Mechanisms of Quinolones Resistance Among *Pseudomonas aeruginosa* Isolates

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One hundred and twelve *Pseudomonas aeruginosa* strains were isolated from 436 different clinical specimens collected from patients attending different hospitals in El-Minia governorate. Antibiotics susceptibility patterns of the isolated *Ps. aeruginosa* isolates to quinolones were studied and results revealed higher percentage of resistance ranged from 22.32% to 75%. gatifloxacin was the most active quinolone against the tested isolates as the percentage of resistance was 22.32% while nalidixic acid was the least active one as the percentage of resistance was 75%. The percentage of resistance to ciprofloxacin, levofloxacin and ofloxacin was 41.96% , 47.32% and 49.1% respectively. Co - resistance to other chemically unrelated antibiotics was also studied and the result showed that, more than 90% of the tested *Ps. aeruginosa* isolates were resistant to amoxycillin, chlormphenicol and tetracyline. On the other hand, variable levels of resistance were obtained for gentamicin, azithromycin, cefoperazone and cefotaxime. Interestingly, all the tested *Ps. aeruginosa* isolates were 100% sensitive to amikacin. *Ps. aeruginosa* isolates showed multiple drug resistance patterns to quinolones and other antibiotics were selected to study the possible mechanisms of resistance to quinolones. Efflux mechanisms was studied flourometerically and the results revealed presence of active efflux pumps in the tested isolates as indicated by the increase in the fluorescence of N-phenyl-1-Naphthylamine (NPN) after preincubation of the cells with the efflux inhibitor carbonyl cyanide m-chlorophenylhydrazone (CCCP). Also, examination of outer membrane proteins in the tested isolates revealed over production of outer membrane proteins of an apparent molecular mass of 50 KDa and 54 KDa suggesting the role of these proteins in quinolones resistance. In addition to the absence of outer membrane protein of molecular mass 46 KDa which aids the intake of the quinolones into the bacterial cells. In conclusion, quinolones resistance in *Ps. aeruginosa* were associated with multidrug efflux pumps and alteration in the outer membrane proteins.

INTRODUCTION.

During the 1980s, a great number of quinolones were developed. These quinolones showed potent activity against Gram-negative bacteria, but not against the Gram-positive bacteria or anaerobes.[1,30] In the 1990s, further alterations of the quinolones resulted in the discovery of novel compounds that not only showed potent activity against Gram-negative bacteria but also against the Gram-positives.[30] In addition, some of the compounds, such as trovafloxacin, also showed promising activity against the anaerobes.[1] The fluoroquinolones have been used to treat a great variety of infections, including gonococcal infections, osteomyelitis, enteric infections or respiratory tract infections, [2-4] and as prophylaxis in neutropenic patients, surgery or to prevent spontaneous bacterial peritonitis in cirrhotic patients, among others. [3,5] Moreover, quinolones, along with other anti-bacterial agents, have been extensively used in veterinary practice, either for medical reasons or as growth promoters.[4]

As a result of their wide spectrum of activity, quinolones have been extensively used and ciprofloxacin was pointed out as the most consumed antibacterial agent world-wide.[4] This high level of use, and to some degree of misuse in the sense of unnecessary use,[6] or use of quinolones with poor activity in some developing countries,[7] has been blamed for the rapid development of bacterial resistance to these agents.

To date, two main mechanisms of quinolone resistance have been established: alterations in the targets of quinolones, and decreased accumulation inside the bacteria due to impermeability of the membrane and/or an overexpression of efflux pump systems. Both of these mechanisms are chromosomally mediated.[30]

The nucleotide and amino acid sequence of gyrA, gyrB, parC and parE genes needed for the synthesis of DNA topoisomerase are very similar to those of

DNA gyrase enzyme. Mutations occur in gyrA and parC genes. This kind of resistance seen with *Ps. aeruginosa*, *E. coli* and *H. Influenzae* is usually against all quinolones. Resistance due to mutations of gyr B is less common and may not be against all quinolones [13]. The second mechanism is the decrease in the amount of quinolones entering the cells because of the defective function of porin channels [14]. The third mechanism is the various efflux systems, localized in the membranes of many bacteria, including *Ps. aeruginosa*, which pump the drug out of the bacteria [12, 14].

Different efflux systems shown to pump out quinolones such as MexAB-OprM, MexCD-OprJ or MexEF-OprN have been described in *Ps. aeruginosa* [31]. A fourth efflux system named MexXY capable of pumping out quinolones has also been described, but no open reading frame corresponding to an outer membrane protein has been found downstream of *mexXY*. In fact, it may be that OprM (which is encoded downstream of MexAB) might act as the outer membrane protein of this efflux system [32, 33]. The aim of this study was to determine the incidence of quinolones resistance among *Pseudomonas aeruginosa* isolates collected from different hospitals in El-Minia governorate, Egypt and to study the possible mechanisms of quinolones resistance among the resistant isolates.

MATERIALS AND METHODS

Tested bacteria:

112 clinical isolates of *Ps. aeruginosa* were recovered from 436 different clinical specimens (urine, stool, ear swabs, sputum, abscess discharges and burn exudates) collected from patients attending different hospitals in El-Minia governorate. These isolates were biochemically identified according to **Koneman *et al***. (1997)[35].

