Comparison between Outer Membrane Protein Profile of Fluoroquinolones Sensitive and Resistant *P. aeruginosa* Isolated from Egyptian Patients

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Abstract: *Pseudomonas aeruginosa* is an important opportunistic pathogen that infects immunocompromised hosts and is characterized by its natural resistance to a variety of antimicrobial agents. The purpose of this study was the assessment of the fluoroquinolones resistance level among *P. aeruginosa* clinical isolates, furthermore to compare between the outer membrane protein profile of fluoroquinolones susceptible and resistant isolates of *P. aeruginosa* using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) technique. Sixty five (43%) were identified as *P. aeruginosa* by conventional culture techniques. MIC of ciprofloxacin, norfloxacin and levofloxacin against pseudomonal isolates were determined by twofold agar dilution technique. Only about 39%, 40% and 42% of these isolates were resistant to ciprofloxacin, levofloxacin and norfloxacin, respectively. Profile of outer membrane protein fraction of the fluoroquinolones resistant isolates showed an additional band with an approximate molecular weight of 50-54 kDa. In conclusion, overproduction of outer membrane protein of approximate molecular weight 50-54 kDa in *P. aeruginosa* was associated with fluoroquinolones resistance.

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1. Introduction:

Pseudomonas aeruginosa is a clinically significant opportunistic pathogen that infects immunocompromised hospitalized patients and is characterized by its innate resistance to a variety of antimicrobial agents. Only a few antimicrobial agents, such as quinolones, show potent antibacterial activity against this species (Zeng, 2004; Pitt and Simpson, 2006). In recent years, a number of clinical *P. aeruginosa* isolates were reported to be resistant to the quinolones, because of the presence of an outer membrane with a low level of permeability and thereby intrinsically resistant to a wide variety of commonly used antibiotics (Lambert, 2002). The quinolones class of antibiotics generally has retained excellent in vitro activity against many common Gram-negative bacterial pathogens (Sárközy, 2001; Scholar, 2003; Van Bambeke *et al.*, 2005). However, as these agents are more frequently prescribed, increasing rates of bacterial resistance are being reported (Hooper, 2001; Ruiz, 2003). Several mutations conferring quinolones resistance have been identified and mapped on *P. aeruginosa* chromosomes. The mutation of *gyrA* (*nfxA*, *nalA*, or *cfxA*) causes an alteration in the subunit A of DNA gyrase (Hsu *et al.*, 2005; Niga *et al.*, 2005), while the *nalB* (*cfxB*) (Li and Poole, 2001; Hocquet *et al.*, 2004), *nfxB* (Chuanchuen *et al.*, 2005; Jeannot *et al.*, 2008), and *nfxC* (Fukuda *et al.*, 1995) mutations decrease the level of quinolones accumulation, and strains with the later mutations show cross-resistance to structurally unrelated antimicrobial agents. The present study aimed to compare between the outer membrane protein profiles of fluoroquinolones susceptible and resistant isolates of *P. aeruginosa.*

2. Materials and methods

Antibiotics:

Ciprofloxacin hydrochloride monohydrate and norfloxacin were provided as a gift from E.I.P.I.CO. Pharmaceutical Company (10th of Ramadan City, Egypt), while levofloxacin hemihydrate standard powder was provided from MUP Pharmaceutical company (Ismailia, Egypt).

Bacterial isolates:

One-hundred and fifty bacterial isolates were isolated from cases admitted to Educational Suez Canal University Hospital, Ismailia, Egypt. The isolates were identified using the conventional culture and biochemical techniques (Cheesbrough, 2000; Goldman and Green, 2009).

Susceptibility testing:

MIC_S were determined by the usual twofold agar dilution technique with Mueller–Hinton agar (*Difco,USA*) and an inoculum size of 10^4 cells according to the guidelines of standard procedures (Schwalbe *et al.*, 2007; CLSI, 2007).

