

Plasmid mediated multidrug resistant of uropathogenic *Proteus mirabilis*

Gulbahar F. Karim

College of Nursing University of Kirkuk

Abstract

A total of 136 urine samples were collected aseptically from patients with urinary tract abnormality from April to June, 2010. Depending on morphological, cultural and biochemical testes 16 (~11.8%) *Proteus mirabilis* isolates were recovered. They were examined for antibiotic resistance. All of the isolates (100%) were resistant to Ampicillin, Amoxicillin, Cephalothin, They showed highly resistant rate to Tetracycline (81%), and Gentamycin (75%). Some of the isolates exhibit resistant to the third generation Cephalosporines as Cefotaxime and Ceftriaxon (Cef). There are 5 Multi drug resistant (MDR) phenotypes have been recognized. It have been found that three of these isolates produced β -lactamase enzymes and each of them was bearing single plasmid of more than 3000bp in size (the largest fragment of the DNA marker). Following curing experiment, the mutant strains lost their plasmids and resistance to all β -lactam antibiotics which included Ampicillin, amoxicillin, cephalothin, cefotaxime and ceftriaxone as well as Tetracycline and Gentamycin. The results of present study highlight that some of MDR phenotype exhibiting by uropathogenic *Proteus mirabilis* attributed to plasmid bearing multiple resistance determinant.

Introduction

Proteus strains occur in the soil, polluted water and in the intestine of human and a wide variety of animals. They form part of normal flora and can be opportunistic human pathogens. (Peliczar, J.R., *et. al.*, 1986).

The genus *Proteus* belong to the family Enterobacteriaceae and is consists of four species: *Proteus vulgaris*, *Proteus penneri*, *Proteus myxofaciens* and *Proteus mirabilis* (Penner, J.L., *et.al.*, 1984). The latter is the causative agent of the majority of *Proteus* infections (Feglo, P.K., *et.al.*, 2010) and is one of bacteria responsible for urinary tract infections in individuals with long term urinary catheters in place or individuals with complicated urinary tract infections (Nabeela, N., *et.al.*, 2004).

The pathogenicity induced by *Proteus mirabilis* due to express of virulence factors including; invasion, adhesion, cytotoxicity, urease,

elastase production and swarming migration (El-baghdadi, K.Z., *et.al.*,2009) as well as antibiotic resistance (Dharmadhikari , S.M. and Peshwe, S.A., 2009;El-baghdadi, K.Z., *et.al.*,2009).

There are many mechanisms were by bacteria confer resistance to the drugs including intrinsic impermeability and acquired resistance as plasmids, transposons and mutations (Gutmann,1985). Yah reported in a study on a wide spread of plasmids resistance genes among *Proteus* species that 44% of antibiotic resistance were plasmid mediated, 32% by chromosome, while 24% of the resistance pattern to antibiotics could not be ascertained.

Transferable resistance has been identified for some antibiotic groups as β -lactams, aminoglycosides, macrolides, sulphonamides, tetracyclins, chloramphenicol, etc. (Verschneren, 1993). However the production of plasmid or chromosomal encoded β -lactamase enzymes is the most common mechanism of resistance in gram negative bacteria causing clinical significant infection (Bush, *et.al.*, 1995).

The aims of the study is to determine the antibiotic susceptibility and plasmid profiles of *Proteus mirabilis* isolated from urinary tract infections in order to provide proper treatment, this will prevent their dissemination and reduce the risk of urinary tract infection complication.

Patients and Methods

A total of 136 urine samples were collected from in and out patients with urinary tract abnormality attending Azadi Teaching Hospital in Kirkuk city. A clean midstream urine samples were collected using sterile containers then transferred promptly to the laboratory for General Urine Examination (G.U.E.) as well as culturing on Nutrient agar ,blood agar and MacConkey agar (Cheesbrough, M. 1991), then incubated at 37°C for 24-48hr. The bacterial species were identified by conventional biochemical tests as described by Atlas (Atlas, *et.al.*, 1995). The isolates subcultured and stored on nutrient agar slants at 4C°for further investigations . The identification of *Proteus mirabilis* isolates was confirmed by using analytical profile index (Api)20E system test. (BiomMerieus, France).

Antibiotic susceptibility test

Antibiotic resistance patterns of the isolates were determined using the Disc diffusion (Kirby Bauer) method; inoculum of tested bacterium was prepared: A single colony was transferred to 5ml nutrient broth then incubated at 37°C for 24hr.