Media and antimicrobial agents:

All strains were cultured on Luria-Bertani medium either for isolation or determination of the MIC's. Culture media such as Luria-Bertani and Mueller-Hinton agar were supplied by LabM. Biochemical tests such as oxidase test, motility test, sugar fermentation test, nitrate reduction test, citrate utilization test, urease test and oxidative-fermentative test were supplied by Oxoid. Different antimicrobial agents such as nalidixic acid (Nal), norfloxacin (Nor), ofloxacin (Ofx), ciprofloxacin (Cip), levofloxacin (Lev), gatifloxacin (Gat), chloramphenicol (Cm), gentamicin (Gm), tetracycline (Tet), clarithromycin (Clr), azithromycin, (Az), amoxycillin (Amx) and ampicillin/sulbactam (Amp/Sul) were supplied by Sigma. cefazoline (Cz), cephradine (Cr), cefotaxime (Cft), cefoperazone (Cpz), cefepime (Cfp) and amikacin (Ak) were supplied by Bristol-Meyer Squibb, USA.

Chemicals used for efflux tests such as monobasic sodium phosphate and dibasic sodium phosphate were supplied by BDH, England. Sodium chloride, magnesium chloride and glucose were supplied by El-Nasr company, Egypt. Carbonyl cyanide m-chlorophenylhydrazone, (CCCP), N-phenyl-1-naphthylamine, (NPN) and HEPES (N-2-hydroxyethyl piperazine N'- [2-ethanesulfonic acid]) were supplied by Sigma, USA. Chemicals for isolation of outer membrane proteins such as sodium dodecyl sulphate, sarkosyl, acrylamide, bis-acrylamide and 2-mercaptoethanol were supplied by Sigma, USA. Protien molecular weight marker was supplied by Fermentas, Lithuania.

Determination of antibiotics susceptibility:

The MICs of each antimicrobial agent were determined, for each strain, by a routine agar plate dilution method with L.B. medium and a final inoculum of 10^6 CFU. All plates were incubated aerobically at 37°C overnight. The MIC of the antibiotic was defined as the concentration (in microgram per milliliter of agar) at which no more than six colonies were detected; a slight haze of growth was ignored. [8]

Studying of hydrophobic antibiotics uptake and efflux mechanisms:

Hydrophobic antibiotics (represented by NPN) uptake and efflux mechanisms were studied according to **Bina, *et al.*** [9] as follows: cultures of tested *Ps. aeruginosa* isolates, grown on Luria-Bertani broth, were harvested and suspended in PBS, washed once in HEPES – Mg Cl_2 buffer (pH 7.2) and centrifuged at 10,000 rpm at 4°C. The pellets were resuspended in HEPES-glucose buffer (pH 7.2) to an optical density of 0.5 at 600 nm, 2 ml of the prepared cell suspension of each *Ps. aeruginosa* isolate were placed in the

quartz cuvette. NPN was added to a final concentration of 10 mM, after mixing, the fluorescence was measured over 6 min to determine the NPN uptake. [9]

To dissipate the proton motive force and inhibit NPN efflux mechanism, the proton translocator CCCP was added to the cell suspension at a final concentration of 40 mM 15 min prior to the assay initiation and the fluorescence was measured over 6 min. [9]

The fluorescence measurements were done on Perkin-Elmer spectrofluorophotometer with an excitation wave length of 350 nm and an emission wave length of 420 nm and slit width of 5nm. All assays were performed at room temperature.

Assay of outer membrane proteins:

Exponentially growing cells in Mueller-Hinton broth were harvested by centrifugation at 7,000 rpm for 10 min at 4°C, suspended in 30 mM Tris-HCl (pH 8) and then broken with a sonicator (Misonix, USA) for 2 min. Unbroken cells were removed by centrifugation at 4°C. Membranes were pelleted by centrifugation at 15,000 rpm for 1 hour at 4°C and were suspended in the same buffer. The inner membrane was solubilized by adding sodium N-lauroyl sarcosinate to the suspension at a final concentration of 1%; this was followed by incubation for 30 min at 30°C. The outer membrane was pelleted by centrifugation at 15,000 rpm for 40 min at 30°C and was suspended in the buffer. The outer membrane fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as reported by Laemmli [10] with 10.7% (wt/vol) acrylamide and 0.3% (wt/vol) N-N'-methylene bis-acrylamide in the running gel. Samples for SDS-PAGE were treated with 2% SDS, 5% 2-mercaptoethanol at 100°C for 5 min, and they were subjected to electrophoresis at a constant current of 25mA at 4°C. [11]

RESULTS

Susceptibility testing:

Antibiotic susceptibility tests were performed using agar dilution method against different quinolones including nalidixic acid, norfloxacin, ofloxacin, ciprofloxacin, levofloxacin and gatifloxacin. Variable patterns of MICs were shown by the tested isolates as in table (1).

The MIC values of each of the tested quinolone against 112 clinical *Ps. aeruginosa* isolates were determined and the data are presented in table (1). As shown in this table, the distribution of the MICs of different members of quinolones is scattered markedly. However, 75% of the isolates had MIC in the resistance range (≥16 µg/ml) for Nal. In contrast, the bulk of the isolates had MIC in the sensitive range (< 4µg/ml) for Gat as the percentage of resistance was 22.32%, it was the most effective quinolone against these isolates where 90% of the tested isolates were inhibited by 8 µg/ml. Although the newer quinolones were highly effective against *Ps. aeruginosa* isolates, they were quantitatively variable with the following increasing order of activity: Lev, Cip and Gat, where MIC_{90} values were 64 µg/ml, 32µg/ml and 8 µg/ml respectively. Ofloxacin and Nor had the same MIC_{90} values (128 µg/ml) while Nal showed four times that value (512 µg/ml) (Table 2).

