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Local Study of *bla$_{CTX-M}$ genes* detection in *Proteus spp.* by using PCR technique

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Abstract

In this study, 25 clinical isolates of *Proteus spp.* were collected from urine, wounds and burns specimens from different hospitals in Baghdad city, all isolates were identified by using different bacteriological media, biochemical assays and Vitek-2 system. It was found that 15 (60%) isolates were identifies as *Proteus mirabilis* and 10 (40 %) isolates were *Proteus vulgaris*. The susceptibility of *P. mirabilis* and *P. vulgaris* isolates towards cefotaxime was (66.6 %) and (44.4 %) respectively; while the susceptibility of *P. mirabilis* and *P. vulgaris* isolates towards ceftazidime was (20%). Extended spectrum β-lactamses producing *Proteus* was (30.7 %). DNA of 10 isolates of *P. mirabilis* and 4 isolates of *P. vulgaris* were extracted and detection of (*bla$_{CTX-M-1}$*, *bla$_{CTX-M-2}$*, *bla$_{CTX-M-8}$* and *bla$_{CTX-M-9}$*) was done by multiplex polymerase chain reaction (PCR). Results showed the presence of *bla$_{CTX-M-2}$* gene, which is responsible for resistance to cefotaxime in these isolates, while no other types of , *bla$_{CTX-M}$* genes were found in them.

Keywords: CTX-M genes, Cefotaxime, *Proteus*, ESBLs producers.

دراسة محلية للتحري عن جينات *bla$_{CTX-M}$* في جنس بكتيريا المتقلبات *Proteus* *spp.* بإستخدام تقنية تفاعل الكوثرة

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الخلاصة

في هذه الدراسة تم جمع 25 عزلة سريرية من جنس بكتيريا المتقلبات*Proteus* من عينات مختلفة من الادرار، والحروق، والجروح من عدد من مستشفيات مدينة بغداد، تم تشخيص العزلات باستخدام الاوساط البكتيريولوجية والاختبارات البايوكيميائية ونظام Vitek-2، شُخصت 15 عزلة (60 %) لنوع *P. mirabilis* و 10 عزلات (40 %) لنوع *P. vulgaris*. كانت نسبة مقاومة عزلات الـ *P. mirabilis* لمضاد السيفوتاكسيم (66.6 %) ونسبة مقاومة عزلات الـ *P. vulgaris* (44.4%) بينما كانت نسبة المقاومة 20%لكلا النوعين لمضاد السيفتازيديم، وكانت نسبة العزلات المنتجة لانزيمات واسعة الطيف 30.7 %. تم استخلاص المادة الوراثية DNA من 10 عزلات من *P. mirabilis* 4 عزلات من *P. vulgaris* والتحري عن المورثات المشفرة لـ (*bla$_{CTX-M-1}$*, *bla$_{CTX-M-2}$*, *bla$_{CTX-M-8}$*, *bla$_{CTX-M-9}$*) بواسطة تقنية تفاعل الكوثرة، اظهرت النتائج وجود المورث المشفر للـ *bla$_{CTX-M-2}$* المسؤول عن مقاومة مضاد السيفوتاكسيم في هذه العزلات في حين لم يتم العثور على الانواع الاخرى التابعة للمورثات المشفرة لـ *bla$_{CTX-M}$*.

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Introduction

Members of *Proteus* genus 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].

Since *Proteus* belongs to the family of *Enterobacteriaceae*, general behaviors are applied on this genus. Bacteria belonging to *Proteus* spp. exhibit swarming growth. This dimorphic bacterium can undergo dramatic morphological and physiological changes in response to growth on surfaces or in viscous environments [1, 3].

The genus *Proteus* currently consists of five named species (*P. mirabilis, P. penneri, P. vulgaris, P. myxofaciens, and P. hauseri*) and three unnamed genomo-species (Proteus genomo-species 4, 5, and 6), *P. vulgaris, P. mirabilis* and *P. penneri* that are opportunistic human pathogens [4, 5]. *P. mirabilis* is the third most common (after *E.coli* and *Klebsiella pneumoniae*) cause of complicated urinary tract infection (UTI) which are also caused by the two other species, *P. vulgaris* and *P. Penneri*, this species best known for its 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 [1, 6]. Besides UTI, *P. mirabilis* and *P. vulgaris* have been described as opportunistic etiological agents in infections 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 food [1].

β-lactamases are the main cause of bacterial resistance to β-lactam antimicrobial agents. Their spread destroyed the utility of most penicillins against some Gram positive bacteria and *Enterobacteria*. The β-lactamases of Gram-positive species are largely extra-cellular; some enzymes may adhere to the cytoplasmic membrane. On the other hand, the β-lactamases of Gram-negative species are largely periplasmic, although some extracellular release may occur that is mediated by leakage rather than secretion [7, 8]. Widespread use of antimicrobial agents' leads to selection of bacteria producing extended spectrum β-lactamases (ESBL), the recent scheme was proposed by Jung Hune Lee and others in 2010 with widen definition of ESBLs.

