

EXTENDED REPORT

Ciprofloxacin susceptibility of *Pseudomonas aeruginosa* isolates from keratitis

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Br J Ophthalmol 2003;87:1238–1240

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Accepted for publication 6 February 2003

Aim: To examine the ciprofloxacin susceptibility of 106 *Pseudomonas aeruginosa* eye isolates from the United Kingdom, Denmark, India, the United States, and Australia, and to determine the molecular mechanisms of resistance.

Methods: Ciprofloxacin susceptibility was tested by an agar dilution method; genomic DNA corresponding to the quinolone target genes *gyrA* and *parC*, and the regulatory genes *mexR* and *nfxB* controlling drug efflux systems, was amplified by PCR and sequenced; multilocus enzyme electrophoresis was performed to examine the genetic relation among resistant strains.

Results: Three out of 90 keratitis isolates (3.3%), one from the United Kingdom and two from India, exhibited MIC values of 16 mg/l or 32 mg/l. The UK isolate had a mutation in *gyrA* (Thr83Ile), whereas the two Indian isolates showed mutations in both *gyrA* (Thr83Ile) and *parC* (Ser87Leu). The remaining isolates from keratitis, endophthalmitis, contact lens associated red eye (CLARE), and contact lens storage cases showed MIC values below 1 mg/l. Several allelic forms of *gyrA* and a single variation in the *mexR* gene product were detected in 10 ciprofloxacin susceptible strains.

Conclusions: The vast majority of eye isolates of *P aeruginosa* from European countries are fully susceptible to ciprofloxacin and the concentration of ciprofloxacin eye drops used for local treatment (3000 mg/l) exceeds MIC values for strains recorded as resistant. Mutations in more than one target gene were associated with higher MIC values.

Ciprofloxacin monotherapy has proved effective in the treatment of keratitis¹ and is now widely used as first line treatment in many countries. In recent years, emerging ciprofloxacin resistance has been reported among Indian, Taiwanese, and American ocular *Pseudomonas aeruginosa* strains,^{2–5} causing concern to ophthalmologists worldwide. However, available data indicate that the prevalence of resistance among *P aeruginosa* isolates varies between countries. Studies of *P aeruginosa* isolates collected between 1994 and 1997 from Italian and Japanese hospitals showed that 83% and 90%, respectively, were resistant.^{6,7} Recent population genetic analyses of clinical isolates of *P aeruginosa* from various infections suggest that the majority of strains causing keratitis are distinct from strains from other infections.⁸

The main mechanism of quinolone resistance in *P aeruginosa* is mutations in the genes *gyrA* and *parC*, encoding the target proteins DNA gyrase and topoisomerase IV, and mutations in the regulatory genes *mexR* and *nfxB* for drug efflux pumps, resulting in reduced intracellular concentration.^{9–10} However, the exact mechanisms of resistance vary among isolates from different infections. Jalal *et al*^{11–12} found that mutations in *nfxB* are more common in resistant *P aeruginosa* isolates from cystic fibrosis (CF) patients than in strains from urine and wounds in which mutations in *gyrA* and *parC* dominate. Japanese hospital isolates with high level of fluoroquinolone resistance showed a predominance of Thr83Ile mutation in *gyrA*.⁷ There is no information on resistance mechanisms in eye isolates.

The purpose of the study reported here was to determine the ciprofloxacin susceptibility of a collection of *P aeruginosa* eye isolates from the United Kingdom, Denmark, United States, India, and Australia and to identify the mutations in target genes responsible for reduced susceptibility.

METHODS

The study included 106 *P aeruginosa* strains isolated between 1984 and 2001, the majority (96%) after 1995. Ninety were isolated from keratitis, including 59 from the United Kingdom, 25 from Denmark, two from India, two from Australia, and two from the United States. The remaining strains were from endophthalmitis in the United Kingdom (n = 5), contact lens associated red eyes in India and Australia (n = 2), contact lens storage cases belonging to patients with keratitis in Denmark (n = 5), and contact lens cases belonging to asymptomatic wearers in India and Australia (n = 4). The quinolone susceptible reference strain PA01 was included for comparison.