Table (2) also describes the frequencies of quinolones resistance among the tested *Ps. aeruginosa* isolates, where 75% of the isolates were resistant to Nal, 55.35% of the isolates were resistant to Ofx, 49.10% were resistant to Nor, 47.32% were resistant to Lev, 41.96% were resistant to Cip and only 22.32% were resistant to Gat.

Table (1): Distribution of minimum inhibitory concentrations of different quinolones among *Pseudomonas aeruginosa* isolates.

Quinolone*	MIC (µg/ml)														
	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	≥1024
	Number of isolates														
Nal	-	-	9	-	-	7	-	12	11	1	29	5	12	19	7
Nor	-	11	8	2	9	5	22	19	8	4	6	15	1	2	-
Ofx	-	5	14	-	27	4	6	2	-	24	12	9	6	3	-
Cip	-	-	12	30	23	5	8	10	7	8	2	3	4	-	-
Lev	-	1	23	16	7	12	11	14	9	5	3	4	7	-	-
Gat	-	5	9	-	26	47	4	12	5	4	-	-	-	-	-

Table (2): Minimum inhibitory concentrations (range, mean and MIC_{90}) and incidence of quinolone resistance among *Pseudomonas aeruginosa* isolates.

Quinolone	MIC ((µg/ml)			Number of resistant isolates (%)
	Range	Mean	MIC_{90}	
Nal	0.25-1024	512.25	512	84 (75%)
Nor	0.125-512	256.125	128	55 (49.10%)
Ofx	0.125-512	256.0625	128	62 (55.35%)
Cip	0.25-256	128.125	32	47 (41.96%)
Lev	0.125-256	128.0625	64	53 (47.32%)
Gat	0.125-32	16.0625	8	25 (22.32%)

Prevalence of co-resistance to other antimicrobial agents among quinolone resistant *Ps. aeruginosa* isolates:

Fluoroquinolones resistant *Ps. aeruginosa* isolates were chosen for studying co-resistance to other antimicrobials. As shown in table (3), high incidences of resistance were observed to all the tested antimicrobials ranged from 56% to 100%.

For Gat-resistant isolates, percentages of resistance to the tested antimicrobials ranged from 56% to 100%. The isolates were 100% resistant to Cm, Gm, Tet, Clr, Amx, Amp/Sul, Cz and Cfp while resistance to other antimicrobials was variable. For Lev-resistant isolates, percentages of resistance to the tested antimicrobials ranged from 62.26% to 100%. The isolates were 100% resistant to

Cm, Tet, Clr, Amx and Cr while resistance to other antimicrobials was variable (Table 3).

For Cip-resistant isolates, percentages of resistance to the tested antimicrobials ranged from 57.44% to 100%. The isolates were 100% resistant to Cm, Gm Tet, Clr, Amx and Cr while resistance to other antimicrobials was variable. For Ofx and Nor-resistant isolates, percentages of resistance to the tested antimicrobials ranged from 58.06% to 100%. The isolates were 100% resistant to Tet and Clr only while resistance to other antimicrobials was variable (Table 3).

On the contrary, all quinolone resistant *Ps. aeruginosa* isolates, were 100% sensitive to Ak indicating that it was the most potent antimicrobial agent of all against these isolates.

Table (3): Prevalence of co-resistance to other antimicrobial agents among quinolone resistant *Ps. aeruginosa* isolates

antibiotics	Prevalence of co-resistance of *Ps. aeruginosa* isolates with the following quinolones				
	Gat (n = 25)	Lev (n = 53)	Cip (n = 47)	Ofx (n = 62)	Nor (n = 55)
Cm	25	53	47	54	50
Gm	23	52	47	57	53
TEt	25	53	47	62	55
Clr	25	53	47	62	55
Az	21	40	42	51	42
Amx	25	53	47	61	53
Amp/Sul	25	52	45	57	54
Cz	25	51	46	58	54
Cr	24	53	47	61	53
Cft	15	33	27	40	35
Cpz	14	34	27	36	33
Cfp	23	47	45	57	52
Ak	-	-	-	-	-

From the above mentioned data, it was observed that amikacin was the most potent antimicrobial agent against the quinolone resistant *Ps. aeruginosa* isolates, in contrast to Tet and Clr which were the least potent ones (to which all isolates were 100% resistant), while other antimicrobials showed variable resistance patterns.

Patterns of co-resistance to other antimicrobial agents among 7 selected quinolone resistant *Ps. aeruginosa* isolates:

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Out of the tested quinolone resistant *Ps. aeruginosa* isolates, 7 multiple quinolone resistant isolates were chosen to report their patterns of resistance to other antimicrobials. These 7 isolates were also used for determination of different quinolones resistance mechanisms. As shown in table (4), co-resistance to many chemically unrelated antimicrobials was observed in all isolates selected

Table (4):Patterns of co-resistance to other antimicrobial agents among the selected quinolone resistant *Ps. aeruginosa* isolates:

Isolate code	Resistance pattern of	
	Quinolones	Other antimicrobial agents
1	Gat Lev Cip Nor Ofx Nal	Az, Cm, Gm, Amx, Clr, Tet, Cr, Cfp, Cpz, Cft, Cz, Amp/sul
2	Gat Lev Cip Nor Ofx Nal	Az, Cm, Gm, Amx, Clr, Tet, Cr, Cfp, Cpz, Cft, Cz, Amp/sul
3	Lev Cip Nor Ofx Nal	Cm, Gm, Amx Clr, Cft, Cz, Amp/Sul,
4	Gat Lev Cip Nor Ofx Nal	Az, Cm, Gm, Amx, Clr Amp/Sul
5	Gat Lev Cip Nor Ofx Nal	Cm, Gm, Amx, Clr, Tet,
6	Gat Lev Cip Nor Ofx Nal	Cm, Amx, Tet Cr, Cfp, Cpz,
7	Gat Lev Cip Nor Ofx Nal	Gm, Amx, Clr, Cr Cpz Amp/Sul

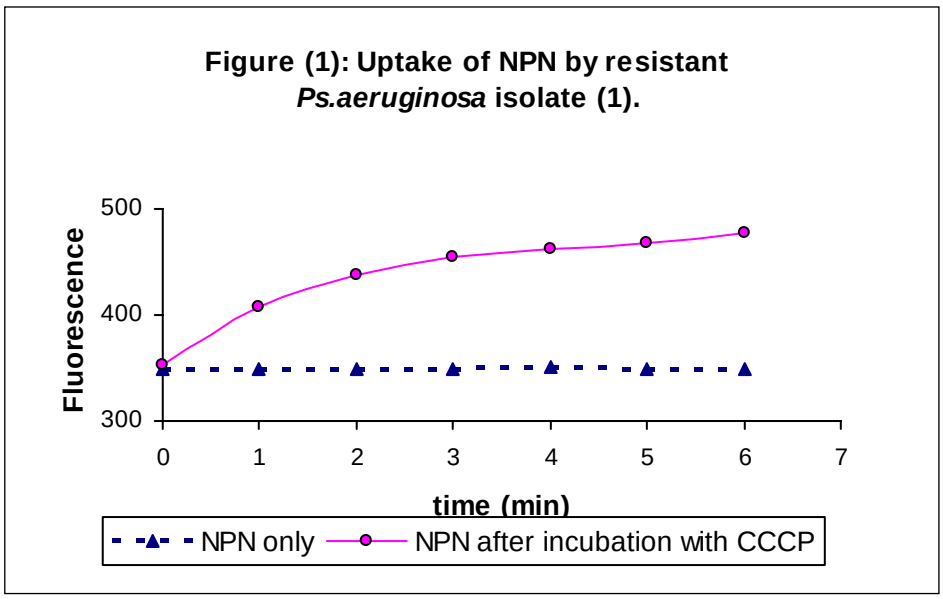
Efflux and uptake of antibiotics by *Ps. aeruginosa* isolates:

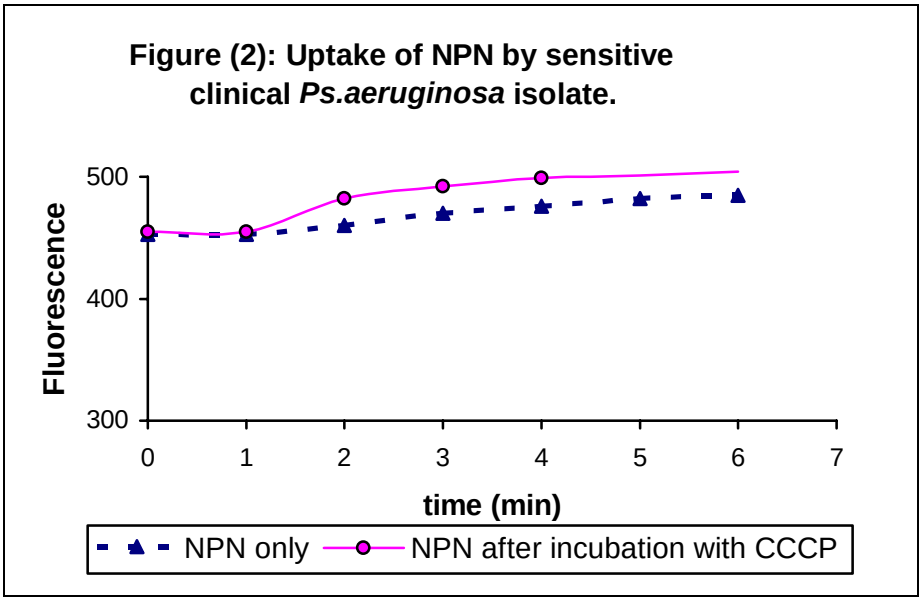
NPN is a fluorescent probe which has been used to define the uptake pathways across the outer membranes of Gram-negative bacteria [34]. NPN fluoresces weakly in aqueous environments but strongly in hydrophobic ones, such as the membrane interior. It is usually excluded from the outer membrane by its outer monolayer, which comprises the polyanion lipopolysaccharide, stabilized by divalent cation cross bridging [9].

When NPN was added to the cell suspension of the tested *Ps. aeruginosa* isolates and the fluorescence was measured over 6 minutes, it was found that the intrinsic uptake of NPN by the tested isolates was relatively low and the fluorescence only increased by 10 units throughout the test (Figure 1).

When the cells were pre-incubated with the efflux inhibitor CCCP, the fluorescence increased after the addition of NPN to the bacterial suspension, rising from 353 to 478, indicating the increased uptake of NPN by the cells after inhibition of the efflux pumps (Figure 1).

