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Research Article

Prevalence and Characterization of Extended-Spectrum ß-Lactamase-Producing *Proteus mirabilis* Isolates

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Abstract

A total of 108 *Proteus mirabilis* isolates were isolated from different clinical specimens including urine, stool, pus and ear discharge. The specimens were collected from different departments in Tanta and Minoufia Universities Hospitals.

Dienes typing experiment was performed and the discriminatory index was calculated. The susceptibility of all 108 *P. mirabilis* isolates to each of 23 different commonly used antimicrobial agents was detected. B- lactamase production among 108 *P. mirabilis* isolates was studied and the prevalence of ESBL was detected, where ESBLs were identified in 13 isolates. The isoelectric focusing points experiment was performed on the ESBL producers. The role of plasmids in resistance of ESBL producers was studied. The Examination of the outer membrane proteins of 9 multiresistant *P. mirabilis* isolates was performed. Five quinolone resistant *P. mirabilis* isolates were selected to study the role of efflux mechanism in resistance by using the hydrophobic fluorescent probe NPN.

Keywords: *Proteus mirabilis; OMPs; Antimicrobial; Extended Spectrum ß-Lactamase; Plasmid Dienes Efflux Pump*

Abbreviations

ESBL: Extended Spectrum B-Lactamase; *P. mirabilis*: *Proteus mirabilis*; NPN: N-Phenylnaphthylamine; NCCLS: The National Committee For Clinical Laboratory Standard; DNA: Deoxyribonucleic Acid; UV: Ultraviolet; *E. coli: Escherichia coli*; µG :Microgram; Ml: Millilitres; Omps: Outer Membrane Proteins; SDS-PAGE: Polyacrylamide Gel Electrophoresis; Min: Minute; Ma: Milliampere; Epis: Efflux Pump Inhibitors; CCCP: Carbonyl Cyanide M-Chlorophenyl Hydrazine; Fig: Figure; AM: Ampicillin AMX: Amoxicillin AMC: Augmentin CZ: Cefazolin; CN: Cephalexin; CXM: Cefuroxime; CRO: Ceftriaxone; CAZ: Ceftazidime; FEP: Cefepime; IPM: Imipenem; AK: Amikacin; GM: Gentamycin; NM: Neomycin; SM: Streptomycin; TOB: Tobramycin; NA: Nalidixic Acid; CIP: Ciprofloxacin; NOR: Norfloxacin; LEV: Levofloxacin; GAT: Gatifloxacin; SXT: Cotrimoxazole; TE: Tetracycline; N: Nitrofurantoin; KBP: Kilobase Pairs; KDA: Kilodalton

Introduction

Proteus mirabilis is opportunistic pathogens, widely distributed in nature (in soil, water, and sewage) and plays a significant ecological role. When present in the niches of higher microorganisms, this organism able to evoke pathological events in different regions of the human body [1]. In hospitals *Proteus mirabilis* is the second most frequently isolated *Enterobacteriaceae* species after *Escherichia coli* [2]. *Proteus mirabilis* is a frequent cause of urinary tract infections that are often persistent and difficult to treat, it infects wounds, burns, the respiratory tract, and other sites [3].

Dienes [4] described a test for discrimination between strains of this species based on the mutual inhibition of different strains as they swarm towards each other on a plate (Sabbuba., *et al*.2003). More recently, Pfaller., *et al*. [5] evaluated the discriminatory power of the Dienes test and they concluded that the Dienes method was just as discriminatory as the genotyping technique and had the advantages that it is simple, inexpensive, and easy to perform.

The determination of antimicrobial susceptibility of a clinical isolate is often crucial for the optimal antimicrobial therapy of infected patients. This need is only increasing with increasing resistance and the emergence of multidrug-resistant microorganisms. Testing is required not only for therapy but also to monitor the spread of resistant organisms or resistance genes throughout the hospital and community [6].

Antibiotic resistance genes were developed especially within and between the Gram-negative bacteria [7]. With the presence of antibiotics selective pressure, these resistant Proteus species tend to persist, enabling the organism to cause extra infections such as septicemia [8]. *Proteus mirabilis* vary in their carriage of genes encoding antibiotic resistance [9,10]. Widespread drug resistance of Proteus species is plasmids mediated [11,12]. Also chromosomally mediated resistance is involved [13].

Materials and Methods

Isolation and typing

A total of one hundred and eight isolates of *P. mirabilis* were obtained from 1311 clinical specimens. The specimens were collected from patients admitted to various departments of Tanta and Minofia Universities Hospitals during the period from March 2006 to August 2007. The data concerning age, sex history of use of antibiotics within the last month was collected. Samples possessed fishy smell, swarming appearance on nutrient agar, and non-lactose fermenting colonies on MacConkey agar, was suspected to be *P. mirabilis.* Then the identification was confirmed by Biochemical tests [14].

Dienes typing

The Dienes typing was performed as described by [15]. Isolates of *P. mirabilis* were plated on MacConkey agar and incubated over night at 37°C. Single colony was then inoculated as macro-colony onto brain heart infusion agar. After overnight incubation at 37°C, those isolates showing a clear band (Dienes demarcation line) between each other were designated different Dienes types, while those with no demarcation line were regarded as the same Dienes type. Each isolate was tested against all other isolates then the Dienes type was designated.

Antibiotic susceptibility

The susceptibility of all 108 *P. mirabilis* isolates to each of 23 different commonly used antimicrobial agents was performed by disk diffusion method. The average of the zone diameters were translated into pattern of antimicrobial sensitivity resistance according to the committee for clinical laboratory standard [16]. The pattern of antimicrobial resistance of each of tested (108) *P. mirabilis* isolates was determined. Isolates showing the same resistance pattern were given one pattern code.

