Original article:

Norfloxacin Induced Cross-Resistance to Fluoroquinolones and Non fluoroquinolone Groups of Antimicrobial Agents in *Pseudomonas aeruginosa*

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Abstract

Objective: To detect whether development of resistance to norfloxacin promotes development of resistance to other fluoroquinolones. ***Material and Methods:*** Four clinical and two environmental isolates of *Pseudomonas aeruginosa* susceptible to norfloxacin (Minimum Inhibitory Concentration in the range of 0.4µg/ml to 0.84µg/ml) were manipulated in vitro to induce resistance to norfloxacin by means of serial transfer up to 14 times through media containing increasing concentrations of norfloxacin. ***Results and Discussion:*** This in vitro manipulation of isolates of P. aeruginosa resulted in increase of MIC from 0.4µg/ml to 0.84µg/ml to 12.5µg/ml to 13.5µg/ml indicating development of resistance to norfloxacin. The antimicrobial susceptibility testing showed a major decrease in the zone diameters of pefloxacin and ofloxacin, and significant decrease in the zone diameters of ciprofloxacin indicating development of cross resistance to other fluoroquinolones. No appreciable change in the zone diameters of non fluoroquinolone groups of antimicrobial agents indicating no development of resistance to structurally unrelated antibiotics. ***Conclusions:*** These results indicate that development of resistance to norfloxacin promotes development of resistance to other fluoroquinolones.

Keywords: P. aeruginosa; norfloxacin; cross resistance; induced resistance

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Introduction

The emergence of organisms resistant to antibiotic may significantly reduce the effectiveness of an antibiotic and *P. aeruginosa* is not an exception to this phenomenon. The causative factor for development of such resistance has been reported to be the uncontrolled and inappropriate use of available antibiotics as well as self medication; thus encouraging the spread of resistant strains over a period of time. The growing problem of substandard and spurious drugs, which lack adequate amounts of active ingredients, further exacerbates the problem.

Antibiotic resistance is a significant problem in the treatment of *P. aeruginosa* infections, partly because of its intrinsic resistance to a wide range of antibiotics due to lower outer membrane permeability and the presence of several drug efflux systems[1]. Clinically significant resistance to fluoroquinolones emerges more readily in Pseudomonads. Resistance to quinolones develops rapidly during the treatment. Development of resistance to norfloxacin and other fluoroquinolones in *P. aeruginosa* isolates during therapy has been reported by various workers. Development of cross resistance among fluoroquinolones is also frequently reported[2-4]. The resistance to fluoroquinolones can also be induced

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in vitro. Modak *et al.* (1988) in their in vitro study found that when *P. aeruginosa* cultures were serially transferred for 10 times through sub inhibitory concentrations of fluoroquinolones like norfloxacin and pefloxacin, an increase in MIC for 40 times was observed[4]. In the majority of patients studied, failure to eradicate *P. aeruginosa* after therapy with norfloxacin was due to the development of resistance rather than to re-infection.

The purpose of the present study was to induce in vitro resistance to norfloxacin in clinical and environmental isolates of *P. aeruginosa* and to study its effect on the susceptibility pattern of other fluoroquinolones and structurally unrelated antibiotics from nonfluoroquinolone groups.

Materials and methods

For this study, six isolates of *P. aeruginosa* with minimum inhibitory concentration (MIC) in the range of 0.4 µg/ml to 0.8 µg/ml and susceptible to ciprofloxacin, pefloxacin, norfloxacin, ofloxacin, netillin, tobramycin, gentamicin, amikacin, piperacillin, carbenicillin, ceftriaxone, cefoperazone, cephotaxime and ceftazidime were selected for the study of development of resistance to norfloxacin. These strains were manipulated in vitro to induce resistance to norfloxacin by means of serial transfer up to 14 times in media containing increasing concentrations of norfloxacin, starting with subinhibitory concentrations[4,5].