The diversity and increasing prevalence of CTX-M-type ESBLs pose a serious threat to the clinical use of third generation cephalosporins for the treatment of severe infections. What is more alarming is the underdetection of ESBL production in clinical isolates, as it is common practice to cefotaxime and ceftazidime resistance as an indicator of ESBLs. CTX-M is considered as a family of plasmid-mediated ESBLs, called *CTX-M* which is hydrolyzes oxy-amino cephalosporins, especially third and fourth generation, and monobactam. It hydrolyze cefotaxime more rapidly than ceftazidime but not cephamycins such as cefoxitin and carbapenems including imipenem, ertapenem, meropenem, or doripenem in addition. They are generally susceptible to β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam. CTX-M β-lactamases are typical ESBLs that belong to Bush's group 2be and Ambler's class A [1, 9, 10].

The first clinical isolate with a cefotaximase property was reported from *Escherichia coli* grown from ear discharge of a 4-month child in Munich, Germany, in 1989. It was named CTX-M-1 (CTX for cefotaximase and M for Munich) [11]. Sequencently, several cefotaximase have been observed in worldwide, as CTX-M-2, -3, -4, -9, -10, -14, -15, and -45. They were in different Gram negative bacteria and obtained from different clinical samples [12, 13]. These CTX-M- clusters were classified according to the amino acids sequences and then phylogenic tree of CTX-M β-lactamases [8].

According to this, there are at least 128 of CTX-M types that have been described. They are divided into five clusters (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25). Naming the cluster was based on the first member of the group that described by [18] and each group consist of members have >94% amino acid relatedness and ≤90% relatedness across the groups. As a result the *bla* $_{CTX-M}$ gene encodes a 291 amino acid enzyme. Members of different lineages differ at 10-30% of the amino acids residue, while most lineages include a number of minor variants that may differ from each other by one or a few amino acids substitutions. A single amino acid change in bla_{CTX-M} constitutes a new CTX-M type [12, 14].

Enzymes closely related to the CTX-M group include the chromosomal β-lactamases of *Klebsiella oxytoca, Proteus vulgaris, Citrobacter diversus, serratia fonticola* and *Kluyvera cryocrescencs*. Mobilization of bla_{CTX-M} genes to another bacterial genera have occurred through recombinatorial events mediated by genes such as IS*CR1*, IS*Ecp 1* or transfrable plasmids may be associated with complex class 1 integrons related to In6 and In7 [12]. Plasmid encoded bla_{CTX-M} enzymes represent an

important sub-group of class A β-lactamases which hydrolyse broad-spectrum β-lactam antimicrobial agents causing an ESBL phenotype, which is increasingly found in enterobacterial species [15].

Organisms that produce CTX-M enzymes have become the most prevalent type of ESBLs described recently. At present, the CTX-M family comprises more than 40 enzymes [12, 14]. The rapid and wide spread of CTX-M enzymes worldwide is mainly due to the frequent association of bla_{CTX-M} genes with genetic mobile elements, such as epidemic plasmids, transposons, and/or insertion sequences located upstream bla_{CTX-M} also responsible for the expression of gene [16]. Strains of *Enterobacteriaceae* with CTX-M enzymes normally appear to be resistant or exhibit reduced susceptibility to cefotaxime (also to ceftriaxone and cefpodoxime) in addition to ceftazidime [13]. The aim of this study is to detect the genotypes of bla_{CTX-M} genes in *Proteus* from different clinical isolate.

Materials and methods:

Isolation and identification of *Proteus spp.*

The twenty five isolates of *Proteus spp.* isolates were isolated from clinical samples of urine, wounds, and burns of patients attending to several hospitals in Baghdad, Iraq during the period from November 2014 to February 2015. The samples were streaked on blood, MacConkey agar plates. The plates were incubated aerobically at 37° C for 24 h. The isolates were identified bacteriologically, biochemically according to methods described by [17]. In addition, the morphological features on culture media such as Swarming on blood agar, Non lactose fermented growth on MacConkey agar were examined, then identification of bacteria was confirmed by using Vitek 2 identification system (Biomerieux/ Frane) [18].

Antibiotic susceptibility test

Antibiotic susceptibility test was carried out for cefotaxime and cftazidime by Kiruby-Bauer method according to [18]. The isolate was interpreted as susceptible, intermediate or resistant to particular antibiotics by comparison with standard inhibition zones according to Clinical Laboratories Standards Institute (CLSI).