When NPN was added to the cell suspension of the tested *Ps. aeruginosa* sensitive isolate and the fluorescence was measured over 6 minutes, it was found that the intrinsic uptake of NPN by the tested isolates was relatively higher than the resistant ones and the fluorescence only increased by 30 units throughout the test, (Figure 2). When the cells were pre-incubated with CCCP, the fluorescence showed a slight increase after the addition of NPN to the bacterial suspension, rising from 455 to 504 (about 50 units), which is not a significant increase as shown by the resistant isolate.





Outer membrane protein profiles of multiple-drug-resistant *Pseudomonas aeruginosa* isolates:

The outer membrane proteins of the seven selected multiresistant *Ps. aeruginosa* were prepared and polyacrylamide gel electrophoresis of the protein contents was performed. The isolates selected were multiple drug resistance to all tested quinolones.

Gels were photographed and the molecular size of each detected band was determined using the standard molecular size protein markers, injected along with the test samples. Visual examination of the obtained electrophoretic profile of the tested *Ps. aeruginosa* isolates revealed that, there were changes in the amounts of more than one protein among the tested isolates as indicated by differences in the band intensities (Figure 3).

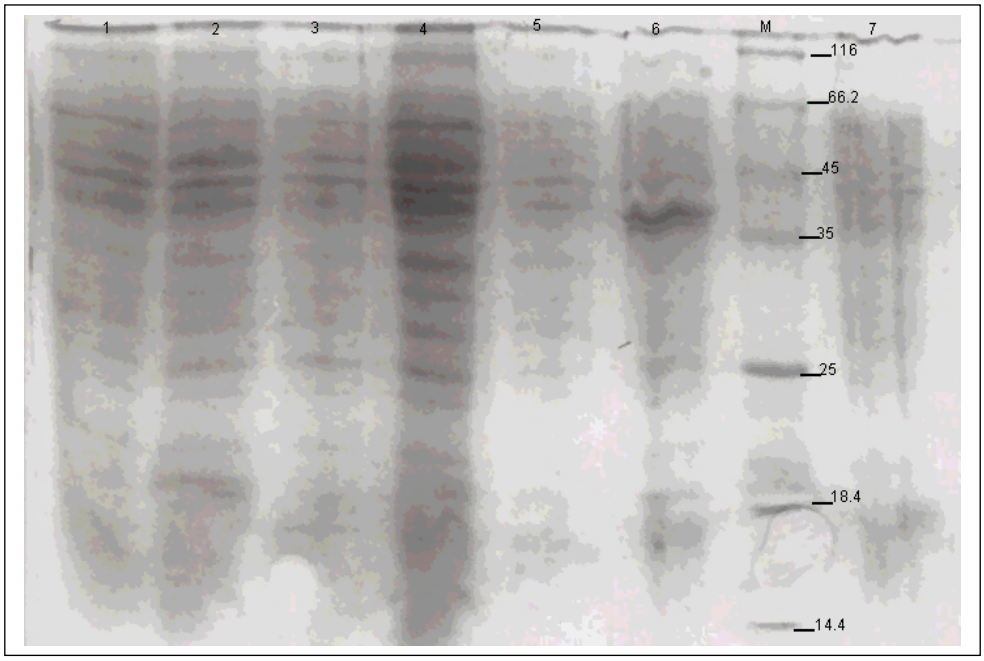


Figure (3): Outer membrane protein profiles of the seven selected *Pseudomonas aeruginosa* isolates.

The amounts of outer membrane protein with an apparent molecular mass 50 KDa (OprM) were increased in all isolates. Also the amounts of outer membrane protein with an apparent molecular mass 54 KDa (OprJ) were increased in all of them. Another outer membrane protein with an apparent molecular mass 46 KDa (OprF) was absent in all isolates.

DISCUSSION

Fluoroquinolones are bactericidal, rapidly acting antimicrobial drugs with wide spectrums. They are very effective against many gram negative bacterial pathogens *in vitro* [12]. Their effect against Gram negative bacilli, including *Ps. aeruginosa*, is one of their most important features. The main mechanism in the development of resistance to fluoroquinolones is the decrease in binding of the target quinolones to enzymes because of changes in DNA gyrase enzyme and / or the topoisomerase enzyme(s).

Susceptibility testing:

In this study, the isolated *Ps. aeruginosa* isolates were tested for their susceptibility to quinolones (Nal, Nor, Ofx, Cip, Lev and Gat) and 22.32%-75% were found to be resistant to different quinolones. Gat was the most active where only 22.32% of *Ps. aeruginosa* isolates were resistant to it, while Cip came next (41.96%), followed by Lev (47.32%), Nor (49.10%), Ofx (55.35) and Nal (75%).

In various studies investigating the resistance of *Ps. aeruginosa* to ciprofloxacin, the proportion was reported to be 0% to 89%. In a study from Spain, ciprofloxacin resistance, evaluated in 1014 *Ps.aeruginosa* strains obtained from 136 different hospitals, was found to be 23% [15], whereas in France, the proportion evaluated in 738 *Ps.aeruginosa* strains from 15 education hospitals was 40% [16], while a study in Turkey investigated the sensitivity of 136 *Ps. aeruginosa* strains, isolated from various clinical materials, to fluoroquinolones, The resistance rate to ciprofloxacin was 12.5% and it was 23.4% in Intensive care unit (ICU) [17]. In the present study, ciprofloxacin resistance was 41.96%.