Strains under study were inoculated into peptone water and incubated overnight at 37^0C. After achieving the final bacterial concentration of $1x10^5$ CFU/ml approximately (turbidity matched with McFarland No.1), 200 µl of peptone water culture was inoculated into 10 ml of brain – heart infusion broth (BHI) containing 0.05 µg/ml of norfloxacin and incubated at 35^0C for 16 hours with intermittent shaking. After incubation, 200 µl of BHI broth was transferred to 10 ml of BHI broth containing 0.1 µg norfloxacin per ml. In this way, all six strains ware transferred serially for 14 times through increasing concentrations of norfloxacin. The different increasing concentrations of norfloxacin used were 0.05 µg/ml, 0.1 µg/ml, 0.2 µg/ml, 0.4 µg/ml, 0.6 µg/ml, 0.8 µg/ml, 1 µg/ml, 1.2 µg/ml, 1.4 µg/ml, 1.6 µg/ml, 1.8 µg/ml, 2 µg/ml, 2.2 µg/ml and 2.5 µg/ml.

After serial transfer for 14 times through drug containing media, MIC of all six isolates against norfloxacin was determined by agar dilution method to determine the increase in MIC levels and development of resistance[6]. For this 21 different concentrations of norfloxacin were incorporated into different Mueller – Hinton agar plates, one plate for each concentration to be tested. The various concentrations of norfloxacin used were 3 µg/ml, 3.5 µg/ml, 4 µg/ml, 4.5 µg/ml, 5 µg/ml, 5.5 µg/ml, 6 µg/ml, 6.5 µg/ml, 7 µg/ml, 7.5 µg/ml, 8 µg/ml, 8.5 µg/ml, 9 µg/ml, 9.5 µg/ml, 10 µg/ml, 10.5 µg/ml, 11 µg/ml, 11.5 µg/ml, 12 µg/ml, 12.5 µg/ml and 13 µg/ml. The MIC was determined by agar dilution method.

Rate of variant isolation was detected by standard plate count method. For this, a culture of test strain exposed to norfloxacin (BHI showing growth at highest concentration) was serially diluted for 10 times. One ml of BHI broth exposed to antibiotic was mixed with nine ml of sterile distilled water (1:10), this was further diluted to 1:20, 1:30, 1:40, 1:50, 1:60, 1:70, 1:80, 1:90 and 1:100 dilutions. Then one ml of each dilution was mixed with 15 ml of Mueller – Hinton agar containing 12.0 µg/ml of norfloxacin at 50^0C and poured in a sterile petriplate. The plates were incubated at 35^0C for 72 hours. Assuming that each bacterium forms one colony, the numbers of bacteria were counted by counting colonies and from this the rate of variant isolation was determined.

Antibiotic susceptibility pattern of all six isolates was studied before and after the development of resistance to norfloxacin to note the changes induced by norfloxacin in the susceptibility pattern of other antibiotics. The method used was Kirby – Bauer disc diffusion method[7]. The antibiotics used were ciprofloxacin, pefloxacin, norfloxacin, ofloxacin, netillin, tobramycin, gentamicin, amikacin, ticarcillin, piperacillin, carbenicillin, ceftriaxone, cefoperazone, cephotaxime and ceftazidime.

Strict technical precautions such as standard inoculum size, diffusion time, incubation temperature and time, etc. were taken to avoid disparity in the results of antibiotic susceptibility testing before and after the induced resistance to norfloxacin. The results of zone diameters in millimeter were recorded before and after the development of resistance to find out the changes induced by norfloxacin in the susceptibility pattern of other antibiotics.

This study was ethically approved.

