PREVALENCE OF \( \text{blaTEM} \) and \( \text{qnr} \) GENES IN CLINICAL ISOLATES OF PROTEUS VULGARIS FROM PATIENTS WITH URINARY TRACT INFECTION, BABIL, IRAQ

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ABSTRACT

Proteus vulgaris resistant to beta-lactam and quinolone drugs, is widely recognized as an opportunistic pathogen causing urinary tract and septic infections; often nosocomial. The resistance property is obtained by acquisition of mobile element-encoded \( \text{blaTEM} \) and \( \text{qnr} \) genes. The aim of this study was to document the prevalence of these resistant genes in Proteus vulgaris. The susceptibility of isolates to a variety of antibiotics has been investigated. It has been found that all the isolates had the highest percentage of resistance (100%) to ampicillin, amoxicillin, cephalothin, chloramphenicol and ceftazidime, and some isolates had lower frequencies of resistance to ceftriaxone (89%), ciprofloxacin (82%) and cotrimazole (92.8%). Whereas, all the isolates were sensitive (100%) to amikacin, gentamycin, nitrofurantion, imipenem, meropenem, and norfloxacin. \( \text{blaTEM} \) and \( \text{qnr} \) genes were detected in 100% and 50% of isolates, respectively. The results suggest the spread of resistance genes among strains of Proteus vulgaris.

Keywords: Proteus vulgaris, \( \text{bla Tem} \) gene, \( \text{qnr} \) gene.

INTRODUCTION

The etiology of UTI and the antibiotic resistance of uropathogens have been changing over the past years, both in community and nosocomial infection [1, 2]. The genus Proteus is a member of a large gram-negative bacilli family, Enterobacteriaceae[3]. Proteus vulgaris is one
of the most commonly isolated members of *Proteus sp.*, along with *Proteus mirabilis*. *P. vulgaris* is a rod-shaped, Gram negative bacterium that inhabits the intestinal tracts of humans and animals. It can also be found in soil, water and fecal matter. It is an opportunistic pathogen of human, it is known to cause urinary tract infections and wound infections [3, 4]. *Proteus spp.* are generally susceptible to broad-spectrum cephalosporins, aminoglycosides, and imipenem[5]. Otherwise, *P. vulgaris* is also susceptible to cefoxitin, cefepime, and aztreonam. The resistance to ciprofloxacin may develop with unrestricted use. *P. vulgaris* is resistant to piperacillin, amoxicillin, ampicillin, cefoperazone, cefuroxime, and cefazolin. Resistance to B-lactam among *Proteus* is emerging. Several mechanisms explain the emergence of drug resistance have been discovered in the past decades, the best known being beta-lactamase and quinolone resistance mechanisms [6]. Beta-lactamases are enzymes that break the beta-lactam ring and deactivate this class of antimicrobial drugs. Beta-lactamases are encoded by either chromosomes or plasmids. The highly mobile nature of beta-lactamase genes remains an important problem in UTI treatment [7, 8, and 9].

A variety of transferable genes encoding b-lactamase activity have been described in clinical environments including *blaCTX-M, blaGES, blaHER, blaOXA, blaOXY, blaSED, blaSHV, blaSPM, blaVEB, blaVIM, and ampC* alleles. Among the most common *bla* genes is the *blaTEM* gene, the first described *bla* gene and a representative of the *blaTEM* group that now consists of almost 150 different alleles, all encoding different amino acid polymorphisms that extend their substrate range. The newer variants of the *blaTEM* alleles have only been found in clinical isolates and are likely emerging as a result of point mutations and directional selection [10].