A French study reported that out of 47 *Ps. aeruginosa* isolates, 26% were resistant and 2% were intermediately resistant to gatifloxacin and MIC_{90} was 32µg/ml [28]. In our study, 22.32% of *Ps. aeruginosa* isolates

were resistant to gatifloxacin and MIC_{90} was 8µg/ml.

Levofloxacin has some added advantages such as its effectiveness against gram positive bacteria, its ability to concentrate more in the urine and its use of daily single dose [17]. Yamane *et al.* [18] reported that the activity of levofloxacin was generally two-fold greater than ofloxacin and was equal to or slightly less potent than ciprofloxacin. Levofloxacin resistance was reported in 36% of strains isolated from nosocomial infections [19] and it was also reported that the overall resistance to levofloxacin was found to be 16.9% but it was 29.7% in intensive care unit (ICU) [17]. In our study, levofloxacin resistance was 47.32%.

Ofloxacin resistance has been reported to be 31% and 62.5% in different studies [12, 20] and in another study, the overall resistance was 19.9% but it was 32.8% in ICU [17], in this study, ofloxacin resistance was higher (55.35%). Norfloxacin resistance was reported to be 14.7% in a study conducted on Turkey [17] while in our study it was 49.10%.

A Swedish study reported that the MIC range for the susceptibility of 20 *Ps. aeruginosa* to nalidixic acid was > 32µg/ml. Also the MIC_{90} and MIC_{50} for these isolates were also > 32µg/ml [29]. In this study, the MIC_{90} was 512µg/ml and 75% of the isolates were resistant to nalidixic acid.

Efflux and uptake of antibiotics by *Ps. aeruginosa* isolates:

Although exclusion from the cell due to reduced outer membrane permeability was thought to play a key role in the intrinsic resistance of *Ps.aeruginosa* and related bacteria to many antimicrobial compounds, this is now attributed to synergy between a low-permeability outer membrane and active efflux from the cell [21]. To date, five families of bacterial efflux systems have been identified [22]. Also it was reported that efflux mechanism and alteration of outer membrane proteins lead to multiple antibiotic resistance in *Ps.aeruginosa* [23].

A Swedish study reported that 17 out of 20 strains overproduced mRNA for one or more pump proteins (MexB, MexD, MexF, or MexY), which caused multidrug resistance phenotype in more than half the strains [24]. **Jalal *et al.,*** (2000) reported that alterations in two efflux systems, MexCD-OprJ and MexEF-OprN, were the predominant mechanisms of fluoroquinolone resistance in *Ps.aeruginosa* strains from the lungs of cystic fibrosis patients [25], while **Li *et al.,*** (1994) reported that the efflux mechanism(s) are likely to contribute significantly to the intrinsic resistance to *Ps.aeruginosa* isolates to tetracycline, chloramphenicol and fluoroquinolones, as does the low permeability of the outer membrane [26].

In the present study, the presence of the putative efflux pumps in *Ps. aeruginosa* isolates was demonstrated by the increase in the fluorescence of NPN after pre-incubation with the proton translocator, CCCP. The accumulation of Nor was studied **(Li *et al.*, 1994)** using ^{14}C labeled compound. The resistant strain showed a much lower steady state accumulation level than the susceptible isolate and the accumulation level showed a dramatic increase upon addition of CCCP in the former strain, which coincides with the results obtained in this study but using NPN as the substrate.

Outer membrane protein profiles of multiple-drug-resistant *Pseudomonas aeruginosa* isolates:

In this study, we found an increase in the amounts of outer membrane proteins of apparent molecular mass 50 KDa and 54 KDa (which were much more overexpressed in isolate No. (4) and the absence of proteins of apparent molecular mass 46 KDa in all the tested *Ps. aeruginosa* resistant isolates. These isolates showed multiple drug resistance to quinolones and other chemically unrelated antimicrobials (Table 4).

These results were concomitant with a Japanese study that reported 3 types of *Ps. aeruginosa* mutants were isolated, the first type mutants showed cross resistance to meropenem, cephems and quinolones. They overproduced an outer membrane protein with an apparent molecular mass of 50 KDa (named OprM). Type 2 mutants showed cross resistance to quinolones and new cephems, i.e., cefpirome and cefozopran, concomitant with an overproduction of an outer membrane protein with an apparent molecular mass of 54 KDa (named OprJ). Type 3 mutants showed cross resistance to carbapenems and quinolones. They produced decreased a mounts of 46 KDa protein and increased 50

KDa protein [11]. Al-Domany, 1999 reported that a *Ps. aeruginosa* isolate was resistant to aminoglycosides, ampicillin, imipenem, nalidixic acid and norfloxacin. This isolate overproduced a protein of apparent molecular mass 50 KDa [27].

Overexpression an outer membrane protein of apparent molecular mass 50 KDa was reported to be associated with decreased susceptibility to a number of antimicrobial agents including quinolones, aminoglycosides, β-lactams, tetracycline and chloramphenicol [26]. In conclusion, our study revealed that the higher incidence of resistance among the tested *Ps. aeruginosa* were associated with the presence of active efflux pumps that extrude the drug out of the cells and alteration in the outer membrane proteins. Further studies are required for the detection of mutation in gyr A, gyr B and par C genes.