Results

Table No. 1 shows MIC and zone diameters of clinical and environmental isolates of *P. aeruginosa* against fluoroquinolones before and after the development of resistance to norfloxacin. By serial passage of six isolates in media containing increasing concentrations of norfloxacin, the resistance to norfloxacin was induced in all isolates. Norfloxacin MIC in the range of 0.4 to 0.8 µg/ml of original strains of *P. aeruginosa* was increased to 12.5 to 13.5 µg/ml. The spontaneous resistant mutants were recovered on M-H plate containing 12 µg/ml of norfloxacin at frequencies of 3 x 10^{-6} to 7 x 10^{-6}. The antimicrobial susceptibility pattern of newly isolated norfloxacin induced mutants showed a major decrease in the zone diameters of norfloxacin and major decrease in the zone diameters of pefloxacin and ofloxacin (Complete inhibition - no zone of inhibition seen). Decrease in the zone diameters of ciprofloxacin was also observed but there was no complete decrease in zone diameters was observed.

Table No. – 1 : MIC and Zone Diameters of Fluoroquinolones of P. aeruginosa Isolates Before and After Induced Resistance to Norfloxacin In vitro.

Source	Strain No.	Zone Diameters in mm to Antimicrobial Agents										
		Nt	Tb	G	Ak	Ti	Pc	Cb	Ci	Cs	Ce	Ca
Cheatle forcep fluid Sink Pus Urine Pus Sputum	PAO -1	20	20	28	30	14	29	28	16	21	21	22
	PAM -1	21	22	25	25	16	24	22	22	21	18	20
	PAO -2	30	24	30	30	21	29	28	25	25	23	22
	PAM -2	28	22	28	29	22	25	28	24	24	25	20
	PAO -3	20	25	26	29	16	21	21	19	21	20	19
	PAM -3	21	23	23	25	00	14	20	09	18	12	20
	PAO -4	17	20	20	19	00	19	22	17	20	20	22
	PAM -4	18	22	20	20	00	20	24	18	20	18	20
	PAO -5	11	20	21	23	16	26	27	18	28	22	25
	PAM -5	15	27	27	26	15	30	29	20	29	26	23
	PAO -6	24	16	20	20	23	25	25	30	30	34	17
	PAM -6	22	18	20	21	23	24	22	28	22	25	20

Note : PA - *P. aeruginosa* , O -original, M -mutant
Nt - netillin, Tb - tobramycin, G - gentamicin, Ak - amikacin, Ti - ticarcillin,
Pc - piperacillin, Cb - carbenicillin, Ci - ceftriaxone, Cs - cefoperazone,
Ce – cephotaxime, Ca - ceftazidime

Table no. 2 shows antibiotic susceptibility pattern of clinical and environmental isolates of *P. aeruginosa* against nonfluoroquinolone antimicrobial agents (structurally unrelated antibiotics) before and after the development of resistance to norfloxacin. No appreciable differences were seen in the zone diameters before and after the development of resistance to norfloxacin, except for significant difference in zone diameters of ticarcillin, piperacillin, cephotaxime and ceftriaxone were seen in one of the isolates of *P. aeruginosa* from pus.

Table No. 2 : Zone Diameters of *P. aeruginosa* Isolates to Nonfluoroquinolone Group of Antibiotics Before and After Induced Resistance to Norfloxacin In vitro

Source	Strain No.	Zone Diameters in mm to Antimicrobial Agents										
		Nt	Tb	G	Ak	Ti	Pc	Cb	Ci	Cs	Ce	Ca
Cheatle	PAO-1	20	20	28	30	14	29	28	16	21	21	22
forcep fluid	PAM-1	21	22	25	25	16	24	22	22	21	18	20
Sink	PAO-2	30	24	30	30	21	29	28	25	25	23	22
	PAM-2	28	22	28	29	22	25	28	24	24	25	20
Pus	PAO-3	20	25	26	29	16	21	21	19	21	20	19
	PAM-3	21	23	23	25	00	14	20	09	18	12	20
Urine	PAO-4	17	20	20	19	00	19	22	17	20	20	22
	PAM-4	18	22	20	20	00	20	24	18	20	18	20
Pus	PAO-5	11	20	21	23	16	26	27	18	28	22	25
	PAM-5	15	27	27	26	15	30	29	20	29	26	23
Sputum	PAO-6	24	16	20	20	23	25	25	30	30	34	17
	PAM-6	22	18	20	21	23	24	22	28	22	25	20