Detection of ESBLs

Double-disk synergy test method was used to detect ESBLs-producing isolates [10], 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°C for 18 -24 h. After incubation, synergism activity between the central disk and any one of surrounding antimicrobial discs was noted to detect the ESBLs producing isolates.

Extraction of DNA from *Proteus* and Estimation the concentration and purity of extracted DNA

DNA was extracted from 14 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_{CTX-M} by Multiplex Polymerase Chain Reaction

Multiplex PCR was performed for detection of 304-1179 CTX-M genes in *Proteus* by using specific primers, as in the Table-1.

Table 1- Multiplex specific primers of CTX-M genes according to Mahrouki *et al., 2012*

Gene	Sequences	Gene size
$bla_{CTX-M-1}$	F 5' ATGGTTAAAAAATCACTGCGTC 3' R 5' TTGGTGACGATTTTAGCCGC 3'	864
$bla_{CTX-M-2}$	F 5' ATGATGACTCAGAGCATTCG 3' R 5' TGGGTTACGATTTTCGCCGC 3'	866
$bla_{CTX-M-8}$	F 5' ACTTCAGCCACACGGATTCA 3' R 5' CGAGTACGTCACGACGACTT 3'	877
$bla_{CTX-M-9}$	F 5' ATGGTGACAAAGAGAGTGCAA 3' R 5' TCACAGCCCTTCGGCGATGATTCTCGC 3'	876

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 according to the following conditions :Initial denaturation at 94 °C for 7 min., denaturation at 94 °C for 1 min., annealing for ($bla_{CTX\text{-}M\text{-}1}$ 60 °C for 1 min.), ($bla_{CTX\text{-}M\text{-}2}$ 54 °C for 1 min.), ($bla_{CTX\text{-}M\text{-}8}$ 60 °C for 1 min.), ($bla_{CTX\text{-}M\text{-}9}$ 55 °C for 1 min.), extension at 72 °C for 3 min. and final extension for 72 °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**.**

Results and Discussion:

Identification of 25 isolates of *Proteus* was done by using morphological features on Blood agar media and MacConkey agar media in addition to microscopic examination by using Gram stain showed that all isolates were appeared polymorphic Gram negative rods [17, 19]. Moreover, vitek 2 identification system was used to confirm the identification of these isolates. The results showed that 15 isolates (60 %) were *P. mirabilis*, and 10 isolates (40 %) were *P. vulgaris.* Although Cefotaxime and ceftazidime (the third generation of cephalosporins) have important role as a drug of choice to treat the most of *Protous* infections, *Proteus* resistance to these drugs are increased progressively in recent years [20, 21]; so the susceptibility of *P. mirabilis* isolates to cefotaxime and ceftazidime were investigated. It was noticed that 10 of 15 (66.6%) *P. mirabilis* isolates were resistant to cefotaxime while 4 of 10 (44.4%) *P. vulgaris* isolates were resistant to the same antibiotic. Resistancy for cftazidime was proximate for the two species; 3 of 15 isolates (20%) of *P. mirabilis* and 2 of 10 isolates (20%) of *P. vulagris*, as in Figure-1.

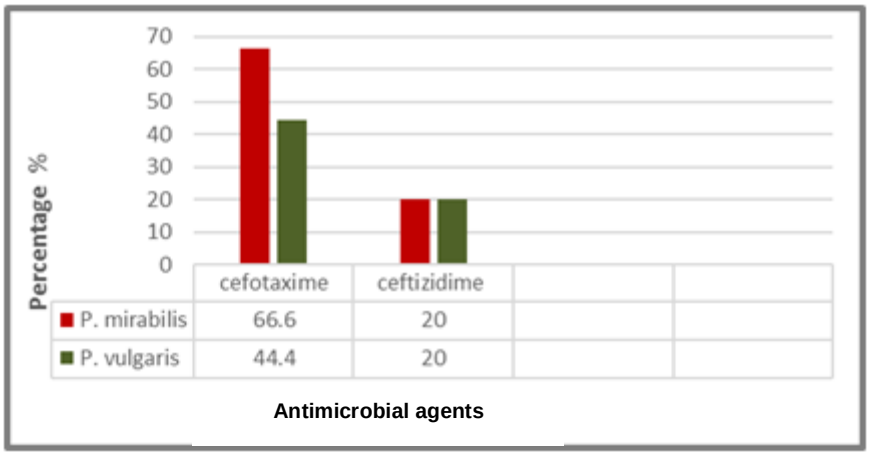

Figure 1- Resistance of *Proteus mirabilis* and *Proteus vulgaris* to cefotaxime and cftazidime

The presence of high percentage of isolates that expressed ESBLs may due to the high proportion of antimicrobial agents usage and increase the resistant to antibiotic treatment, so ESBLs producing *Proteus* test was done and the results showed that 5 among 14 (30.7 %) isolates of *Proteus* were able for production ESBLs. Thereafter, 10 isolates of *P. mirabilis* and 4 isolates of *P. vulgaris* were selected for detection of $bla_{CTX\text{-}M}$ gene that resist to cefotaxime 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 was ranged from 45.8 ng/µl to 98.3 ng/µl, while purity was ranged from 1.3 to2.0, the DNA purity was confirmed by using gel electrophoresis technique.

