Genetic fingerprinting and antimicrobial susceptibility profiles of *Pseudomonas aeruginosa* isolates from eye infections

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**ABSTRACT**

**Background:** As *Pseudomonas aeruginosa* is known the most common etiologic agent in microbial keratitis associated with contact lens use, this study was designed to study the distribution and patterns of resistance to antimicrobial agents of keratitis isolates in Iran. In this study, also the suitability of enterobacterial repetitive intergenic consensus (ERIC)-PCR to rapidly type *P. aeruginosa* strains isolated from patients with keratitis was examined.

**Patients and methods:** For this purpose, 57 clinically isolates of *P. aeruginosa* from keratitis patients referred to Farabi hospital were analyzed by antimicrobial susceptibility test using the disc diffusion method. Polymerase chain reaction with enterobacterial repetitive intergenic consensus primers (ERIC-PCR) was used to establish clonal relationship between the different isolates.

**Results:** All the strains showed resistance to at least 4 antibiotics, but all were susceptible to fluoroquinolones. Multidrug resistance was found in two isolates (3.5%) which were resistant to more than one category of antibiotics including aminoglycoside (gentamicin) and β-lactam (cefazoline). ERIC-PCR produced 53 different ERIC fingerprints, 49 of which contained only 1 strain. Eight of the isolates had 100% similarity, forming four real clones but considering 85% similarity cut off between isolates, 8 clones containing 25 isolates (43.8%) could be considered.

**Conclusion:** Fluoroquinolones appeared to be the most effective agent against ocular *P. aeruginosa* isolates. Comparison of ERIC-PCR profiles revealed a low level of similarity among the strains analyzed. ERIC-PCR seems to be an inexpensive, fast, reproducible, and discriminatory DNA typing tool for effective epidemiologic surveillance of *P. aeruginosa* isolates potentially transmissible between patients with ocular infections.

**Keywords:** *Pseudomonas aeruginosa*, Keratitis, ERIC-PCR.


**INTRODUCTION**

*Pseudomonas aeruginosa* is the species type of the genus Pseudomonas. It is an opportunistic human pathogen and is frequently isolated from the hospital environments, clinical specimens, and soil and water samples (1). *P. aeruginosa* has also remained the most common cause of contact lens-related keratitis (2). The remarkable ability of *P. aeruginosa* to adapt to a wide variety of environments may be due to its extensive genetic...
versatility that ensures its pathogenic potential (3). Evidence that bacteria related to eye infections belong to specific subpopulations of P. aeruginosa would support further development of strategies for better control of the disease.

Investigations of the epidemiology of P. aeruginosa have been hampered by the inadequate discriminatory capacity of classical phenotypic methods such as serotyping, phage typing, pyocin typing and biotyping (4). Genetic typing techniques have been shown to be more discriminatory than phenotypic methods for typing P. aeruginosa isolates (5).

Rep-PCR assays utilize primers targeting highly conserved repetitive sequence elements in the bacterial genome. One of such groups of repetitive elements is the enterobacterial repetitive intergenic consensus (ERIC) sequences common to Gram-negative enteric bacteria (6). The products of the ERIC-PCR, with chromosomal DNA of different bacterial strains as template, were found to generate very characteristic patterns when separated on agarose gels. Thus, it was proposed that ERIC-PCR may constitute a useful method to fingerprint bacterial genomes (7).

In this study, we characterized 57 eye clinical isolates collected from patients attending Farabi Hospital in Tehran by ERIC to determine the genetic diversity of these P. aeruginosa strains in our population.

PATIENTS and METHODS

A collection of 57 P. aeruginosa clinical isolates were obtained between September 2009 and February 2010 from patients attending Farabi Hospital in Tehran because of conjunctivitis and keratitis. All isolates were identified as P. aeruginosa according to colony morphology, Gram’s stain appearance, oxidase reaction, and growth at 41°C (8).