Detection of B-lactamases

Detection of B-lactamases producing *P. mirabilis* isolates was done using the iodometric overlay method [17]. Isolates showing colorless zones at bluish background indicating the destruction of penicillin G and the reaction of the resultant penicilloic acid with the soluble starch were considered positive β-lactamase producers.

Detection of extended spectrum B-lactamase (ESBL)

The ESβL type of enzymes was detected by using the double disk synergy test [18]. The inoculum suspension of 57 β-lactamases producing *P. mirabilis* isolates was prepared as in disk diffusion method. A swab was dipped into the inoculum suspension and spread over the dried agar plate. On the surface of the agar plates, disks containing (30 mg) of aztreonam, ceftazidime, cefepime, ceftriaxone and cefotaxime were placed around a disk of AMC (20 mg of amoxicillin plus 10 mg of clavulanate) in a distance of 30 mm center to center. Enhancement of the inhibition zone toward the AMC disk indicating synergy between clavulanic acid and any of the tested antibiotics was taken as presumptive evidence of ESBL production.

Determination of isoelectric focusing points

Isoelectric focusing was performed with polyacrylamide gels containing ampholines with a pH range of 3.5 to 10 as described by Abo-kamar (1995).

Plasmid DNA analysis

Plasmids DNAs of the ESβL producing *P. mirabilis* isolates (13 isolates) were prepared by a modified Brinboim and Doly's method [19]. Agarose gel electrophoresis was performed using 0.8% agarose. Samples of 20 ml volumes of plasmid preparations of the tested *P. mirabilis* isolates as well as of reference (*Shigella flexneri* F49) strain containing known molecular weight plasmids were loaded into wells in the gel, made by a comb inserted during the casting of the gel. Electrophoresis was performed on horizontal apparatus and it was run at 60V for about 6 hrs or 15V overnight. Plasmid DNA was visualized using a UV transilluminator. Photographs were taken by a digital camera through a tiffen 15 orange filters. The molecular weight of the resultant plasmid DNA bands was determined on the basis of its mobility through agarose gel compared with the mobility of the reference strain containing plasmids of known molecular sizes.

Conjugation studies

Mating techniques, [19] Direct transfer of resistance into nalidixic acid-resistant strain *E. coli* UB5202 (recipient) was performed by overnight mating of logarithmic-phase cells at 37°C on drug-free liquid and solid Mueller-Hinton medium. Transconjugants were selected on Mac Conkey agar plates containing one of the antimicrobials to which the donor strain is resistant in addition to 25 mg/ml of nalidixic acid, to which the recipient is resistant. Such medium is selective for *E. coli* transconjugants.

Appearance of lactose fermenting colonies growing on the antimicrobial selective media indicated the transfer of its corresponding resistance marker. The susceptibility of the transconjugants to different antimicrobials was detected by the disc diffusion method, as described before. Plasmid analysis of the transconjugants was carried out as described before.

Analysis of outer membrane proteins (OMPs)

Outer membrane proteins were prepared by the method reported by [20]. The protein preparations were analyzed electrophoretically with one dimensional SDS- polyacrylamide gel electrophoresis (SDS-PAGE) as described by, using 12% separating acrylamide gel and vertical PAGE apparatus. The protein samples were injected into the gel and covered by the run buffer then electrophoresis was carried out for 80 minutes at 20 mA. The molecular weight of the detected OMPs was determined on the bases of its mobility through polyacrylamide gel as compared with the mobility of known molecular weight proteins present in the protein marker.

Studying the efflux mechanism of resistance

Isolates primarily resistant to quinolones were selected to study the efflux mechanism according to [21]. Identification of efflux pump and confirmation by efflux pump inhibitors (EPIs) of the 5 selected quinolone resistant *P. mirabilis* isolates was studied by studying the efflux of NPN in the absence and presence of CCCP (EPIs) in comparison to a quinolone sensitive control *E. coli* (NCTC 10418).

Results

Source of the isolates

In the present study 108 *P. mirabilis* isolates 8.2% were isolated out of 1311 clinical specimens (Table 1).

Clinical sample (number)	No (%)
Ear discharge (149)	16(15)
Pus (162)	18(17)
Urine(913)	67(62)
Stool (87)	7(6)
Total (1311)	108(100%)

Table 1: *Distribution of P. mirabilis isolates among different clinical samples.*

Dienes typing

In the present study the Dienes typing of 108 *P. mirabilis* isolates was detected, it was observed that there is 69 different Dienes types among 108 *P. mirabilis* isolates. The discriminatory index for each typing method was calculated using the generalized formula proposed by Hunter [22] and it was 0.997.

Resistance of P. mirabilis strains

As shown in figure 1, all isolates (108) were resistant to cotrimoxazole. On the other hand all "except one" of the tested isolates were sensitive to imipenem. Also, among ß-lactam drugs the highest resistance (88%) was recorded to amoxicillin followed by ampicillin (81.4%). Presence of clavulanate with amoxicillin markedly decreased the number of resistant isolates to 14 instead of 95 in case of amoxicillin alone. Resistance of the tested isolates to aminoglycoside drugs ranged between 7.4% and 60.2%, with streptomycin showing the highest resistance. Comparing to other tested antimicrobials, quinolone drugs were relatively more active against the tested *P. mirabilis* isolates, where only 5.5% to 43% of isolates exhibited quinolone resistance. Multi-resistance was markedly observed among our tested isolates, where all isolates were resistant to at least 2 antibiotics, and 92 (85%) of isolates were resistant to 4 - 18 antibiotics. The maximum number of isolates exhibiting the same pattern (SXT-TE) was only 10.