The TEM β-lactamases represent one of the most clinically significant families of β-lactamases. The first in this group to be discovered, TEM-1, is considered broad spectrum and hydrolyzes the early cephalosporins, in addition to many penicillins. TEM-1 has become the most commonly encountered β-lactamase and is ubiquitous among *Enterobacteriaceae*[10]. TEM-3 was the first of the extended-spectrum β-lactamases (ESBLs), which have an increased substrate spectrum, including third-generation cephalosporins, but are susceptible to β-lactamase inhibitors such as clavulanic acid [10]. Quinolones are a class of molecules that are used extensively in the treatment of many infections [11]. Their availability and use have increased in recent years, especially in developing countries [11]. For more than 30 years, the only known mechanisms of resistance
to quinolones were chromosome borne. The two main mechanisms known to account for quinolone resistance are alteration of drug permeation \textit{(i.e.,} decreased uptake mediated by mutations in the structural or regulating gene of porins\cite{11}, active efflux mediated by mutations in active expulsion pumps and target alteration \textit{(i.e.,} mutation in the quinolone-resistance determining regions (QRDRs) of \text{gyr}A-gyrB\text{or} C-parE encoding topoisomerase II \cite{12}. Recently, plasmid-mediated resistance mechanisms have been described. The first plasmid-mediated resistance to quinolones was discovered in 1998 \cite{11}, in a clinical isolate of \textit{Klebsiella pneumoniae} that could transfer low-level resistance to quinolone to \textit{Escherichia coli} or other Gram-negative bacteria. The plasmid-mediated quinolone resistance gene was named “\textit{qnr}”. This gene encoded a 218 amino-acid protein \textit{Qnr} (later named \textit{QnrA}), belonging to the pentapeptide-repeat family. More recently, four other markers (\textit{QnrB} and \textit{QnrS}, \textit{QnrC} and \textit{QnrD}) have been identified in several enterobacterial species \cite{13,14,15,16}. These markers interact with quinolones, the topoisomerases, and DNA, thus limiting the binding of the quinolones to their target \cite{16}.

The main distinction of \textit{qnr} genes is carried on several integrons\cite{17}. These determinants can be easily transferred, accelerating the spread of quinolone resistance through gene transfer mechanisms. In addition, the described integrons can carry genes which encode for resistance to third-generation cephalosporins (ESBL or ESC or derepressed cephalosporinase) \cite{11}. The present study aimed to investigate the occurrence of antimicrobial resistance and the prevalence of \textit{bla}_{TEM} and \textit{qnr} genes in clinical isolates of \textit{P.vulgaris}.

**MATERIALS AND METHODS**

**Bacterial isolates**

\textit{Proteus vulgaris} was isolated from a patient with UTI who were admitted to four hospitals: Babylon Hospital for Maternal and Pediatrics, Al-Hilla Surgical Teaching Hospital, Al-Hashymia hospital and Al-Qasim hospital during the period from 4/2012 to 1/2013. Standard biochemical tests were used for detecting \textit{P.vulgaris} strains\cite{18}.

**Antibiotic sensitivity test**

The antibiotic sensitivity test of the isolates were determined, using Bauer Kirby disc diffusion method on Oxoid-Mueller-Hinton agar. The following antibiotics were used: amikacin (30), amoxicillin (25), ampicillin (10), cephalothin(30), ceftazidime (30), ciprofloxacin (5),cotrimazole(25),gentamycin (10), imipenem (10), meropenem (10), nitrofurantion (10) and norfloxacin (10) (Bioanalyse /Turkey). In this test small filter paper
disks (disc: 6 mm) impregnated with a standard amount of antibiotic are placed onto an agar plate to which bacteria (The inoculate was prepared directly from an overnight agar plates adjusted to 0.5 McFarland standard of National Committee for Clinical Laboratory Standards, (NCCLS- 2000) [19] have been swabbed. Then the plates are incubated overnight at 37°C, and the zone of inhibition of bacterial growth is used as a measure of susceptibility, where large zones of inhibition indicate that the organism is susceptible, while small or no zone of inhibition indicate resistance. An interpretation of intermediate is given for zones which fall between the accepted cutoffs for the other interpretations [20,21].