Assay of outer membrane proteins:

The method of Masuda *et al.* was followed, briefly resistant isolates were isolated by plating on Mueller–Hinton agar (*Difco,USA*) plates containing sub-MIC of each fluoroquinolone. The sub-MICs ranged from 2-4, 8-16 or 4-8 µg/ml for ciprofloxacin, norfloxacin and levofloxacin, respectively. The largest colony in each plate was picked up and recultivated in Mueller–Hinton broth (MHB; Difco, USA) containing the same concentrations of antibiotics, in which they grew. While sensitive isolates were cultivated directly in antibiotic- free Mueller–Hinton broth (MHB; Difco, USA). The overnight growing cells in MHB were harvested by centrifugation at 12300×g for 5 min at room temperature. Harvested cells were suspended in 0.1M Tris-HCl; pH 8.0. Suspended cells were broken with a sonicator for 3 min. Unbroken cells were removed by centrifugation at 4000×g for 10 min at room temperature. Membranes were pelleted by ultracentrifugation at 38000×g for 1 h at 6^0C. The inner membrane was solubilized by adding 2% sodium N-lauroylsarcosinate (sarkosel) in 10mM HEBES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 8 to the pellets, this was followed by overnight incubation at room temperature. The outer membrane was pelleted by centrifugation at 18,000×g for 40 min at 6^0C and resuspended in 0.1M Tris-HCL buffer; pH 8.

Analysis of outer membrane protein:

The outer membrane fraction was analyzed by SDS-PAGE as reported by Sambrook *et al.*, with 12.5 % polyacrylamide resolving gel and a 5% stacking gel. The analyzed samples were treated with reducing sample buffer, pH 6.8 that containing 0.2% SDS and 2% β-mercaptoethanol. Treated samples were immersed in boiling water bath for 5 min, and then they were subjected to electrophoresis at a constant current of 200 mA at room temperature. The gel was stained with silver staining using silver staining kit *(Pharmacia Biotech).*

3. Results and Discussion:

Sixty- five out of one-hundred and fifty (43%) clinical bacterial isolates were identified as *P. aeruginosa*. The sources and numbers of the isolates which grew *P. aeruginosa* were burn – 37; urine – 15 and wound — 13. Results showed that out of the 65 *P. aeruginosa* isolates 25 (39%), 26 (40%) and 27 (42%) isolates were resistant to ciprofloxacin, levofloxacin and norfloxacin, respectively (Table1).

In agreement with Swiatlo *et al.* (2000), Oliphant and Green (2002) and Emami *et al.* (2005) the present study showed that cross-resistance existed between these fluoroquinolones.

Electrophoresis of outer membrane protein (OMP) fraction on SDS-PAGE gel revealed that fluoroquinolones- resistant isolates differed in OMP profile from the susceptible ones. An additional protein band with an approximate molecular mass of 50-54 kDa appeared in each of ciprofloxacin-resistant isolates (lane 2), norfloxacin-resistant isolates (lane 3), and levofloxacin-resistant isolates (lane 4) when compared with sensitive isolates (lanes 5, 6 and 7) (Figure 1).

These results suggested that the outer membrane protein may be involved in *P. aeruginosa* resistance to fluoroquinolones, in agreement with Masuda *et al.* (1995); Alonso *et al.* (1999); Le Thomas *et al.* (2001) and Griffith *et al.* (2006).

The previous was agreed with Le Thomas *et al.* (2001) and Nakajima *et al.* (2002); when they used probing technique for probing the outer membrane of fluoroquinolones-resistant mutant with outer membrane protein-specific antibody, they demonstrated a 2-3 fold overexpression of an outer membrane protein named, OprM. This evidence agreed with Masuda and Ohya (1992), who suggested that overproduction of OprM is associated with resistance to fluoroquinolones in *P. aeruginosa*.

These reports agreed with Köhler *et al.* (1999), Poole (2000) and Griffith *et al.* (2006) who suggested that, the elevated intrinsic resistance in *P. aeruginosa* due to the low outer membrane permeability correlated to the appearance of 50-54kDa outer membrane proteins.

4. Conclusion

From all the previous, we can conclude that fluoroquinolones resistance in *P. aeruginosa* was associated with overproduction of 50-54 kDa outer membrane protein. This protein may be responsible for decreased drug accumulation inside the cells; making antibiotics inefficient in infected sites, and could be responsible for increasing the level of intrinsic resistance, enhancing acquired resistance, and increasing frequency of emergence of *P. aeruginosa* strains highly resistant to fluoroquinolones in clinical settings especially when combined with mutations in the target enzymes (DNA gyrase and topoisomerase IV).

Table 1: Resistant patterns of 65 *Pseudomonas aeruginosa* clinical isolates to the three fluoroquinolones.