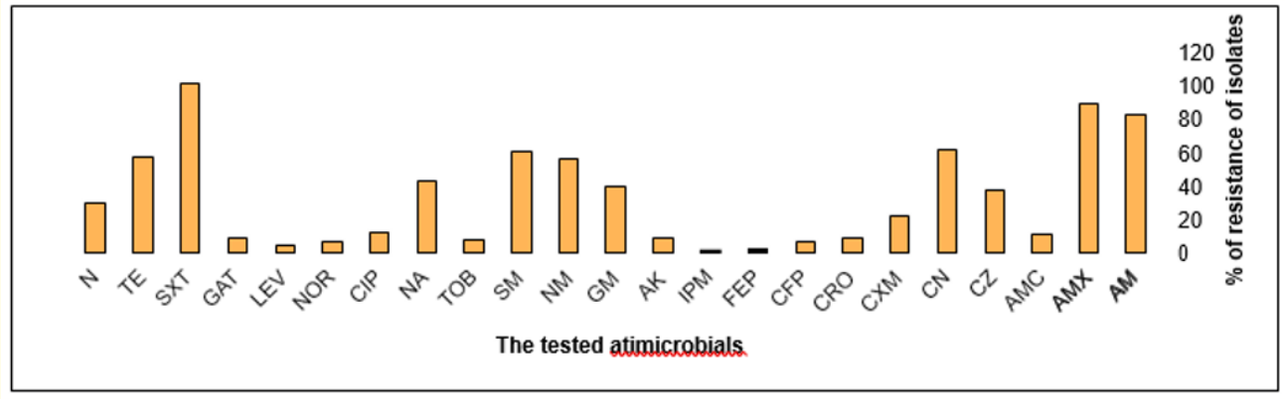

Figure 1: *Histogram showing resistance of P.mirabilis isolates to the tested antimicrobial agents.*
N.B: AM, ampicillin; AMX, amoxicillin; AMC, augmentin ; CZ, cefazolin; CN, cephalexin; CXM, cefuroxime; CRO, ceftriaxon; CAZ, ceftazidime; FEP, cefepime; IPM, imipenem; AK, amikacin; GM, gentamycin; NM, neomycin; SM, streptomycin; TOB, tobramycin; NA, nalidixic acid; CIP; ciprofloxacin; NOR; norfloxacin; LEV, levofloxacine; GAT, gatifloxacine; SXT, cotrimoxazol; TE, tetracyclin, N; nitrofurantoin.

β-Lactamase characterization

All (108) *P. mirabilis* isolates were tested for β-lactamase production using the iodometric overlay method.

The results revealed that 57 (53%) out of 108 *P. mirabilis* isolates were ß-lactamase producer.

The ß-lactamase producing *P. mirabilis* (57) isolates were screened for production of ESβL using the double- disk synergy method. It was found that 13 isolates showed synergy between clavulanate and one or more of the antibiotics cefotaxime (CTX), ceftazidime (CAZ), ceftriaxone (CRO), cefepime (FEP) and aztreonam (AZT) indicating presence of ESBL type of enzymes. The isoelectric focusing of these 13 ESBL producing isolates was determined. It was observed that an enzyme having pI value of 6.3 was detected in 5 out of 13 isolates.

Plasmid study

The ESβL producing *P. mirabilis* isolates (13 isolates) were selected for studying their plasmid profile using agarose gel electrophoresis. The results revealed the presence of a heterogeneous plasmids sizes ranging from (10.5 - 190 kbp). These plasmids were distributed where one or two plasmids were present in each isolates. Table 2 shows the results of the plasmid profiles and isolates antimicrobial resistance patterns of 13 selected ESβL producing *P. mirabilis*. It was observed that plasmids having large molecular size of 150 - 190 kb were common where they were detected in 8 out of 10 plasmid harboring isolates. No relation was observed between molecular sizes and resistance profiles of the isolates

Isolate code	Antimicrobial Resistance Pattern	Plasmid profiles (kbp)
pr55	AM-AMX-AK-CRO-CZ-CN-CXM-CIP-CAZ-GM-IPM-N-NM-NOR-NA-SM-SXT-TE-TOB	190 - 45
pr33	AM-AMX-CZ-CN-CXM-CAZ-CIP-GM-GAT-N-NA-NM-NOR-SM-SXT-TE	190
pr79	AM-AMX-NM-CN-GAT- GM- LEV-N-NA-SM-SXT	190-25
pr60	AM-AMX-AMC-AK-CZ-CN-CRO-CXM-CIP-GM-CAZ-NM-NA-NOR-SXT-SM-TE	180
pr62	AM-AMX-CIP-CN-CZ-CXM-CRO- N-NA-NOR-SXT-TE	180
pr3	AM-AMX-AK-CIP-N-NM-GM-GAT-NA-SM- SXT-TOB	170
pr101	AM-AMX-AK-CZ-GM-N-NM-SM-SXT-TE-TOB	-
pr17	AM-AMX-AMC- CN-CZ-GM-N-NM-NA-SM-SXT-TE	170-10.5
pr35	AM-AMX-CN-CZ-CXM-NM-SM-SXT-TE	-
pr48	AM-AMX-AK-GAT-LEV-N-NM-NA-SM-SXT-TE-TOB	150
pr91	AM-AMX-GM-SM-SXT-TE	-
pr47	AM-AMX-GM-N-NM-SM-SXT-TE	70
pr15	AM-AMX-CN-CZ-NM-SXT-TE	45

Table 2: *Plasmid profiles and antibiotic resistance patterns of P. mirabilis isolates.*

Conjugation

The plasmids that could be transferred by conjugation from ESBL producing *P. mirabilis* isolates to the recipient *E. coli* UB5202 were studied. Three out of 10 plasmid harboring isolates were conjugative. The transconjugants were tested for their sensitivity to different antimicrobials. Table 3 represents the antimicrobial resistance patterns and the plasmid profiles of these 3 *P. mirabilis* isolates and their corresponding transconjugants. It was observed that the transfer of plasmids was accompanied by the transfer of different antimicrobials resistance markers to their corresponding transconjugants. It was observed also that quinolones resistance was not transferred to any of the transconjugants.