Note : PA- *P. aeruginosa*, O-original, M-mutant
Nt - netillin, Tb - tobramycin, G - gentamicin, Ak - amikacin, Ti - ticarcillin, Pc - piperacillin, Cb - carbenicillin, Ci - ceftriaxone, Cs - cefoperazone, Ce – cephotaxime, Ca - ceftazidime

Discussion and conclusion

Repeated exposure of organisms to antimicrobial agents in sub lethal doses enhances the development and maintenance of resistance. Also the presence of antimicrobial agent in sub lethal concentrations makes an environment suitable for step wise mutations resulting in the development of resistance. In the present study, by exposing susceptible clinical and environmental isolates of *P. aeruginosa* to sub lethal concentrations of norfloxacin followed by repeated exposure to increasing concentrations, it was possible to induce resistance to norfloxacin in all six Isolates of *P. aeruginosa*, as evidenced by increase in MIC of all six isolates of *P. aeruginosa* to norfloxacin.

A major decrease in the zone diameters of norfloxacin after exposure to norfloxacin in in vitro study also indicates development of resistance to norfloxacin. A major decrease in the zone diameters of pefloxacin and ofloxacin, and significant decrease in the zone diameters of ciprofloxacin, indicates development of cross resistance to these agents following exposure to norfloxacin. No major change in the zone diameters of aminoglycosides, cephalosporins and antipseudomonal penicillins, except for one of the isolates of *P. aeruginosa* from pus that to only in betalactam antibiotics, indicates that no cross resistance was developed to these structurally unrelated antimicrobial agents.

The development of resistance to norfloxacin in *P. aeruginosa* isolates during therapy has been reported by various workers[2-3]. Modak *et al.* induced resistance to norfloxacin in vitro by serial transfer of *P. aeruginosa* for 10 times through sub inhibitory concentrations of norfloxacin[4]. In the present study also, it was possible to induce resistance to norfloxacin in vitro in all six isolates of *P. aeruginosa*.

The finding that development of resistance to one fluoroquinolone also results into development of cross resistance to other fluoroquinolones is similar to earlier reports who also reported development of cross – resistance among the quinolones more and more frequently during therapy[2,8,9].

In the present study, there was no major change in the zone diameters of various nonfluoroquinolones antimicrobial agents (structurally unrelated antibiotics) following the exposure to norfloxacin

in vitro was observed. This indicates that there was no development of high level of resistance to structurally unrelated antibiotics following the development of norfloxacin resistance, except for one strain, in which minor differences in zone diameters of beta-lactam antibiotics was observed. This finding is not consistent with earlier findings that quinolone resistance developed in in vitro is associated with cross – resistance to several groups of antimicrobial agents (structurally unrelated antibiotics), particularly beta- lactam antibiotics[8, 10]. The possibility of development of low level resistance cannot be excluded as the development of low level resistance to these agents may not produce a major change in the zone diameters in vitro study (unless MIC goes very high, no major change occurs in zone diameters). From these results, it can be safely concluded that there was no development of high level of resistance to non fluoroquinolones antimicrobial agents - structurally unrelated antibiotics, which can be appreciated just by doing antibiotic susceptibility testing by disc diffusion test.

The results of present study indicate that the development of resistance to norfloxacin simultaneously results into development of cross resistance to other fluoroquinolones. This may be attributed to structural similarity and similar mode of action of norfloxacin with other fluoroquinolones.

From the present study, it is concluded that due to the tendency to develop cross resistance, the care should be exerted in the clinical use of fluoroquinolones for the treatment of pseudomonal infections and their use must be restricted to avoid the possibility of evolution of resistant strains. This study further suggests that fluoroquinolones should be used with caution and only when indicated. Potentially resistant strains could emerge in the future, if proper caution is not exerted in the use of these compounds for the treatment of pseudomonal infections.

Conflict of interest: None declared

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