Fluoroquinolones	NO. of Sensitive Isolates	NO. of Resistant Isolates	% of Resistance
Ciprofloxacin	40	25	39%
Norfloxacin	38	27	42%
Levofloxacin	39	26	40%

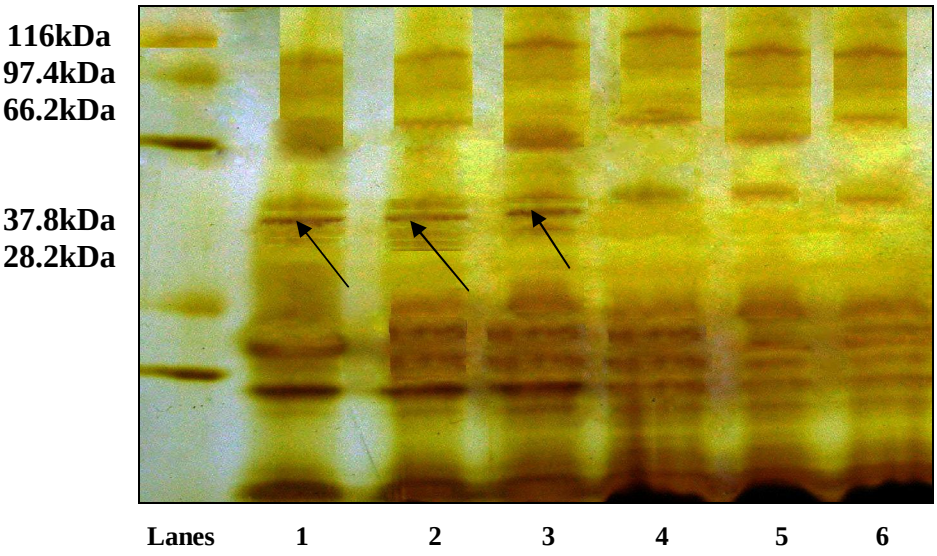

Fig. 1: Silver-stained SDS-PAGE of Sarkosel-insoluble membrane fractions: showing the outer membrane protein profile of ciprofloxacin-resistant isolates (lane: 2), norfloxacin-resistant isolates (lane: 3), and levofloxacin-resistant isolates (lane: 4), When grew in the presence of ciprofloxacin, norfloxacin or levofloxacin, respectively, these lanes obtained an outer membrane protein having electrophoretic mobility corresponding to an apparent molecular mass of approximate 50-54 kDa, arrows showing these bands. Lanes: 5, 6, & 7, showing the outer membrane protein profiles of the fluoroquinolones–susceptible isolates. Lane: 1, showing molecular weights of protein marker components (Jena Bioscience,Germany) in kDa.

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5. References:

1. Alonso A, Campanario E, Martinez J L. Emergence of multidrug-resistant mutants is increased under antibiotic selective pressure in *Pseudomonas aeruginosa*. Microbiology 1999; 145: 2857–62.
2. Cheesbrough M. District laboratory practice in tropical countries. 2nd ed. Cambridge university press. 2000: 2 vols; 7.1-7.18.
3. Chuanchuen R, Murata T, Gotoh N, Schweizer H P. Substrate-dependent utilization of OprM or OpmH by the *Pseudomonas aeruginosa* MexJK efflux pump. Antimicrob Agents Chemother 2005; 49(5): 2133–36.
4. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 17th informational supplement: CLSI/NCCLS document M100–S17. Wayne, PA: Clinical and Laboratory Standards Institute. 2007.
5. Emami S, Shafieeb A, Foroumadib A. Quinolones: recent structural and clinical