DNA Extraction and Genes Amplification

Genomic DNA was extracted by a commercial nucleic acid extraction kit (Bioneer-Korea) according to the manufacturer’s instructions.

PCR Amplification of $bla_{TEM}$ and Qnr Genes

Amplification of $bla_{TEM}$ and QNR genes were performed in thermal cycler (MJ Reasearch, USA) using primers designed by this study using NCBI GenBank and MP Primer design online. The GenBank: ($bla_{TEM}$ gene: GQ983321.1 [22], and (qnr gene: EF488761.1) [23] as shown in Table 1. These primers were provided by (Bioneer Company, Korea). Briefly each reaction was carried out in 25µl reaction volume using 12.5µl of Accustart™ Taq PCR Super Mix(VWR-USA), 1µl of primers, 2µl of DNA template, and 8.5µl of Nuclease free water (ddH2o). Thermocycling parameters were as follows: an initial denaturation of 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 45 s, primer annealing 62 °C for 30 s, and extension at 72 °C for 45s.Finally one extension step at 72°C for 7 min.

Table (1): Primers used in PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Product size</th>
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<tbody>
<tr>
<td>$bla_{TEM}$ gene</td>
<td>5'-AGAGCAACTCGGTCGCGCATA-3'</td>
<td>310bp</td>
</tr>
<tr>
<td></td>
<td>5'-GCAGCATCGGTTTAGGCTTTCGCA-3'</td>
<td></td>
</tr>
<tr>
<td>qnr gene</td>
<td>5'-ACGCGAGGAGTTAGGCGACGACG-3'</td>
<td>410bp</td>
</tr>
<tr>
<td></td>
<td>5'-CGCTGAGGTTGGCATTGCTCCA-3'</td>
<td></td>
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</table>
Detection of Amplified Products by Agarose Gel Electrophoresis
Successful PCR amplification was confirmed by agarose gel electrophoresis. The PCR products were assessed by electrophoresis (Amersham Biosciences, USA) in 1% agarose gel with 0.5% ethidium bromide (Alfa Aesar, USA). Agarose gel was prepared by dissolving 0.45 gm of agarose powder in 40 ml of TBE buffer (pH: 8) in a microwave (Kenmore, USA), allowed to cool to 50°C and then ethidium bromide at the concentration of 0.5 mg/ml was added [24]. The comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber filled with TBE buffer that covered the surface of the gel, 5 µl of DNA sample mixed with Gel Loading Dye Blue (Biolabs, USA) was transferred into the wells in agarose gel, and in one well we put 1 µl of DNA ladder (Biolabs, USA). The electric current was allowed at 110 volt for 60 min. UV Trans-illuminater (San. Gabriel, USA) was used for the observation of DNA bands, and then gel was photographed using a Gel Documentation System with a digital camera (Bio Rad, USA).

Statistical Methods
Data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentages). All statistical calculations were done using Microsoft Excel 2007 (Microsoft Corporation, New York, USA).

RESULTS
Antibiotic susceptibility testing
The effect of different antibiotics on P. vulgaris isolates was investigated. These isolates showed different susceptibility towards antibiotics used in this study, as shown in figure (1). It has been found that the majority of the isolates were multidrug resistant since they were resistant to three antimicrobials agents or more. The highest rate (100%) of resistance is seen with ampicillin, amoxicillin, cephalothin, chloramphenicol and ceftazidime, and they are moderately resistant to ceftriaxone; 25/28 (89%), ciprofloxacin 23/28 (82%) and cotrimazole 26/28 (92.8%). Whereas, all the isolates were sensitive (100%) to amikacin, gentamycin, nitrofurantion, imipenem, meropenem, and norfloxacin.
Detection of $bla_{TEM}$ and $qnr$ Genes by PCR

A-$bla_{TEM}$

All $P. vulgaris$ isolates which were resistant for B-lactam antibiotics were have $bla_{TEM}$ gene, this results obtained after amplify the DNA of resistant isolates with specific primers for $bla_{TEM}$ gene by PCR. Amplicons with predicted size of 310bp were generated and the prevalence rate was 100% as show in figure (2).