Isolate code	Donners isolates		Transconjugants	
	Plasmid profiles (kbp)	Resistance patterns	Plasmid profiles (kbp)	Resistance patterns
pr48	150	AM-AMX-AK-N-NM-NA-GAT-LEV-SM-SXT-TE-TOB	150	AM-AMX-AK-NM-SM-SXT-TE
pr3	180	AM-AMX-AK-CIP-N-NM-GM-NA-SM- SXT-TOB	180	AM-AMX-AK-N-NM-GM-SM-TOB
pr55	190-45	AM-AMX-AK-CRO-CZ-CN-CXM-CIP-CAZ-GM-IPM-NM-N-NOR-NA-SM- SXT-TE-TOB	190	AM-AMX-AK-CRO-CZ-CN-CXM-GM-NM-SM-SXT-TE-TOB

Table 3: *Plasmid profiles and resistance patterns of the P. mirabilis donners and their transconjugants.*

The outer membrane proteins study

Nine isolates showing resistance to at least 10 antimicrobials were selected for this experiment. The outer membrane proteins (OMPs) of the 9 selected multiresistant *P. mirabilis* isolates were prepared and polyacrylamide gel electrophoresis of the protein contents was performed. The electrophoretic profile of the selected multiresistant isolates was shown in figure 2.

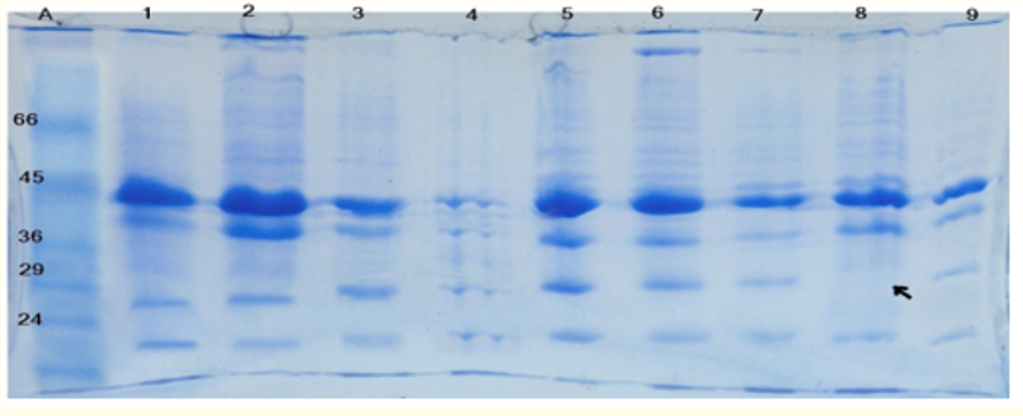

Figure 2: *SDS-PAGE of outer membrane proteins of 9 P.mirabilis Isolates, lane 1: pr101, lane 2: pr7, lane 3: pr38, lane 4: pr16, lane 5: pr48, lane 6: pr3, lane 7: p79, lane 8: pr55, lane 9: pr62 . lane A: molecular weight marker . The arrow points to the lost band.*

As shown in this figure 2 major protein bands of molecular weights 44,41 and 17 kDa were detected in all the tested isolates, another major protein band of molecular weight 26 kDa was absent in only one isolate (pr55). It is to be noted that the latter isolate which lacks the 26 kDa OMP was imipenem resistant strain.

Studying the efflux mechanism

Five quinolone resistant *P. mirabilis* isolates were selected to study the efflux mechanism of resistance by using the hydrophobic fluorescent probe NPN. A quinolone sensitive strain (*E. coli* ATCC 10418) was used as a control. The efflux of NPN by quinolone resistant *P. mirabilis* isolates was determined by measurement of fluorescence in a spectrofluorometer along 5 minutes experimental time. It was found that the fluorescence values after addition of NPN to the cell suspension were increasing in all isolates which indicates the uptake

of this compound by the cell. Regarding isolates Pr33 and Pr62 marked decrease in fluorescence was detected by time indicating the efflux of NPN in these isolates Preincubation of cells of these 2 isolates with CCCP (efflux inhibitor) resulted in markedly higher increases in fluorescence compared to untreated cells.

Discussion

In the present study 108 *P. mirabilis* isolates 8.2% were isolated out of 1311 clinical specimens. This result was in agreement with Neto., *et al*. (2003) and Trules., *et al*. (2006) who found that *P. mirabilis* were isolated in percentage of 8.6, 8.7% respectively.

In the present study, it was found that 62% of *P. mirabilis* isolates were isolated from urine samples, 17% *P. mirabilis* isolates were isolated from wound infection samples, 15% *P. mirabilis* were isolated from ear discharge samples and 6% *P. mirabilis* isolates were isolated from diarrhea stool samples. This result was in the same ranges detected by Mishra., *et al*. (2001) who reported that Maximum isolates were obtained from urine (47.30%) and pus (40.54%). Also, the results obtained by De Champs., *et al*. (2000) showed that most isolates were from urine (70.2%), others came from wounds (11.9%), bronchopulmonary samples (6.1%), blood cultures (2.2%) and various other samples (9.6%). Amigot., *et al*. (2003) found that the percent of *P. mirabilis* came from otitis media was (10.9%). These rates could be explained by the frequency of urinary tract infections observed in these units and by the presence of *P. mirabilis* infections in patients with severe underlying diseases and in those who were chronically ill [1].

The Dienes test is a simple and highly discriminatory tool for epidemiological typing of *P. mirabilis*, and is easy to perform (Ho., *et al*. 2005). The ease of performing the Dienes test coupled with excellent discriminatory power makes it the method of choice for initial epidemiologic characterization of *P. mirabilis* [5].