Minimal inhibitory concentration (MIC)

The MIC for each strain was determined in triplicate using Isosensitest agar medium (Oxoid, Basingstoke, Hants, UK) containing ciprofloxacin in final concentrations of 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.12, 0.06, and 0 µg/ml.¹² The inoculum was about 100 000 colony forming units transferred to the agar surface with a 96 pin applicator from a microtitration plate containing suspensions of the individual strains. The MIC was defined as the lowest concentration inhibiting visible bacterial growth as evaluated after 18 hour incubation at 37°C. Using the reference value for resistance in systemic therapy, strains were regarded resistant to ciprofloxacin if MIC ≥ 2 mg/l.

Polymerase chain reaction (PCR)

Chromosomal DNA was extracted as described.¹³ The quinolone resistance determining region of the genes encoding DNA gyrase (*gyrA*) and topoisomerase IV (*parC*), and the genes encoding regulatory genes for multidrug efflux pump systems MexAB-OprM and MexCD-OprJ, a 385 bp region of

mexR and a 924 bp region including the whole *nfxB* gene (561 bp), were amplified using PCR. The methods and primers used were essentially as described by Jalal *et al.*^{14, 15} except that only primers *gyrA*-1 and *gyrA*-2 were employed to amplify the relevant stretch of the *gyrA* gene. All reactions were performed in Ready-To-Go PCR Beads (Amersham Pharmacia, Biotech Inc, Piscataway NJ, USA) added DNA and the two preboiled primers (10) each at a final concentration of 5 pmol/μl. PCR products were sequenced on both strands using the same primers as employed in the PCRs with a Thermo Sequenase dye terminator cycle sequencing kit (Amersham Life Science, Cleveland, OH, USA) and analysis on an Applied Biosystem DNA sequencer. Numbering of altered amino acids is according to that in the complete proteins for strain PAO1 (www.pseudomonas.com).

Multilocus enzyme electrophoresis (MLEE)

This method determines the electrophoretic mobilities of housekeeping enzymes,¹⁴ and was performed to examine the genetic relation among the resistant strains. The electrophoretic mobility of nine enzymes (malate dehydrogenase, alkaline phosphatase, glutamate dehydrogenase, glucose-6-phosphate dehydrogenase, esterases, phosphoglucose isomerase, leucine isomerase, hexokinase, and alcohol dehydrogenase) was determined as described.⁸

RESULTS

The triplicate analysis of the ciprofloxacin susceptibility of each strain revealed excellent reproducibility. A total of 104 isolates showed identical results in all three determinations or variations within a single step dilution. Two isolates showed variations within two dilutions.

Of the 106 *P. aeruginosa* isolates, three (2.8%) showed in vitro resistance to ciprofloxacin. The resistant isolates were all isolated from keratitis, two out of two isolates from India, and one out of 59 isolates from the United Kingdom. The two strains from India exhibited median MICs of 32 mg/l and the strain from the United Kingdom showed a median MIC of 16 mg/l.

The median MIC of ciprofloxacin for the remaining strains varied between 0.12–0.25 mg/l for 100 isolates, was 0.5 mg/l for three isolates, and 1 mg/l for one strain (PAO1); 95% of the strains showed median MIC values identical to or below 0.25 mg/l.

Mutations in target genes were found in all three resistant isolates (Table 1). All three had a mutation in *gyrA*, corresponding to amino acid change Thr83Ile. The two Indian isolates (*paer31* and *paer32*), in addition, showed a mutation in *parC* corresponding to amino acid change Ser87Leu. The additional mutation in these two strains was associated with a higher MIC value (32 mg/l compared with 16 mg/l for strain MK56). None of the strains had mutations in *nfxB*.

Sequence analysis of the corresponding segments of *gyrA*, *parC*, and *mexR* in 10 fully susceptible isolates that were

phylogenetically related to the resistant strains⁸ revealed several allelic forms of *gyrA* and a single variation in the *mexR* gene product. The following non-synonymous mutations relative to PAO1 were found in *GyrA*: Ala117Pro (GCC→CCC in strains AKH01, MK58, PJ39), Arg121Gln (CGA→CAA in MK30, MK34, MK56 (resistant), MK58, Leu125Val (TTG→GTG in all strains except for PAO1 and the resistant strain *paer32*). The variation observed in *MexR* was: Phe80Val (TTC→GTC in all strains except for PAO1, MK2, and PJ39). None of the two mutations present in resistant strains was detected in the 10 susceptible strains.