B-$qnr$ gene

Quinolones constitute an important group of antimicrobials active against Gram-negative and Gram-positive bacteria. Because of wide clinical use, clinical isolates resistant to fluoroquinolone are emerging and spreading rapidly [25]. In the present study, PCR was used to detect the prevalence of $qnr$ gene in $P. vulgaris$ isolates. As it is evident from figure (2) that $qnr$ gene was detected, with 5 out of the 10 isolates (50%). Amplicons with predicted size of 410bp were generated.

![Figure 1: Antibiotic susceptibility of Proteus vulgaris](image)

Fig (2):- Gel electrophoresis of PCR of bla<sub>TEM</sub> and qnr amplicon product. M: Marker; 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10: no. of <i>P. vulgaris</i> isolates obtained from urine; bla<sub>TEM</sub> gene band size 310bp and qnr gene band size 410bp.

DISCUSSION

The present study revealed the highest resistance of <i>P. vulgaris</i> to B-lactam antibiotics (Fig.1). The emergence of multidrug resistance strains which are resistant to most of the antimicrobials agent tested may be due to the fact that ampicillin, amoxicillin, cephalothin considered the most commonly prescribed antibiotics in the hospital even before the results of urine analyses and also the most easily available in the market without prescription and because they were also very cheap in terms of cost. The widespread use and more often the misuse of antimicrobial drugs has led to a general rise in the emergence of resistant bacteria [26].

Our results were correspond with other results reported by other investigators such as results of Feglo et al. [27] who found (84.6%) of <i>Proteus</i> isolates recovered from different clinical samples were characterized by multidrug resistance phenotype and with Al-Jumaa et al. [28] who have shown that all <i>Proteus</i> isolates were resistance to β-lactam group, where they found (80%) of the isolates were resistant to amoxicillin, (93.3%) of them were resistance to penicillin and all isolates (100%) were resistant to cephalothin.

Emergence and dissemination of β-lactam resistance in nosocomial <i>Enterobacteriaceae</i> became a serious problem worldwide. Gram-negative bacteria pursue various molecular strategies for development of resistance to these antibiotics: (a) generation of extended-spectrum β-lactamases (ESBL) according to the original definition due to extension of the spectrum of already widely disseminated plasmid-encoded β-lactamases by amino acid substitution; (b) acquisition of genes encoding ESBL from environmental bacteria (c) high-level expression of chromosome-encoded β-lactamase (<i>bla</i>) genes as bla<sub>OXA</sub> or bla<sub>ampC</sub> genes.
due to modifications in regulatory genes, mutations of the β-lactamase promoter sequence as well as integration of insertion sequences containing an efficient promoter for intrinsic bla genes; (d) mobilization of bla genes by incorporation in integrons and horizontal transfer into other Gram-negative species; (e) non-expression of porin genes and/or efflux pump-based antibiotic resistance[29].

A significant increase in resistance of pathogenic strains to ampicillin and cephalothin has been found worldwide [30] but older agents like gentamicin still show high efficacy against UTI pathogens because of its multiple mechanisms of action seem to have enabled it to retain potent activity against P. vulgaris [26].

The production of β-lactamase remains the major mechanism of resistance in gram-negative bacilli to β-lactam antibiotics. In recent years, extended-spectrum β-lactamases (ESBLs) have become progressively widespread due to extensive use of third generation cephalosporins in hospital settings [31,32]. Proteus has an intrinsic resistance to ampicillin and cephalosporin due to extended spectrum β-lactamase (ESBLs). B-lactamases (ESBLs) are enzymes that compromise the efficacy of all β-lactams by hydrolysis of the β-lactam ring[33].