The inoculum was adjusted to 0.5McFarland standard of National Committee for Clinical Laboratory Standard(NCCLS,2000). The tested antimicrobial agents were Ampicillin (Am) 10µg,Amoxicillin(AX)25µg, Cephalothin (KF) 30 µg, Cefotaxime(CTX) 30 µg, Ceftriaxon(cef)30µg, Gentamicin (GM) 10 µg, Tetracycline (T) 30 µg, Nalidixic acid (NA)30 µg, and Ciprofloxacin (CIP) 5 µg. These were aseptically placed on the inoculated Muller Hinton agar(Oxoid Company) and incubated overnight. The zones of inhibition were measured and interpreted according to (NCCLS, 2000).

Rapid Iodometric Method

Rapid iodometric test was carried out for detection of β-lactamase enzymes (WHO, 1978). MacConkey's agar was inoculated with tested bacteria and incubated at 37°C for 24hr, then a number of colonies were picked with a sterile loop and transferred to epindorf tube containing 100µl penicillin G solution and incubated for 30 min. 50µl starch solution was added and mixed well with other contents followed by 20µl iodide solution, then mixed for 1min. Dark blue color developed, rapid change of color from blue to white was considered positive result, any result was disregarded after 4 min.

Plasmid DNA Extraction

Plasmid DNA was extracted and purified from 5ml overnight culture of the selected isolates grown in LB broth medium containing 100µg/ml Ampicillin using a QIA prep spin Miniprep kit(Qiagen) according to the manufacturer's instructions.

Agarose Gel Electrophoresis

1% (w/v) agarose gel was made by adding 1 gm of agarose to 100 ml of 1X TBE buffer solubilized by heating at boiling temperature, then the agarose was left to cool at 55 C° before pouring in a tray to solidify. A comb was placed near one edge of gel, and gel was left to harden. 1X TBE was poured into gel tank and the gel tray was placed horizontally in

electrophoresis tank, 3µl of loading buffer was mixed with 10 µl DNA sample, and then samples were added carefully to individual wells. Power was turned on at 45 Volts for 15minutes and 85 Volts for 4-5 hour to run DNA. Agarose gels were stained with ethidium bromide by immersing them in distilled water containing the dye of final concentration of 0.5µg/ml for 30-45 minutes. DNA bands were visualized by U.V. illumination at 366nm wavelengths on U.V. transilluminator.

Gel was destained in distilled water for 30-60 minute to get rid of the back ground staining. Then, photographing was done using Digital camera (Sambroock *et al.*, 1989).

Plasmid Curing

Proteus mirabilis strains harboring plasmids were cured of their plasmids according to Garriga (Garriga, *et al.*,1993). An overnight culture of each *Proteus* isolate standardized to 10⁸ cfu/ml with phosphate buffered saline (pH 7.2), then it was subculture at 10⁵ cfu/ml in to 30 ml of nutrient broth (pH 7.6) containing 1.25% ethidium bromide solution. Cultures were incubated for 72hr. with continues shaking (120 rpm) under aerobic condition. Cell pellets were then obtained by centrifugation (5000rpm, 10min.,4C°). Curing is indicated by loss of plasmid that confirmed by plasmid extraction and agarose gel electrophoresis.

Result and Discussion

Out of 136 urine samples 16(11.8%) isolates were diagnosed as *Proteus mirabilis* depending on morphological and cultural properties on Blood agar, MacConky agar and Nutrient agar as well as conventional biochemical tests as shown in table (1) and confirmed by analytic profile index(Api)20E system as shown in figure (1).

This finding might be due to its existence as part of normal intestine flora and uropathogenic *Proteus mirabilis* virulence facors (Rozalski,A.,*et.al.*,1997; stankowska,D.,*et.al.*, 2008).

The results of the current study were in accordance with other investigators (warren, J.W., *et.al.* , 1987) who recorded that (12%) of UTI cases due to *Proteus mirabilis*. Other researchers also stated that proteus species are the third etiological agents of UTI after *E. coli* and *Kleibsella pneumoniae* (Nabella,N.M.A., *et.al.*, 2004).

Antibiotic susceptibility test for (9) different antibiotics by Disc diffusion method recommended by (NCCLS) guide line was performed for the studied isolates.

Table (1) Biochemical tests for identification of *Proteus mirabilis* isolates

| Biochemical test | | The results |
|---------------------|-----------------------------|-------------------------|
| Indole | | - |
| Methyl red | | + |
| Voges proskauer | | - |
| Citrate utilization | | - |
| Urease production | | + |
| Oxidase | | - |
| Catalase | | + |
| Kleigler | Gas production | + |
| | H ₂ S production | + |
| | Slope | Alkaline |
| | Bottom | Acid |
| Motility | | + (swarming migration) |

As shown in table (2), All of the isolates (100%), were resistant to Ampicillin, Amoxicillin, Cephalothin, this might be due to the production of β -lactamase enzymes by the isolates or due to other mechanisms as mutation in penicillin binding site, or inability to diffuse across the bacterial outer membrane (George, *et. al.*,2005).