In the present study the Dienes typing of 108 *P. mirabilis* isolates was performed, it was observed that there

are 69 different Dienes types among 108 *P. mirabilis* isolates with discriminatory index of 0.997.

The result obtained by Pfaller., *et al*. [5] showed that discriminatory power of Dienes typing was 0.98.

Ho., *et al*. (2005) found that there is 54 different Dienes types among 92 *P. mirabilis* isolates. Sabbuba., *et al*. (2003) described that typing of *P. mirabilis* by the Dienes method is simple culture based phenotyping inhibition test, and reported that 55 *P. mirabilis* isolates were grouped into 43 Dienes types with discriminatory index of 0.98.

In developing countries, however, antibiotic resistance is highly linked to inappropriate use of antibiotics, lack of health care personnel with continual health education on antibiotics and poor quality drugs. Therefore, continual surveillance of antibiotic resistance in developing countries is important to alleviate morbidity and mortality rate of various microbial infections [13].

Monitoring sensitivity to antimicrobials in Egypt is necessary for optimum selection of effective antimicrobials and elimination of antimicrobials with little therapeutic value (El-Banna and Eissa 1997, Sonbole., *et al*. 2000).

In the present study the 108 *P. mirabilis* isolates were tested for their antimicrobial susceptibility against 23 different antimicrobials using disk diffusion method. It was found that 81.4% of isolates were resistant to ampicillin and 88% were resistant to amoxicillin. Nicolas., *et al*. (1997) and Chanal., *et al*. (2000) reported that 48.5% and 42.6% of *P. mirabilis* was resistant to amoxicillin and ampicillin respectively. Abo sayed., *et al*. (1990) reported that 84.6% of *P. mirabilis* was resistant to ampicillin. Ahmed., *et al*. (2000) found that 78% and 85% of *P. mirabilis* isolates were resistant to amoxicillin and ampicillin respectively. Grant., *et al*. (1986) reported that the use of ampicillin must be discouraged because of development of resistance, which may be explained by the repeated use of this antimicrobial leading to alteration of bowel flora, and resistance of Gram -ve pathogens. Amoxicillin resistance in *P. mirabilis* was almost (97%) associated with TEM-derived B- lactamases, most of which evolved via TEM-2 (Chanal., *et al*. 2000).

The combination of amoxicillin with a B-lactamase inhibitor, clavulanic acid reduces the incidence of resistance to 13%. Stratchounski., *et al*. (1998) and Szymczyk., *et al*. (2005) observed that 20% and 32% of *P. mirabilis* isolates were resistant to amoxicillin/clavulanic acid (augmentin) respectively. Chanal., *et al*. (2000) and Anoh., *et al*. (2006) reported that 100% of *P. mirabilis* isolates were sensitive to augmentin and claimed that the addition of clavulanic acid to amoxicillin increase the sensitivity. *P. mirabilis* were highly resistant to first generation cephalosporin, 39% resistant to cefazolin, 57.4% resistant to cephalexin, while 20.4% of *P. mirabilis* isolates were resistant to the second generation cephalosporin cefuroxime. Ahmed., *et al*. (2000) found that 15% and 4% of *P. mirabilis* isolates were resistant to cefuroxime and cephalexin respectively. Park., *et al*. showed that 29.1% and 17.9% were found to be resistant to cefuroxime and cephalothin respectively.

The third generation cephalosporin was highly active against *P. mirabilis* only 6.5% and 5.6% were resistant to ceftriaxone and ceftazidime respectively. Stratchounski., *et al*. (1998) reported that 17% of *P. mirabilis* isolates were resistant to ceftriaxone and only 1% was resistant to ceftazidime. Karlowsky., *et al*. (2003) found that 0.6% and 1.6% of *P. mirabilis* isolates were resistant to ceftriaxone and ceftazidime respectively. Rhomberg., *et al*. (2006) showed that 10% *P. mirabilis* isolates were resistant to ceftazidime.

In this study only four isolates were resistant to the fourth generation cephalosporin cefepime with percentage 1.8%. These result is in accordance with other authors Cao., *et al*. (2002) ; Decre., *et al*. (2003) and Mutnick., *et al*. (2002) who reported that 3%, 5% and 8% of *P. mirabilis* isolates were resistant to cefepime respectively, while Wu., *et al*. (2006) and Luzzaro., *et al*. (2006) reported that 100% of *P. mirabilis* isolates were sensitive to cefepime.

The resistance to B-lactams is more commonly due to the development of ESBLs, which are derivatives of older TEM-1, TEM-2 and SHV-1 B-lactamases and are capable of hydrolyzing the newer cephalosporins (Arakawa., *et al*. 1995; DuBois., *et al*. 1995; Senda., *et al*. 1996).

The activity of the carbapenem (imipenem) against *P. mirabilis* was very high; only one strain was 0.93% resistant to imipenem. Cao., *et al*. (2002) and Mutnick., *et al*. (2002) detected that 3% and 8% of *P. mirabilis.*

isolates were resistant to imipenem respectively. Park., *et al*. (2006) reported that 100% of *P. mirabilis* isolates were susceptible to carbapenems.

The resistance to carbapenems is due to the metallo-B-lactamase efficiently hydrolyses both carbapenems and cephalosporins. This enzyme was found in Gram-negative rods (Arakawa., *et al*. 1995; DuBois., *et al*. 1995; Senda., *et al*. 1996). Neuwirth., *et al*. (1995) reported that imipenem resistance in *P. mirabilis* might result from altered PBPs, while Villar., *et al*. (1997) reported that imipenem resistance in *P. mirabilis* might arise from alteration in outer membrane proteins.