The characterization of various plasmid mediated TEM-type β-lactamase in Proteus are evidence of the wide diversity of β-lactamases produced by this species and of its possible role as β-lactamase-encoding plasmid reservoir [34]. So, in the present study we selected important gene that confer resistant to B-lactam antibiotics, the blaTEM gene.

As shown in figure (2), the prevalence of blaTEM was (100%), showing its presence in all resistant isolates of P. vulgaris. These results are in agreement with previous results reported by Dallenne et al. [35], Tissera and Mae Lee[36] who found blaCTX-M genes with the highest occurrence in clinical Proteus isolates in studies conducted in France and in a Chinese urban river. Similarly, blaTEM genes were of note-worthy high occurrence too. Where, blaTEMs often co-occur with other chromosomal (AmpC) or plasmidic (SHV, OXA, CTX-M) β-lactamases [37, 38] and they are common in commensal bacteria inhabiting the human gut [39].

The increasing variety of β-lactamases produced by isolates of the family Enterobacteriaceae raises concern about our dependence on β-lactam drugs and the emergence of pan-resistant species [40]. Proteus has an intrinsic resistance to ampicillin and cephalosporin due to
extended spectrum β-lactamase [33]. Resistance to expanded spectrum cephalosporins may develop through the expression of chromosomally encoded class C beta-lactamases. Horizontal gene transfer mediated by R plasmids, transposons and integrons is largely responsible for increasing the incidence of antibiotic-resistant infections worldwide [41].

In terms of danger to human health, previous research highlight that potential ESBL species such as K. pneumonia and E. coli have a high tendency to possess and transfer bla genes [42]. Transfer may occur by conjugation because the genes are often found on mobile elements like transposons and integrons [36]. Some of these species may be pathogenic strains that have the potential to cause life-threatening diseases and widespread outbreaks. For instance, blaCTX-M and blaTEM genes in opportunistically pathogenic enterobacteriaceae have been associated with nosocomial infections [43].

Resistance to ciprofloxacin (Fig.1), may be due to one of the three mechanisms of resistance to quinolones which are: mutations that alter the drug targets, mutations that reduce drug accumulation, and plasmid-mediated qnr genes that protect cells from the lethal effects of quinolones. These genes are found mainly in Enterobacteriaceae and affect the dynamics of development and acquisition of quinolone resistance [12, 44]. In this study, we used chromosomal DNA from resistant isolates of P. vulgaris to detect the presence of qnr gene, which may be the reason for ciprofloxacin resistant.

As shown in figure (2), qnr genes were detected in (50%) of P. vulgaris isolates. Our results were corresponding with previous study by Enabulele et al. [45] who found the average resistance of the gram negative isolates to the various quinolones ranged from 42.7% to 66.7%. Klebsiella were the most resistant isolates with a mean resistance of 66.7% while Proteus were the less resistant isolates with a mean resistance of 42.7%. Also, it was similar to the results obtained by Wallace et al. [46] and Daini et al. [47] who referred to that all gram negative strains resistant to any antimicrobial agents were also resistant to ciprofloxacin.

Resistance to the quinolones often emerges at low-levels by acquisition of an initial resistance-conferring mutation or gene. Acquisition of subsequent mutations leads to higher levels of resistance to the first-generation quinolone, nalidixic acid and a broadening of the resistance spectrum to include second-generation quinolones (first-generation fluoroquinolones) such as ciprofloxacin, followed by newer second- and third-generation
fluoroquinolones[48].

This work describes the prevalence of qnr genes among ESBL strains of P. vulgaris identifies the presence of qnr genes in quinolone-susceptible strains which could lead to in vivo selection of ciprofloxacin-resistant strains.

CONCLUSIONS
In this study, the resistance of P. vulgaris isolates to β-lactam and fluoroquinolones antibiotics belong to the expression of chromosomally encoded TEM-type β-lactamase and qnr genes.

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REFERENCES