The electrophoretic mobilities of the nine housekeeping enzymes from *paer31* and *paer32* were identical, strongly suggesting that they belong to the same clone. The UK isolate MK56 showed different mobilities for the esterases, glucose-6-phosphate dehydrogenase, and leucine aminopeptidase, indicating that this strain is clonally unrelated to the two resistant isolates from India.

DISCUSSION

This study shows that ciprofloxacin resistance is still rare among *P. aeruginosa* isolates from eye infections in Europe. Only one out of 102 isolates (0.98%) from Europe revealed an MIC value of 16 mg/l, thus exceeding the value normally regarded as reference value for resistance in systemic therapy (MIC ≥2 mg/l). The finding that both of two isolates from India were resistant is in accordance with previous studies that revealed a resistance rate of 15.6–30.7% among eye isolates in India.^{2, 15} Significantly lower rates have been observed in the United States (2.1%) and Japan (3.4%).^{4, 7} Conceivably, differences in susceptibility between countries and between different environments within countries reflect different degrees of selection pressures. In contrast with nosocomially acquired *P. aeruginosa* infections, eye infections arise in healthy contact lens wearers in the community with less selection for resistance and with clonally distinct isolates.⁸

Evaluation of the susceptibility of *P. aeruginosa* keratitis isolates is usually made with reference to concentrations used in systemic therapy. Nevertheless, the concentrations achieved on the ocular surface by local application of antibiotic eye drops or ointments are significantly higher.^{16, 17} The concentration of ciprofloxacin in eye drops used for local treatment (3000 mg/l) far exceeds in vitro MIC values (16–32 mg/l) even for the three strains recorded as resistant in this study. Whether the increased resistance of bacteria in biofilms will affect their susceptibility to treatment in vivo is not clear.

The three eye isolates with reduced susceptibility had previously recognised mutations in different combinations of target genes. Mutations in more than one gene were associated with a higher level of resistance. The frequently observed mutation at position 83 in *gyrA*, associated with a high level of resistance in *P. aeruginosa*, *Escherichia coli*, and *Yersinia pestis*, was found in all three isolates.^{7, 10, 18} In addition,

Table 1 Mutations detected in fluoroquinolone resistant strains of *Pseudomonas aeruginosa* in the target genes *gyrA* and *parC*

Isolate	MIC (mg/l) of ciprofloxacin	Amino acid change (nucleotide change)	
		<i>gyrA</i>	<i>parC</i>
PAO1	1.0		
MK 56	16	T83I (ACC→ATC)	
<i>Paer 31</i>	32	T83I (ACC→ATC)	S87L (TCG→TTG)
<i>Paer 32</i>	32	T83I (ACC→ATC)	S87L (TCG→TTG)

L = leucine; S = serine; I = isoleucine; T = threonine; K = lysine; F = phenylalanine; E = glutamic acid; N = asparagine; A = alanine. All changes are relative to reference strain PAO1, which is quinolone susceptible.

the two Indian strains shared a previously described mutation in *parC* (Ser87Leu). *GyrA* and *ParC* with these two mutations show an increased ability to supercoil DNA in the presence of ciprofloxacin.¹⁹ The finding of identical mutations in the two Indian isolates is in agreement with their clonal identity as revealed by MLEE analysis. Sequence analysis of 10 additional isolates revealed several allelic variations in *gyrA* that were not associated with decreased susceptibility to ciprofloxacin.

In conclusion, ciprofloxacin resistance of *P aeruginosa* eye isolates from countries with a restrictive usage of antibiotics is still rare and the problem is considerably smaller than that observed in certain hospital environments. These findings are relevant also for the third and fourth generation fluoroquinolones, as there is cross resistance between ciprofloxacin and these new compounds among Gram negative bacteria.²⁰

ACKNOWLEDGEMENT

This study was supported by Alcon Denmark.

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doi: 10.1136/bjo.87.10.1238

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