For aminoglycosides the incidences resistance of *P. mirabilis* isolates to streptomycin, neomycin, gentamycin, amikacin and tobramycin was 60.2%, 52%, 39%, 7.4% and 7.4% respectively. Ahmed., *et al*. (2000) found that 37% of *P. mirabilis* isolates were resistant to gentamicin. Szymczyk., *et al*. (2005) reported that 52% of *P. mirabilis* were resistant to neomycin. Park., *et al*. (2006) reported that 28.4% of *P. mirabilis* was resistant to gentamicin, and 7.5% was resistant to amikacin.

The mechanism of resistance to aminoglycosides may be due to modification at amino groups by AAC enzymes or at hydroxyl groups by ANT or APH enzymes lose their ribosome-binding ability and thus no longer inhibit protein synthesis. Besides aminoglycoside-modifying enzymes, efflux systems and rRNA mutations have been described (Paulsen., *et al*. 1997; Schmitz., *et al*. 1999; Shaw., *et al*. 1993).

The introduction of the fluoroquinolones (FQs) in the 1980s provided clinicians with a class of broad- spectrum agents applicable to a range of gram-negative infections including urinary tract infections, gastrointestinal infections, respiratory tract infections, sexually transmitted diseases, bone and joint infections, and infections of the skin and soft tissue. Targeted microorganisms include the family Enterobacteriaceae (Poole, 2000).

In the present study the activity of quinolones was tested and the resistance percentage to nalidixic acid was 43%. El sheriff., *et al*. (1985) reported that 30% of *P. mirabilis* isolates were nalidixic acid resistance. De Champs., *et al*. (2000) found that 24.8% were nalidixic acid resistance. In the present study it was found that the resistance percentage decreased to 19%, 6.5%, 5.5% and 14% for ciprofloxacin, norfloxacin, levofloxacin and gatifloxacin respectively. This result agreed with Livermore., *et al*. (2002) who reported that Fluoroquinolone (FQ) antimicrobial drugs were a major therapeutic advance because they have 100-fold greater activity than their parent compound, nalidixic acid.

It was observed that the resistance percentage to fluoroquinolone in the present agreed with the results mentioned by other authors as; Park., *et al*. (2006), who reported that 21.6% of *P. mirabilis* isolates were ciprofloxacin resistance, Also Szymczyk., *et al*. (2005) reported that 22% of *P. mirabilis* were resistant to ciprofloxacin and norfloxacin and Yah., *et al*. [13] found that 19.6% and 5.2% of *P. mirabilis* were resistant to norfloxacin and ciprofloxacin respectively.

It is unfortunate that resistance to FQs has increased in a number of gram-negative organisms (Acar., *et al*. 1997; Köhler and Pechere, 1998). The primary mechanisms of resistance to fluoroquinolones are mutations that result in alteration of the target proteins, DNA gyrase (encoded by gyrA and gyrB) and topoisomerase IV (encoded by parC and parE), and decreased intracellular drug accumulation due to drug efflux or changes in outer membrane proteins [3].

In the present study100% of *P. mirabilis* isolates were resistant to cotrimoxazole. Ahmed., *et al*. (2000) and Jones., *et al*. (2004) found that 81% and 40% of *P. mirabilis* isolates were resistant to cotrimoxazole respectively. El-Banna and Eissa (1997) reported that the high incidence of resistance to trimethoprim in developing countries is likely to be due to the wide excessive use of such agent in human and veterinary medicine.

Regarding nitrofurantoin and tetracycline activity, 28.7% and 57% of *P. mirabilis* isolates were resistant respectively. Ahmed., *et al*, (2000); Szymczyk., *et al*. (2005) and Yah., *et al*. [13] notified that 59%, 54% and 13.4% of *P. mirabilis* were resistant to nitrofurantoin respectively. Abo sayed., *et al*. (1990) studied the activity of tetracycline and reported that 92.8% of *P. mirabilis* was resistant to it.

Interestingly, multi-drug resistances were observed in most of *P. mirabilis* isolates in the present study. About 85.2% of the tested isolates were resistant to 4 - 18 antimicrobial agents. Resistant to amoxicillin, ampicillin, cephalexin, cotrimoxazole, streptomycin, tetracycline were common among these isolates. These results were supported by Ashraf., *et al*. (2007) who reported that *P. mirabilis* showed the typical multidrug resistance, as it was resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline, in addition to trimethoprim and nalidixic acid. Also, Chow., *et al*. (1979) reported that *P. mirabilis* showed multidrug resistance to tetracycline, chloramphenicol and aminoglycosides.

Multiple antibiotic resistance markers of Proteus strains were identified as plasmid mediated (Khan and musharraf 2004). Transposons are well known for their capacity to carry multiple antimicrobial resistance genes; also Integrons are a special case of multidrug resistance. Multidrug resistance can also often be caused by reduced expression of porins and changes in the cell, which cause reduced uptake or the expression of efflux pumps [6].

In this study, detection of B-lactamases by the iodometric method revealed that 53% of the isolates were B- lactamases producers. De Champs., *et al*. (2000) reported that 48.5% of *P. mirabilis* isolates were B-lactamases producers, while Pagani., *et al*. (2002) found that 52% of *P. mirabilis* isolates produce B-lactamases.

Recognition of ESBL-positive strains appears to be critical for the clinical management of patients with systemic *P. mirabilis* infections (Endimiani., *et al*. 2005). In the present study the prevalence of ESBL producers was 12% this result within the same range that reported by Canal., *et al*. (2000) and Yong., *et al*. (2006) who found that ESBL prevalence was 14.2% and 17%, while De Champs., *et al*. (2000); Pagani., *et al*. 2002; Endimiani., *et al*. (2005) and Luzzaro., *et al*. (2006) reported that ESBL prevalence was 6.9%, 48% 44%, 56.7 respectively.

