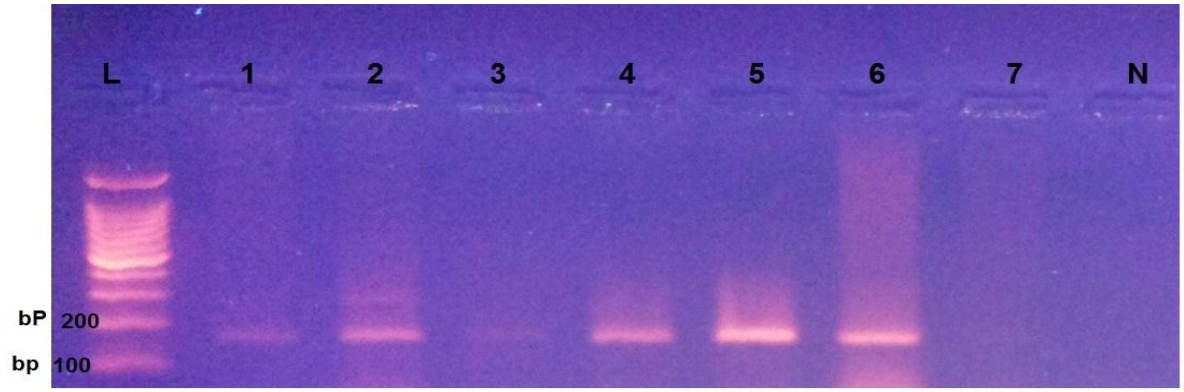

Figure 3- Agarose gel electrophoresis (1%) for *blaoxa10* gene of pseudomonas agarose gel. Lane L: 100 bp DNA ladder, lane 1,2,3,4,5 and 6: positive 170 bp PCR product for *blaoxa10* gene. Lane 7 negative results. Lane N: negative control.

According to present study, it can be concluded that *bla OXA-10* gene has a moderare frequency in the *P. aeruginosa* strains isolated from hospitalized patients in Baghdad compared to another countries and the drug of choice is the Imipenem. It recommended a proper infection control practices are essential to prevent the spreading of ESBL-producing *P. aeruginosa* in hospitals.

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