REFERENCES

1. **Spangler, S. K.; Jacobs, M. R and Appelbaum, P. C. (1996):** Susceptibility of anaerobic bacteria to trovafloxacin: comparison with other quinolones and non-quinolone antibiotics. *Infect. Dis. in Clin. Practice*. **(5):** Suppl. 3, S101–109.
2. **Vila, J.; Ruiz, J.; Sanchez, F.; Navarro, F.; Mirelis, B.; de Anta, M. T. and Prats, G. (1999):** Increase in quinolone resistance in a *Haemophilus influenzae* strain isolated from a patient with recurrent respiratory infections treated with ofloxacin. *Antimicrob Agents Chemother.* **(43)**: 161-2.
3. **Davis, R.; Markham, A. and Balfour, J. A. (1996):** Ciprofloxacin. An updated review of its pharmacology, therapeutic efficacy and tolerability. Drugs. 6: 1019–1074.
4. **Acar, J. F. and Goldstein, F. W. (1997):** Trends in bacterial resistance to fluoroquinolones. *Clin. Infect. Dis.* **(24),** Suppl. 1, S67–S73.
5. **Grange, J. D.; Roulot, D.; Pelletier, G.; Pariente, E. A.; Denis, J.; Ink, O.; Blanc, P.; Richardet, J. P.; Vinel, J. P.; Delisle, F.; Fischer, D.; Flahault, A. and Amiot, X. (1998):** Norfloxacin primary prophylaxis of bacterial infections in cirrhotic patients with ascites: a double-blind randomized trial. *J Hepatol.* **(29)**: 430-6.
6. **Solomon, D. H.; Van Houten, L.; Glynn, R. J.; Baden, L.; Curtis, K.; Schrager, H. and Avorn, J. (2001):** Academic detailing to improve use of broad-spectrum antibiotics at an academic medical center. *Arch Intern Med.* **(161)**: 1897-902.
7. **Hart, C. A. and Kariuki, S. (1998):** Antimicrobial resistance in developing countries. *British Med. J.*317, 647–50.
8. **Everett, M. J.; Jin, Y. F.; Ricci, V. and Piddock, L. J. V. (1996):** Contributions of individual mechanisms of fluoroquinolone resistance in 36 *E. coli* strains isolated from humans and animals. *Antimicrob. Agents Chemother.* **(40):** 2380–2386.
9. **Bina, J. E.; Alm, R. A.; Uria-Nickelsen, M.; Thomas, S, R.; Trust, T. J. and Hancock, R. E. W. (2000):** *Helicobacter pylori* uptake and efflux: Basis for intrinsic susceptibility to antibiotics in vitro. *Antimicrob. Agents Chemother.* **(44)**: 248–254.
10. **Laemmli, U. K. (1970):** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680-685.
11. **Masuda, N.; Sakagawa, E. and Ohya S. (1995):** Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **(39):** 645–649.
12. **Swiatlo, E.; Moore, E.; Watt, J. and McDaniel, LS. (2000):** In vitro activity of four fluoroquinolones against clinical isolates of *Pseudomonas aeruginosa* determined by the E test. *Int. J Antimicrob. Agents.* **(15)**: 73-76.
13. **Tunger O and Aydemir S. (2000):** Quinolones. *Turk J Antibiotics.* **(1)**: 18-24.
14. **Hooper DC. (2000):** Quinolones. In: Principles and Practice of Infectious Disease 5th ed. (Mandell, GL, Bennet, JE, Dolin, R). 404-423.
15. **Bouza, E.; Garcia-Garrotte, F.; Cercenato, E.; Marin, M. and Diaz M.S. (1999):** *Pseudomonas aeruginosa*: A survey of resistance in 136 hospitals in Spain. *Antimicrob. Agents Chemother*. **(43)**: 981-982.