developments. Iranian J Pharmaceut Res 2005; 3: 123-36.
6. Fukuda H, Hosaka M, Iyob S, Gotoh N, Nishino T, Hirai K. nfxC-type quinolone resistance in a clinical isolate of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 1995; 39(3): 790–2.
7. Goldman E, Green L H. Practical handbook of microbiology. 2nd ed. Boca Raton: Taylor and Francis Group, LLC. 2009.
8. Griffith D C, Corcoran E, Lofland D, Lee A, Cho D, Lomovskaya O, Dudley M N. Pharmacodynamics of levofloxacin against *Pseudomonas aeruginosa* with reduced susceptibility due to different efflux pumps: Do elevated MICs always predict reduced in vivo efficacy. Antimicrob Agents Chemother 2006; 50(5): 1628–32.
9. Hocquet D, Llanes C, Patry I, El Garch F, Plésiat P. Two efflux systems expressed simultaneously in clinical *Pseudomonas aeruginosa*. Pathol Biol (Paris) 2004; 52: 455–61.
10. Hooper D C. Emerging mechanisms of fluoroquinolone resistance. Emerg Infect Dis 2001; 7(2): 337-41.
11. Hsu D I, Okamoto M P, Murthy R, Wong-Beringer A. Fluoroquinolone-resistant *Pseudomonas aeruginosa*: risk factors for acquisition and impact on outcomes. J Antimicrob Chemother 2005.
12. Jeannot K, Elsen S, Ko¨Hler T, Attree I, Van Delden C, Ple´Siat P. Resistance and virulence of *Pseudomonas aeruginosa* clinical strains overproducing the MexCD-OprJ efflux pump. Antimicrob Agents Chemother 2008; 52(7): 2455–62.
13. Ko¨Hler T, Epp S F, Curty L K, Pechère J-C. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. J Bacteriol 1999; 181(20): 6300–5.
14. Lambert P A. Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. J R Soc Med 2002; 95(41): 22–6.
15. Le Thomas I, Couetdic G, Clermont O, Brahimi N, Plesiat P, Bingen E. In vivo selection of a target/efflux double mutant of *Pseudomonas aeruginosa* by ciprofloxacin therapy. J Antimicrob Chemother 2001; 48: 553-5.
16. Li X-Z, Poole K. Mutational analysis of the OprM outer membrane component of the MexA-MexB-OprM multidrug efflux system of *Pseudomonas aeruginosa*. J Bacteriol 2001; 183(1): 12–27.
17. Masuda N, Ohya S. Cross-resistance to meropenem, cephems, and quinolones in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 1992; 36(9): 1847-51.
18. Masuda N, Sakagawa E, Ohya S. Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 1995; 39(3): 645–9.
19. Nakajima A, Sugimoto Y, Yoneyama H, Nakae T. High-level fluoroquinolones resistance in *Pseudomonas aeruginosa* due to interplay of the MexAB-OprM efflux pump and DNA gyrase mutation. Microbiol Immunol 2002; 46(6): 391-5.
20. Niga T, Ito H, Oyamada Y, Yamagishi J-I, Kadono M, Nishino T, Gotoh N, Inoue M. Cooperation between alteration of DNA gyrase genes and over-expression of MexB and MexX confers high-level fluoroquinolones resistance in *Pseudomonas aeruginosa* strains isolated from a patient who received a liver transplant followed by treatment with fluoroquinolones. Microbiol Immunol 2005; 49(5): 443-6.
21. Oliphant C M, Green G M. Quinolones: a comprehensive review. Am Fam Physician 2002; 65(3): 455-64.
22. Pitt T L, Simpson A J H. *Pseudomonas* and *Burkholderia spp.* In: Gillespie S H, Hawkey P M, 2nd ed. Principles and practice of clinical bacteriology. West Sussex: John Wiley and Sons Ltd. 2006: 427-43.
23. Poole K. Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria. Antimicrob Agents Chemother 2000; 44(9): 2233–41.
24. Ruiz J. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. J Antimicrob Chemother 2003; 51: 1109–17.
25. Sambrook J, Fritsch E F, Maniatis M. Molecular clonining: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press. 1989: 1 vols; 6.36-6.50.
26. Sárközy G. Quinolones: a class of antimicrobial agents. Vet Med–Czech 2001; 46(9–10): 257–74.
27. Scholar E M. Fluoroquinolines: past, present and future of a novel group of antibacterial agents. Am J Pharm Educ 2003; 66: 164-72.
28. Schwalbe R, Steele-Moore L, Goodwin A C. Antimicrobial susceptibility testing protocols. Boca Raton: CRC press: Taylor and Francis Group, LLC. 2007.
29. Swiatlo E, Moore E, Watt J, Mcdaniel L S. In vitro activity of four fluoroquinolones against clinical isolates of *Pseudomonas aeruginosa* determined by the E test. Int J Antimicrob Agents 2000; 15: 73–6.

30. Van Bambeke F, Michot J-M, Van Eldere J, Tulkens P M. Quinolones in 2005: an update. Clin Microbiol Infect 2005; 11: 256–80.
31. Zeng L. *Pseudomonas aeruginosa*: pathogenicity and antibiotic resistance. Academic Dissertation (PhD). University of Florida 2004.

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