This difference in the results may be due to the different methods used for the detection of ESBLs, regardless of the used method, it is important to note that none of the methods that rely on phenotypic expression of the B-lactamase will detect every ESBL-producing isolate. Also there have been reports of false- positive results for ESBL phenotypic screening tests that can occur with strains that do not possess an ESBL. Nevertheless, increased awareness of the ESBL problem among clinical microbiology laboratory and infection control personnel will help in the interpretation of these tests (Bradford, 2001). Although infection control procedures continue to play a central role, changes in antibiotic policy may play an even greater role in this setting (Safdar., *et al*. 2002). Determination of the isoelectric point was usually sufficient to identify the ESBL that was present (Bradford, 2001).

In this study, the isoelectric focusing of 13 ESBL producing isolates were determined. All the detected *P. mirabilis* isolates produced one or two of enzymes with pI (5.6, 5.2, 6, 6.3, 6.9, 7.4, 7.8, 8.2, 8.3). Ho., *et al.* (2005) detected two B-lactamase of pIs 7.5, 5.4. Pagani., *et al*. (2002) detected 3 B-lactamase of pIs 5.2, 5.6,5.9, Nass., *et al*. (2000) detected two B-lactamase of pIs 7.4, 6.1. Bonnet., *et al*. (1999) found B- lactamase of pIs 5.6, 6, 6.3, 6.5 in *P. mirabilis* isolates. Canal., *et al*. (2000) and Neuwirth., *et al*. (2001) detected only one B-lactamase of pI 6.3 in *P. mirabilis* isolates. Bonnet., *et al*. (2002) also found one B- lactamase of pI 6.9 in *P. mirabilis* isolates.

TEM-1 was the main ß-lactamase produced by *Proteus* spp (Manageiro., *et al*.2007). The amino acid substitutions that occur within the TEM enzyme occur at a limited number of positions. The combinations of these amino acid changes result in various subtle alterations in the ESBL phenotypes, such as a change in their isoelectric points, which can range from a pI of 5.2 to 6.5 (Paterson., *et al*. 2003). Regarding SHV-1 ß- lactamase, Sequencing showed that the ß-lactamase differed from SHV-1, by replacement of glycine by serine at the 238 position. This mutation alone accounts for the extended-spectrum properties of this ß-lactamase, designated SHV-2 (Paterson., *et al*.2003). ß-lactamase related to SHV-1 varies in pI from 7 to 8.2 (Philippon., *et al*.1989).

Pitout., *et al*. (1998) reported that the enzymes with pIs of 5.4, 5.6, 7.6, and 8.2 aligned with TEM-1 (pI 5.4), TEM-10 or TEM-26 (pI 5.57), SHV-1, SHV-2, or SHV-8 (pI 7.6), and SHV-5 (pI 8.2), also AmpC B-lactamase with a pI of 8.0 was detected.

Plasmids have been known to play a major role in the dissemination of antibiotics resistance genes in a microbial population [13] the resistance genes are often found on plasmids, which spread easily from one bacterium to another and even from one species of bacterium to another (Hawkey, 1998).

Plasmid analysis of the 13 ESBL producing isolates of *P. mirabilis* isolates in the current study was carried out to clarify the role of the plasmids in the resistance. Nine plasmids with different molecular sizes were detected among 10 out of 13 ESBL producing isolates, and they ranged between 1 to 2 plasmid per isolate. The sizes of the plasmids ranged between the low molecular sized plasmid (10.5 kbp) to the high molecular size plasmid (190 kbp).

Bonnet., *et al*. (1999) reported that ESBL producing *P. mirabilis* isolate resistance was mediated by 170 kbp and 180 kbp plasmids. Nass., *et al*. (2000) isolated plasmids having molecular sizes of 35, 150 and 190 kbp from ESBL producing *P. mirabilis* isolate. Neuwirth., *et al*. (2001a) detected 100 and 25 kbp plasmids in ESBL producing *P. mirabilis* isolates. Ho., *et al*. (2005) revealed that *P. mirabilis* isolates had one to four plasmids with size ranging from 1.5 to 300 kbp. The sizes of the plasmids varied and resistance markers showed that there was no plasmid epidemic involved in the antibiotic resistance of Proteus species [13].

In the current study no relation was observed between molecular sizes and resistance profiles of the isolates. This observation was in agreement with Yah., *et al*. [13] who found that plasmids with the same relative distance on electrophoresis, size and molecular weights had varied antibiotic resistance patterns, revealed that there was no correlation between the number of antibiotics that a plasmid mediates to and its size.

According to Miranda., *et al*. (2004) these clinically derived plasmids do not belong to distinct plasmid lineages but exhibit evidence of broad scale inter-plasmid gene transfer probably involving a range of mechanisms such as recombination, transposition and integration. It is necessary to elaborate and enhance ways of controlling this phenomenon [13].

In the present study 3 ESBL producing *P. mirabilis* isolate were plasmidless. The antimicrobial resistance in plasmidless isolates was predominantly due to chromosomally mutations. Bonnet., *et al*. (2002) reported that class D B-lactamase OXA-23 was chromosomally mediated. Bret., *et al*. (1998) found that class A and C enzymes (GN-79 and CMY-3), which are generally plasmid encoded, have been observed in the chromosome of *P. mirabilis* strains. Yah., *et al*. [13] reported that most of the isolates were highly mediated by chromosomal resistant, where 44% percent of the antibiotics were plasmids mediated, 32% by chromosome while 24% of the resistant pattern to the antibiotics could not be ascertained.