Multiplex PCR technique was used for detection of ($bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}8}$ and $bla_{CTX\text{-}M\text{-}9}$) genes in 14 *Proteus* isolates susceptible to cefotaxime (10 isolates of *P. mirabilis*, followed by 4 isolates of *P. vulgaris*). The results showed that $bla_{CTX\text{-}M\text{-}2}$ gene with 780 bp was found in all isolates of *P. mirabilis* and *P. vulgaris*, after confirmed by gel electrophoresis technique with molecular weight of $bla_{CTX\text{-}M\text{-}2}$ according to Nagano *et al.* (2003) by using 100bp DNA ladder [13] Figure-2.

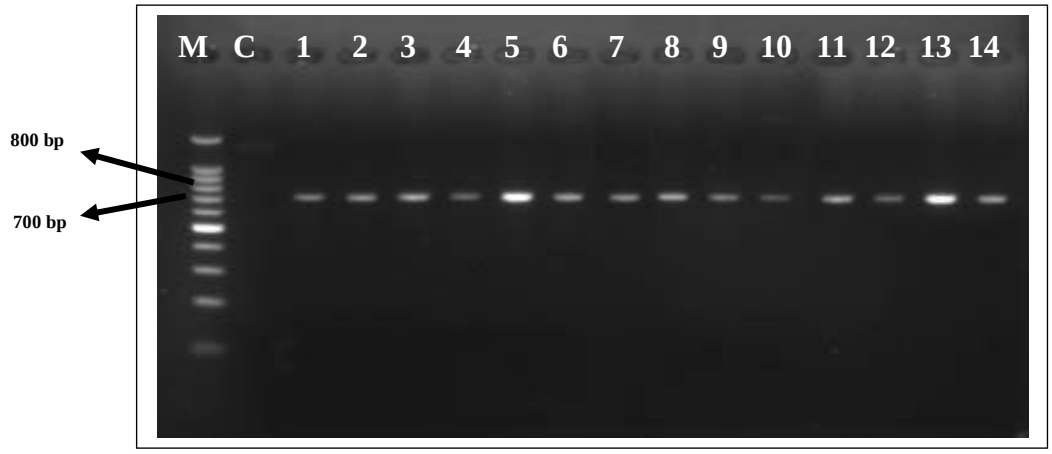

Figure 2-PCR reaction with specific primers for $bla_{CTX\text{-}M\text{-}2}$ (1,2,3,4,5,6,7,8,9,10 are *P. mirabilis isolates* while 11,12,13,14 are *P.vulgaris* isolates) with DNA marker 100 bp ladder (M) and Control of ionized distilled water (C), in 1.5 % agarose gel at 5 volt/cm for 1-2 h, visualized by E-graph gel documentation equipped by UV light source.

While detection of other $bla_{CTX\text{-}M}$ genes ($bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}8}$ and $bla_{CTX\text{-}M\text{-}9}$) in the 10 isolates of *P. mirabilis* and 4 isolates of *P. vulgaris* showed the absence of DNA bands that represented the $bla_{CTX\text{-}M}$ genes, Figure-3.

Figure 3- PCR reaction with specific primers for $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}8}$ & $bla_{CTX\text{-}M\text{-}9}$ (1,2,3,4,5,6,7,8,9,10 are *P. mirabilis isolates* while 11,12,13,14 are *P. vulgaris* isolates) with DNA marker 100 bp ladder (M) and Control of ionized distilled water (C), in 1.5 % agarose gel at 5 volt/cm for 1-2 h, visualized by E-graph gel documentation equipped by UV light source.

The results showed that the isolates of specific species of bacteria (*Proteus*) have one genotype responsible of resistant for cefotaxime, in spites of that the different among these related genes may occurs in one amino acids sequence but the susceptibility towards the antibiotic is restricted by specific class of gene, this may be return to the distribution of bacteria according to the geographic area or race in addition to increase bacterial infections with similar genetic structure due to the transmittance of microorganism with same environment and health conditions of population in specific area.

Conclusions:

The results showed the presence of blaCTX-$_{M\text{-}2}$ gene, which is responsible for resistance to cefotaxime in the isolates of *Proteus*, while no other types of $bla_{CTX\text{-}M}$ genes were found in them. This may be because the geographic area or the development of increased antibiotics activity.

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