Evaluation of biofilm-specific antimicrobial resistance genes in \textit{Pseudomonas aeruginosa} isolates in Farabi Hospital

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\textbf{Abstract}

\textbf{Background.} Biofilm produced from \textit{Pseudomonas aeruginosa} is the cause of infection induced by contact lenses, trauma and post-surgery infection. The aim of this study was to evaluate biofilm formation and the presence of the genes \textit{ndvB} and \textit{tssC1} in ocular infection isolates of \textit{P. aeruginosa}.

\textbf{Methods.} A total of 92 \textit{P. aeruginosa} strains were collected from patients with ocular infection referred to Farabi Hospital between March 2014 and July 2015. Antibiotic susceptibility patterns were evaluated by the agar disc-diffusion method according to CLSI guidelines. PCR assays were used to detect \textit{ndvB} and \textit{tssC1}, genes associated with resistance in biofilm-producing \textit{P. aeruginosa} isolates. Biofilm formation ability was examined by crystal violet microtitre plate assay.

\textbf{Results.} During the period of study, 92 \textit{P. aeruginosa} were isolated from ocular infections including keratitis (n=84) and endophthalmitis (n=8). The highest resistance rates were seen against colistin (57.6 \%) and gentamicin (50 \%) and the lowest resistance rates were seen against imipenem (3.3 \%), aztreonam (4.3 \%), piperacillin-tazobactam (4.3 \%), ceftazidime (4.3 \%) and ciprofloxacin (5.4 \%). Biofilm production ability was found in 100 \% of the isolates. PCR assays showed that of the 92 \textit{P. aeruginosa} isolates, 96.7 and 90.2 \% harboured the genes \textit{ndvB} and \textit{tssC1}, respectively.

\textbf{Conclusions.} Our results showed a considerable ability of biofilm production, as well as the occurrence of biofilm-specific antimicrobial resistance genes (\textit{ndvB} and \textit{tssC1}), in \textit{P. aeruginosa} isolates from ocular infections in Farabi Hospital.

\section*{INTRODUCTION}

Bacterial cells as a member of biofilm are usually more resistant to antibiotics in comparison to planktonic cells [1–3]. An important factor contributing to the pathogenesis of \textit{Pseudomonas aeruginosa} in causing infections is its potential to form biofilms on biotic and abiotic surfaces [3]. The biofilms cause wound infections, urinary tract infection, ear and ocular infection [4, 5]. According to a publication by the National Institutes of Health, more than 80 \% of all bacterial infections are related to biofilms [6]. Bacteria growing in biofilms produce one or more extracellular polymeric matrices which keep the cells of the biofilm together. Polysaccharides are important components of the biofilm matrix, as they contribute to the overall biofilm architecture and to the resistance of biofilm-grown bacteria to certain antibacterial agents [2]. The genes \textit{ndvB} and \textit{tssC1} are among the genes that do not influence biofilm formation but are implicated in biofilm-specific antibiotic resistance [7]. The gene \textit{ndvB} is related to the synthesis of periplasmic glucans, which interact physically with aminoglycoside antibiotics [8]. Keratitis is a serious ocular infection and if left untreated or treated with unsuitable drugs, it can lead to corneal ulcers [9]. Several elements can induce keratitis, such as ocular trauma, superficial ocular problems and cornea surgery [10]. Using contact lenses is also another predisposing factor for the development of keratitis in healthy eyes [5, 11]. \textit{P. aeruginosa} is responsible for 60–70 \% of the contact-lens-related ocular infections [5, 12]. It is believed that the biofilm produced from \textit{P. aeruginosa} is the main cause of infection induced by contact lenses [13, 14]. Phenotypic features of biofilms are also responsible for drug resistance in contact lens infection [15, 16]. Unfortunately, a significant rise in multidrug-resistant (MDR) strains has made it difficult to choose the proper treatment for infectious diseases [17]. According to different studies, MDR \textit{P. aeruginosa} strains are those which are resistant to at least three classes of antibiotics. These classes include aminoglycosides, penicillin, carbapenems, cephalosporins and quinolones [17, 18]. Regular analysis of drug resistance can help physicians to choose the appropriate antibiotic. The aim of...
this study was to evaluate biofilm formation and the presence of the genes ndvB and tssC1 in ocular infection isolates of P. aeruginosa in Farabi Hospital.

METHODS

Bacterial isolates
A total of 92 P. aeruginosa strains were collected at Farabi Hospital in Tehran between March 2014 and July 2015. Of the 92 strains, 58 (63 %) were from females and 34 (37 %) were from males, and the age of the patients ranged from 1 to 91 years. Clinical strains were isolated from cornea, vitreous chamber, aqueous humour, nasolacrimal duct and conjunctiva.

Sample collection and processing
Samples were inoculated on blood agar, MacConkey agar and cetrimide agar, and pure colonies with appropriate morphologies were further tested by conventional biochemical tests including catalase, oxidase, motility, oxidative/fermentative (OF) and indole tests and growth at 42 °C, leading to identification as P. aeruginosa.