The results of conjugation experiment in the current study revealed that plasmids of molecular weights 190, 180 and 150 kbp were transferred by conjugation. In this study the resistance to ampicillin, amoxicillin, amikacin, neomycin and streptomycin, were 100% transferred to the transconjugants, while 67% of the transconjugants were resistant to cotrimoxazole, tetracycline, gentamycin and tobramycin. It was observed also that quinolones resistance was not transferred to any of the transconjugants. Plasmid-mediated quinolone resistance remained unknown even after 30 years of nalidixic acid usage (Livermore., *et al*. 2002).

This results are in accordance with other authors, e.g. Bonnet., *et al*. (1999) found that a plasmid of 170, 25 kb was transferred by conjugation, the study revealed that the transconjugant acquired resistance against kanamycin, tobramycin, amikacin, netilmicin, tetracyclines, trimethoprim, and sulphonamides. Nass., *et al*. (2000) reported that the transferable plasmid with the molecular size 190 kb mediate resistance to chloramphenicol, B-lactamase, gentamycin, streptomycin, tobramycin, kanamycin, sulphonamides and spectinomycin was transferred to transconjugants. De Champs., *et al*. (2001) reported that the phenotype of resistance to aminoglycosides (kanamycin, tobramycin, and gentamicin) observed by the diffusion method was co-transferred with the ESBL phenotype. Yah., *et al*. [13] reported that resistance to ampicillin, gentamicin, norfloxacin, chloramphenicol, nalidixic acid, cotrimoxazole and nitrofurantoin was transferred from the *P. mirabilis* isolates to the transconjugants.

In contrast, Decré., *et al*. (2002) and Ho., *et al*. (2005) reported that the attempts to transfer ß-lactam resistance by conjugation from the *P. mirabilis* isolates were unsuccessful. Also, Wachino., *et al*. (2006) reported that the aminoglycoside resistance of *P. mirabilis* strain could not be transferred to the recipients by conjugation under the experimental conditions used in this study. However, the recipient was successfully transformed by electroporation with the plasmid with molecular size of > 100 kb. These transferable resistance markers between different bacterial isolates may go unnoticed by infection control methods, therefore undermining hospital infections control policies (Nashwan., *et al*.2005).

OMPs are potential virulence factors with a wide range of possible functions related to the development of infections (Sosa., *et al*. 2006).

In the present study when OMPs profile was analyzed using SDS-PAGE. A predominant 44,41 and 17 kDa bands were observed in most of the examined isolates. Kappos., *et al.* (1992) reported that the major proteins of *P. mirabilis* isolates migrate in the region of the gel corresponding to 35 to 45 kDa. Rotten., *et al*. (1992) found three major OMPs 39, 36 and 17 kDa in *P. mirabilis* isolates. Sosa., *et al*. (2006) demonstrated that all isolates showed similar patterns, a predominant 39 and 36 kDa bands was observed in *P. mirabilis* isolates.

In the present study it was observed that 26 kDa OMP was lost only from the imipenem resistant *P. mirabilis* strain. Villar., *et al* (1997) also detected 44, 41, 26 kDa OMP in *P. mirabilis* isolates and demonstrated that 26 kDa OMP was lost from the imipenem resistant *P. mirabilis* mutant. Mehtar., *et al*. (1991) noted the absence of a 24 kDa OMP from an imipenem resistant *P. mirabilis* isolate and imipenem selected laboratory mutants. This association suggested that the protein might be a porin and that resistance might be contingent on impermeability (Villar., *et al*. 1997).

Though wild-type strains of *P. mirabilis* are usually susceptible to fluoroquinolones, a progressive increase in fluoroquinolone resistance has been seen in clinical isolates of the bacterium (De Champs., *et al*. 2000; Hernandez., *et al*. 2000).

Weigel., *et al*. (2002) reported that alteration of the gyrase B subunit is a relatively frequent event in the acquisition of fluoroquinolone resistance by *P. mirabilis*, reducing the inhibiting effects of these agents on the target protein, DNA gyrase.

The rule of efflux mechanism in quinolone resistance was estimated in the present study. Two isolates out of the 5 selected quinolone resistant *P. mirabilis* exhibited efflux Mechanism of resistance. The accumulation of the fluorescent NPN was decreased leading to marked decrease in fluorescence in these 2 isolates only. Upon treating the isolates with the efflux inhibitor CCCP. The accumulation of the fluorescent NPN inside these two isolates was relatively higher than that in the CCCP untreated isolates. Fernandez., *et al*. (1999) studied the effect of carbonyl cyanide m-chloro-phenylhydrazone (CCCP), used to inhibit active efflux and they suggest that FQ resistance in *P. mirabilis* is associated with efflux mechanism. Saito *et al*. (2006) reported that efflux pump might synergistically contribute to a highest level of resistance to fluoroquinolones in clinical isolates of *P. mirabilis*.

Indeed, given the incredible chemical diversity of substrates accommodated by efflux systems, it is likely that many novel or yet to be discovered antimicrobials will themselves be efflux substrates and, as such, efflux inhibitors may become an important component of Gram-negative antimicrobial therapy (Poole, 2004) [23].

Conclusion

In conclusion, this study showed that among different methods used for typing *P. mirabilis* isolates, Dienes test is simple, inexpensive, and easy to perform and has good discriminatory powers, it should be the method of choice for the epidemiological characterization of *P. mirabilis* isolates. The increase in clinical prevalence of ESßL-producing *P. mirabilis* isolates has been noted in this study as well as other's, hence more precise methods should be used to detect these enzymes in laboratories. *P. mirabilis* isolates developed different mechanisms of resistance against different antibiotics leading to an alarming increase in the antibiotic resistance among the isolates, therefore culture sensitivity testing is strongly recommended before antibiotic prescription. It was found in this study that cefepime and imipenem were the most effective antibiotics against *P. mirabilis* isolates, so they should be used under control to avoid the development of new resistance mechanisms against them.

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Conflict of Interest

There is no any conflict of interest exists.

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