There were a high resistant rate expressed by the isolates against Tetracycline (81%),and aminoglycoside antibiotics as Gentamycin 13(75%). So rendering these drugs inactive for treating UTI caused by *Proteus mirabilis*.

Figure (1) Astrip of Api20E system show the biochemical results of *Proteus mirabilis*



It has been shown that there were some resistance arising to the third Generation Cephalosporines as Cephalexin and cephtriaxon despite their potency. The random and over use of these antibiotics might stimulate the bacterial population to develop different defense mechanisms as production of extended spectrum β -lactamase (ESBLs) enzymes. Other investigator (Philipon, 1989) stated that ESBLs enzymes resist the new Cephalosporines as Cephalexin and Cephtriaxon as well as the old Penicillins and Cephalosporines.

Table (2) Antibiotic resistance pattern of all *Proteus mirabilis* isolates

| Antibiotic | R | R% |
|----------------|----|-------|
| Ampicillin | 16 | 100 |
| Amoxicillin | 16 | 100 |
| Cephalothin | 16 | 100 |
| Cefotaxim | 5 | 31.25 |
| Ceftriaxone | 5 | 31.25 |
| Tetracycline | 13 | 81.25 |
| Gentamicin | 12 | 75 |
| Ciprofloxacin | 1 | 6.25 |
| Nalidixic acid | 1 | 6.25 |

R: Resistant

Most of isolates 15 (93.75%) were sensitive to Ciprofloxacin and Nalidixic acid, hence these drugs must form a part of the empirical antibiotics for the treatment of *Proteus mirabilis* infections.

It has been found that the majority of the isolates 14(87.5%) were multidrug resistant since they were resistant to three antimicrobials agents or more. The list of 5 MDR phenotypes recognized in table 3. Most of the sample study (7/16) were resistant to 5 antibiotics followed by (4/16) isolates resistant to 7 antibiotics. However only one isolate was resistant to all 9 antibiotics used in the study. This result was in accordance with other investigators (Feglo, P.K., et. al., 2010) where they recorded that (84.6%) of *Proteus mirabilis* isolates recovered from different clinical samples were characterized by MDR phenotype.

Table (3) Antimicrobial resistance pattern of the MDR isolates

| <i>Number of patterns</i> | <i>Resistanace pattern</i> | <i>Number of MDR isolates</i> |
|---------------------------|--|-------------------------------|
| <i>1</i> | <i>Am, Ax, KF, CTX, Cef,GM, T, Cip, NA</i> | <i>1</i> |
| <i>2</i> | <i>Am, Ax, KF, CTX, Cef,GM, T,</i> | <i>4</i> |
| <i>3</i> | <i>Am, Ax, KF, GM, T</i> | <i>7</i> |
| <i>4</i> | <i>Am, Ax, KF, T,</i> | <i>1</i> |
| <i>5</i> | <i>Am, Ax, KF,</i> | <i>3</i> |

Am: Ampicillin, Ax: Amoxicillin , KF: Cephalothin, CTX: Cefotaxim , Cef: Ceftriaxone ,GM: Gentamicin , T: Tetracycline, Cip: Ciprofloxacin , NA: Nalidixic acid.

According to the rapid Iodometric method 3(3/16) isolates produced β -lactamase enzymes, so the MDR phenomenon of these organisms might be contributed to the plasmid or chromosomal encoded β -lactamase enzymes. However other isolates not produce these enzymes they were exhibit other mechanisms for circumvent drug actions as cell wall permeability or pencillin binding site not inhibit by β -lactam antibiotics (Obrien,1997). It have been reported that MDR in bacteria generally due to multiple transposons and plasmids bearing genetic determinants for different mechanisms of resistance(Gold and Moellering, 1996).

Plasmid extraction for all *Proteus mirabilis* isolates was carried out and visualized by migration through 1% agarose electrophoresis containing 100bp DNA ladder sample as a control.

As shown in (figure.2), three of the isolates that produce β -lactamase enzymes were have one plasmid ,they seem to have the same size of more than 3000bp (the size of the largest fragment of the DNA

marker), These plasmids might be responsible for the antibiotic resistance exhibit by the studied isolates. This result was in agreement with the finding of Adeniyi (Adeniyi, B.A., *et.al.*, 2006) who reported that E.coli, Proteus species and Pseudomonas species isolates carrying plasmid of molecular size above 2.1kb. Other investigators (Stankowska, D., *et.al.*, 2008), also detected two plasmids harbored by some of analyzed strains of Proteus mirabilis of about 6 and 93kb.

The plasmid were able to move genetic antibiotic resistant materials among various bacterial strains (Yah, S.C., *et.al.* , 2007) and contribute to overall pathogenic potential of disease causing bacteria (Dharmadhikari , S.M. and Peshwe, S.A., 2009).