Antibiotics used in susceptibility tests included β-lactams (imipenem, ceftazidime and cefazoline), aminoglycosides (amikacin and gentamicin), fluoroquinolones (ciprofloxacin, norfloxacin and ofloxacin) and chloramphenicol, vancomycin and trimethoprim–sulfamethoxazole. Bacterial susceptibilities to these antibiotics were determined using the disc diffusion method in accordance with the CDS (Calibrated Dichotomous Sensitivity) method standard (9).

ERIC PCR was carried out using the primer sequences ERIC-1R, 5’-CACTTAGGGGTCCTCGGAATGTA-3’ and ERIC-2, 5’-AAGTAAGTGACTGGGGTGAGCG-3’ to amplify the regions in the bacterial genome positioned between the ERIC sequences (10). The PCR mix contained 4 mM magnesium chloride and the temperature program was as follows: initial denaturation at 95°C for 10 min followed by 30 cycles of PCR consisting of denaturation at 90°C for 30 s, annealing at 52°C for 1 min, and extension at 65°C for 8 min with the exception of the last cycle, which had a final extension time of 16 min (11). Ten microliters of the amplification product was analyzed in 2% agarose gel stained with ethidium bromide. PCR patterns were visually evaluated, and a matrix was compiled, in which each taxon was reported in a separate column and the presence/absence of DNA fragments of the same length was reported as 1/0 in rows. The matrix was analyzed with FreeTree software (12). Dice and Jaccard formulae were used for distance calculation, and the unweighted pair group method with arithmetic mean was applied as a clustering method. Similarity dendrograms were then displayed with Treeview software (13). The criterion for related clones for all assays was taken as profiles with 85% or more similar bands. These isolates differed in one to two bands (14).

RESULTS

The susceptibility of 57 ocular isolates of P. aeruginosa to 11 antibiotics, belonging to three categories β-lactam, aminoglycoside, and
fluoroquinolone, were examined in our setting. All strains were resistant to vancomycin, cefazoline, chloramphenicol and trimethoprim–sulfamethoxazole. On the other hand, all isolates were susceptible to aminoglycosides and fluoroquinolones, except two strains which showed resistance to gentamicin (table 1). Therefore among aminoglycosides, amikacin was more effective than gentamicin. None of the isolates were resistant to ciprofloxacin, ofloxacin and norfloxacin (table 1). None of the strains was resistant to only one of the antibiotics tested. Multidrug resistance was found in two isolates (3.5%) which were resistant to more than one category of antibiotics including aminoglycoside gentamycin and β-lactam cefazolin. The fingerprints obtained from ERIC typing of the 57 *P. aeruginosa* eye isolates, consisted of 3 to 14 amplification bands ranging in size from 100bp to 3kb (fig. 1). Genetic analysis of *P. aeruginosa* strains disclosed 53 different ERIC fingerprints, 49 of which contained only 1 strain.

Figure 2 shows the dendrogram of the ERIC-PCR profiles created by UPGMA. Three major clusters (A, B & C) were identified with a small dissimilarity between clusters. Only 4 isolates (7.1%) were placed in clusters A and C. Cluster B contained the majority of the isolates (n=53, 92.9%). In this cluster some isolates were allocated in groups of higher or lower similarity and most strains were discriminated. Eight of the isolates had 100% similarity, forming four real clones but considering 85% similarity cut off between isolates, 8 clones containing 25 isolates (43.8%) could be considered. Over all comparison of ERIC-PCR profiles revealed a low level of similarity among the strains analyzed.

**Table 1. Results of antibiotic susceptibility testing of ocular isolates of *P. aeruginosa***

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Number of clinical isolates (%)</th>
<th>Resistant</th>
<th>Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin</td>
<td>57(100)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cefazoline</td>
<td>57(100)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>57(100)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Trimethoprim–sulfamethoxazole</td>
<td>57(100)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>2(3.5)</td>
<td>55(96.5)</td>
<td></td>
</tr>
<tr>
<td>Amikacin</td>
<td>0</td>
<td>57(100)</td>
<td></td>
</tr>
<tr>
<td>Imipenem</td>
<td>0</td>
<td>57(100)</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0</td>
<td>57(100)</td>
<td></td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>0</td>
<td>57(100)</td>
<td></td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>0</td>
<td>57(100)</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