16. **Cavallo, J.D.; Leblanc, F.; Fabre, R, and Fourticq-Esqueoute, A. (2001):** Survey of the antibiotic sensitivity of *Pseudomonas aeruginosa* in France and the distribution of beta-lactam resistance mechanism. *Pathol Biol*. **(49):** 534-539.
17. **Algun U., Arisoy A. and Ozbakkaloglu B. (2004):** The resistance of *Pseudomonas aeruginosa* strains to fluoroquinolones group of antibiotics. *Indian J. med. Microbial*. **(22):**112-114.
18. **Yamane, N.; Jones, R.N.; Frei, R.; Hoban, D.J.; Pignatari, A.C. and Marco F. (1994):** Levofloxacin in vitro activity. Results from an international comparative study with ofloxacin and ciprofloxacin. *J Chemother*. **(6)**:83-91.
19. **Topkaya, A.; Kucukercan, M. and Oguzoglu, N., (2001):** Inducible beta-lactamase production and susceptibility rates of *Pseudomonas aeruginosa* strains to aminoglycosides and quinolones. *X.Turkish Microbiology and Infectious Diseases Congress Book*; 272.
20. **Prosser, BLT and Beskid, G. (1995):** Multicenter in vitro comparative study of fluoroquinolones against 25.129 Gram-positive and Gram-negative clinical isolates. *Diagn Microbiol Infect Dis*. **(21):**33-45
21. **Poole, K. and Srikumar, R. (2001):** Multidrug efflux in *Pseudomonas aeruginosa*: components, mechanisms and clinical significance. *Curr. Topics Med. Chem.* **(1):**59-71.
22. **Putman, M.; van Veen, H. W. and Konings, W. N. (2000):** Molecular properties of bacterial multidrug transporters. *Microbiol. Mol. Biol. Rev*. **(64)**: 672-693.
23. **Zhao, Q., Li, X.Z.; Srikumar, R. and Poole, K. (1998):** Contribution of outer membrane efflux protein OprM to antibiotic resistance in *Pseudomonas aeruginosa* independent of MexAB. *Antimicrob. Agents Chemother*. **(42):** 1682-1688.
24. **Oh, H.; Stenhoff, J.; Jalal, S. and Wretlind B. (2003):** Role of efflux pumps and mutations in genes for topoisomerase II and IV in fluoroquinolones-resistant *Pseudomonas aeruginosa* strains. *Microb. drug resist.* **(9):** 323-328.
25. **Jalal, S.; Ciofu, O.; Høiby N.; Gotoh, N. and Wretlind B. (2000):** Molecular mechanisms of Fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Antimicrob. Agents Chemother.* **(40):** 710-712.
26. **Li, X-Z., Livermore, D.M. and Nikaido, H. (1994):** Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: Resistance to tetracycline, chloramphenicol and norfloxacin. *Antimicrob. Agents Chemother.* **(38)**: 1732-1741.
27. **Al-Domany, R. (1999):** Aminoglycoside resistance among nosocomial bacterial isolates. Msc. Thesis. Microbiology department, Faculty of Pharmacy, Tanta University.
28. **Decousser, J. W.; Pina, P.; Picot, F.; Delalande, C.; Pangon, B.; Courvalin, P.; Allouch, P.; and the ColBVH study group. (2003):** Frequency of isolation and antimicrobial susceptibility of bacterial pathogens isolated from patients with bloodstream infections: a French prospective national survey. *J. Antimicrob. Chemother.* **(51)**: 1213–122.
29. **Ragnar Norrby, S. and Jonsson, M. (1983):** Antibacterial activity of norfloxacin. . *Antimicrob. Agents Chemother.* **(23)**: 15-18.
30. **Ruiz, J. (2003):** Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. *J. Antimicrob Chemother.* **(51)**: 1109-17.
31. **Nikaido, H. (1996):** Multidrug efflux pumps of gram-negative bacteria. *J Bacteriol.* **(178)**: 5853-9.
32. **Mine, T.; Morita, Y.; Kataoka, A.; Mizushima, T. and Tsuchiya, T. (1999):** Expression in *Escherichia coli* of a new multidrug efflux pump, MexXY, from *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* **(43)**: 415-7.
33. **Masuda, N.; Sakagawa, E.; Ohya, S.; Gotoh, N.; Tsujimoto, H. and Nishino, T. (2000):** Contribution of the MexX-MexY-oprM efflux system to

intrinsic resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. **(44)**: 2242-6.

34. **Li, X. Z.; Nikaido, H. and Poole, K. (1995):** Role of *mexA-mexB-oprM* in antibiotic efflux in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. **(39)**: 1948-53.

35. **Koneman, E. W.; Allen, S. D.; Janda, W. M.; Schreckenberger, P. C. and Winn, W. C. (1997)**: Colour Atlas and Textbook of Diagnostic Microbiology. 4th ed., J.B. Lippincott Company, USA, Philadelphia.

دراسة آلية مقاومة عزلات السودوموناس ارجينوزا للكينولونات

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تم عزل ١١٢ عزلة سودوموناس ارجينوزا من ٤٣٦ عينة اكلينيكية مأخوذة من مرضى من مستشفيات مختلفة بمحافظة المنيا.

تم دراسة حساسية هذه العزلات للكينولونات وأسفرت النتائج عن وجود نسبة مقاومة عالية تراوحت بين ٢٢,٣٢% و٧٥%. وقد لوحظ أن مضاد الجاتيفلوكساسين كان أكثرها فاعلية (٢٢,٣٢%) بينما كان مضاد حمض النالديكسيك أقلها فاعلية (٧٥%) وكانت نسبة المقاومة للسيبروفلوكساسين ، الليفوفلوكساسين و الاوفلوكساسين ٤١,٩٦% ، ٤٧,٣٢% و ٤٩,١% على الترتيب.

وقد تم دراسة المقاومة المصاحبة لمضادات أخرى مختلفة كيميائيا وأوضحت النتائج أن أكثر من ٩٠% من العزلات المختبرة كانت مقاومة للأموكسيسيلين والكلورامفنيكول والتتراسيكلين. وعلى الجانب الآخر لوحظ وجود نسب مقاومة مختلفة لكل من الجنتاميسين والازثيروميسين والسيفوبيرازون والسيفوتاكسيم. والجدير بالذكر أن كل العزلات المختبرة كانت حساسة لمضاد الأميكاسين بنسبة ١٠٠%.

وبدراسة آلية مقاومة العزلات المختبرة للكينولونات تبين وجود آلية إرجاع نشط لها (efflux) عن طريق المضخات تم التعرف عليها عن طريق زيادة وميض مادة NPN بعد فترة تحضين للخلايا مع مثبط الإرجاع النشط (CCCP) وتم أيضا فحص البروتينات الخلوية لبعض العزلات المقاومة للكينولونات وتبين وجود البروتينات الخلوية ذات الوزن الجزيئى ٥٠و٥٤ كيلو دالتون مما يؤكد دورها فى المقاومة. هذا بالإضافة إلى اختفاء البروتين الخلوى ذو الوزن الجزيئى ٤٦ كيلو دالتون والذى يساعد على دخول الكينولونات إلى الخلية.

ومن ثم نستنتج من هذه الدراسة أن مقاومة عزلات السودوموناس ارجينوزا للكينولونات كانت مرتبطة بوجود مضخات إرجاع نشط لعديد من المضادات الحيوية ووجود تغيرات فى بروتينات الغشاء الخارجى.