Curing experiment was carried out for the three isolates that bearing plasmids for detecting the location of antibiotic markers on plasmid or on the chromosomal DNA. As a result they lost their plasmids and resistant to β -lactam antibiotics as Ampicillin, Amoxicillin, Cephalothin, Cefotaxim and Ceftriaxone as well as Tetracycline and Gentamycin which confirmed by plasmid extraction technique, agarose gel electrophoresis, antibiotic susceptibility test and Rapid iodometric technique respectively.

M 1 2 3 4 5 6 7

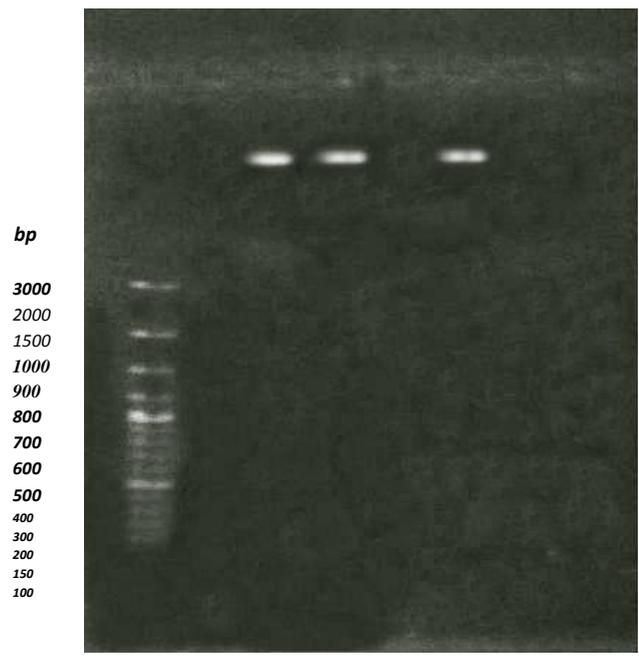


Figure (2) Plasmid pattern of *Proteus mirabilis*, M =molecular marker (100 base pair DNA ladder, largest fragment is 3000bp), Lane 2,3 and 5 represent plasmids exhibit by three *Proteus mirabilis* isolates.

The result revealed that the β -lactamase gens located on plasmids for the analyzed strains. This result was in agreement with the finding of other investigators (Dharmadhikari,S.M, and Peshwe,S.A.,2009) were they confirmed the location of antibiotic markers on R-plasmid by treating the cells with curing agents. The characterization of various plasmid mediated TEM-type β -lactamase in *Proteus mirabilis* are evidence of the wide diversity of β -lactamases produced by this species and of its possible role as β -lactamase-encoding plasmid reservoir (Bonnet, R., *et.al.*, 1999).

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دور البلازميد في المقاومة المتعددة لادوية لبكتيريا *Proteus mirabilis* المعزولة من الإدرار

كولبهار فتح الله كريم

جامعة كركوك – كلية التمريض

الخلاصة

جمعت مئة وستة وثلاثون نماذج إدرار من أشخاص يعانون من أعراض خمجات المجاري البولية من مستشفى ازادي التعليمي في مدينة كركوك من الفترة نيسان الى حزيران 2010. بعد إجراء فحص الإدرار العام لجميع العينات وبالاعتماد على نتائج الزرع البكتيري على الاوساط التفريقية والفحوصات الكيموحياتية تم عزل وتشخيص 16 (~11.8%) بكتيريا على إنها *Proteus mirabilis*.

تم دراسة مقاومة العزلات لبعض المضادات الحيوية، أظهرت جميع العزلات مقاومة (100%) تجاه كل من Amoxicillin و Ampicillin و Cephalothin و (81%) و (75%) تجاه كل من

Gentamycine وTetracycline على التوالي . كما وظهرت بعض العزلات مقاومة تجاه سيفالوسبورينات الجيل الثالث مثل Cefotaxim وCeftriaxon. وصنفت العزلات الى خمسة مجاميع إعتقاداً على القابلية على المقاومة لعدة ادوية (MDR). أظهرت الدراسة بأن ثلاث عزلات قيد الدراسة منتجة لانزيمات β -lactamase وتحمل كل منها بلازميد واحد حجمه يزيد على 3000 bp (حجم اكبر حزمة من حزم الDNA القياسي) . لوحظ بأن العزلات الطافرة بعد إجراء فحص الPlasmid Curing فقدت البلازميد والمقاومة للمضادات الحيوية نوع ال β -lactam بالإضافة الى Gentamycine وTetracycline. نتائج الدراسة أظهرت وجود بعض سلالات بكتيريا Proteus mirabilis المعزولة من خمجات المجاري البولية تحمل بلازميدات مسؤولة عن صفة المقاومة للأدوية المتعددة (MDR).