Keratitis is a serious ocular infection that can lead to corneal scarring and severe visual disability if aggressive and appropriate therapy is not promptly initiated (2). *P. aeruginosa* is one of the most prevalent species isolated from patients suffering from keratitis (15). Patients with infectious keratitis should be followed carefully, and adequate modification of antimicrobial therapy may be required. This case emphasizes the importance of recognizing the possible limitations of these antibiotic agents when treating patients (16). In the last 15 years, the second-generation fluoroquinolones, and more specifically, topical ciprofloxacin, supplanted the aminoglycosides and became the “gold standard” for treating ocular infections with *P. aeruginosa*. Because of its excellent activity against other commonly encountered Gram-positive and Gram-negative ocular pathogens, ciprofloxacin has become a leader in effective empiric monotherapy for bacterial keratitis (17). However, reports of ciprofloxacin resistant *P. aeruginosa* have recently appeared, threatening its seemingly panacea-like power (18,19).

Our study confirmed that the detection of ocular isolates of *P. aeruginosa* with multiple antibiotic resistances is common in Iran, too; however, all the strains except two isolates which showed resistance to gentamicin were found to be sensitive to aminoglycosides and fluoroquinolones. The rate of multidrug resistance in our isolates was 3.5%, and this was significantly lower than what reported (29%) in some countries like India (20).

None of our isolates revealed resistance to fluoroquinolones. This shows that fluoroquinolones resistance is still rare among *P. aeruginosa* isolates from eye infections in Iran. Our results are in accordance with many of previous studies from different countries which show very low rates of resistance to fluoroquinolones in non-nosocomially acquired *P. aeruginosa* infections (21-23). It is concluded 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 (24).

Previous studies have shown that different strains of *P. aeruginosa* were in correlation with specific diseases including keratitis and cystic fibrosis (17,24). Rapid identification of the bacterial strains correlated with specific diseases would greatly aid clinical studies correlating genes and gene expression with clinical outcome. The use of the DNA-based typing methods for investigating diversity is gradually becoming more popular (11). ERIC elements have been highly conserved within the eubacterial kingdom for at least hundreds of millions of years. ERIC-PCR allows clear distinctions between different bacterial species and strains which contain these repetitive elements (7).

Figure 2. ERIC-PCR generated dendrogram showing relationships between the ocular *P. aeruginosa* strains. The dendrogram was constructed by FreeTree program. Dice and Jaccard formulae were used for distance calculation, and UPGMA was applied as a clustering method. Similarity dendrograms were then displayed with Treeview software.
This technique has been used for genotyping of different species and different strains of bacteria. ERIC-PCR has also been used for *P. aeruginosa* isolates from patients with cystic fibrosis and keratitis in some countries (17,24). But as far as we are aware, ERIC-PCR has not previously been used to compare *P. aeruginosa* isolates from patients with contact lens–related keratitis in Iran. In this study the isolates were typed by ERIC-PCR to identify prevalent clones within the collection of strains isolated from patients with keratitis. This enabled 57 isolates to be separated into 53 unique ERIC types. Only 8 of isolates were clustered in four real clones and 25 isolates (43.8%) were clustered in 8 clones with 85% similarity cut off. On the whole a high diversity index was found among our isolates which was revealed by the high number of ERIC-PCR patterns detected. This result is not surprising, because the situation presented here did not represent an outbreak and one would expect unrelated patients to have unrelated strains. These data was consistent with Pinna et al. results that performed ERIC-PCR typing for keratitis isolates in Italy (17).

Although its ability to measure the rate of molecular evolution of the genome of *P. aeruginosa* depends on the choice of primers but different studies have shown that ERIC-PCR typing is reproducible (6,25). In our research this technique has allowed the fine discrimination at the strain level and showed that *P. aeruginosa* strains are genetically diverse. It also allowed the construction of a phylogenetic tree, showing the relative relatedness of the different strains.

Our results suggest that DNA typing tools such as ERIC-PCR may play an important role in routine epidemiological surveillance, outbreak surveillance and in the identification of the source of transmission of *P. aeruginosa* in keratitis patients.

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REFERENCES