Antimicrobial susceptibility testing
Susceptibility testing of isolates was performed by disc-diffusion method according to CLSI guidelines [19], using antibiotic discs manufactured by the Mast. Antibiotic discs used included the following (µg per disc): ceftazidine (30), imipenem (10), cefepime (30), tobramycin (10), piperacillin-tazobactam (100), ciprofloxacin (5), gentamicin (10), amikacin (30), aztreonam (30) and colistin (10). P. aeruginosa ATCC 27853 was used as quality control in each antimicrobial susceptibility assay. The results were interpreted as susceptible, intermediate or resistant according to the criteria recommended by the CLSI and the manufacturer’s protocols.

Biofilm formation assays
The isolated P. aeruginosa strains were analysed for their ability to produce biofilm using the microtitre dish biofilm formation assay [20]. In this method, the P. aeruginosa isolates were grown overnight at 37 °C in tryptic soy broth (TSB) containing 0.25 % glucose. The cultures were diluted 1 : 100 in TSB medium. Sterile flat-bottomed 96-well polystyrene microtitre plates were inoculated with 125 µl bacterial suspension and incubated for 24 h at 37 °C without agitation. The wells were washed in triplicate with 300 µl distilled water, dried in an inverted position at room temperature and finally stained with 125 µl of 0.1 % crystal violet solution in water for about 10–15 min. After staining, the wells were washed three times with distilled water, and then destained with 125 µl of 30 % acetic acid in water. A new, sterile, flat-bottomed 96-well polystyrene microtitre plate was prepared with 125 µl destaining solution in each well. The absorbance of the destaining solution was measured at 570 nm using an ELISA reader (Stat Fax-2100). Each test was performed in triplicate. As control, un inoculated medium was used. Based on the optical density of the samples (ODi) and on the average of the optical density of the negative control (ODc), the samples were classified as strong (4×ODc<ODi), moderate (2×ODc<ODi ≤4×ODc), weak (ODc<ODi ≤2×ODc), or non-producer of biofilm (ODi<ODc).

The experiment was performed in triplicate and repeated three times; data were then averaged and standard deviation was calculated. To compensate for background absorbance, OD570 readings from sterile medium, fixative and dye were averaged and subtracted from all test values. The infection ocular mean OD570 value obtained from the medium control well was deducted from all the test OD570 values.

Molecular detection of the genes ndvB and tssC1
The genes ndvB and tssC1 were amplified by PCR using a specific set of primers (Table 1). Bacterial DNA for the PCR analysis was prepared using the boiling method [19]. Amplification of ndvB and tssC1 were performed in a 50 µl reaction mixture containing 18 µl distilled water, 1 µl forward primer, 1 µl reverse primer (Gen Fanavaran), 25 µl Master mix (Gen Fanavaran) and 5 µl bacterial DNA. The amplification was performed as follows: initial denaturation step at 95 °C for 2 min (one cycle), followed by 30 cycles consisting of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 90 s, and final extension at 72 °C for 10 min. The PCR products were visualized following electrophoresis on 2 % agarose gels and staining with ethidium bromide.

Ethical consideration
Approval of the study protocol was received from the Ethical Review Board of Kashan University of Medical Sciences (no. 5348).

Statistical analyses
Statistical Package for Social Sciences (SPSS) software (SPSS v. 19) was used for statistical analyses. Fischer exact test or χ² test was used for the analysis of the categorical data. P<0.05 was considered statistically significant.

RESULTS

In this study, 92 P. aeruginosa strains were isolated from patients who suffered from ocular infections and were

Table 1. Primers used for PCR assays for detection of ndvB and tssC1 in P. aeruginosa isolated from ocular infections in Farabi Hospital in Tehran, Iran

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>PCR product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ndvB</td>
<td>F</td>
<td>GGCCTGAACGATC-</td>
<td>157</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTCTTCACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GATCTTGCCGACC-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTAAGGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tssC1</td>
<td>F</td>
<td>CTCCAACGAGCGGA-</td>
<td>150</td>
<td>[35]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TGGGTGTGTTGAC-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAGGTA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
referred to Farabi Eye Hospital. The ocular specimens included keratitis \((n=84)\) and endophthalmitis \((n=7)\) (Table 2).

The antibiotic susceptibility patterns of the \textit{P. aeruginosa} isolates are shown in Table 3. A high rate of resistance was seen against gentamicin \((50\%)\) and colistin \((57.6\%)\), whereas a low rate of resistance was seen against imipenem \((3.3\%)\), aztreonam \((4.3\%)\), piperacillin-tazobactam \((4.3\%)\), ceftazidime \((4.3\%)\), cefepime \((4.3\%)\) and ciprofloxacin \((5.4%)\). Of all \textit{P. aeruginosa} isolates, 6/92 \((6.52\%)\) were identified as MDR, and were distributed in patients between 50 and 91 years.

PCR assays showed that of 92 \textit{P. aeruginosa} isolates, 96.7\% harboured the gene \textit{ndvB}. Also 90.2\% of the studied \textit{P. aeruginosa} isolates were found to carry \textit{tssC1} (Figs 1 and 2). Quantitative biofilm determination using the microtitre assay revealed that all 92 isolates \((100%)\) produced biofilm, of which 88 and 12\% showed weak and moderate biofilm production, respectively.

**DISCUSSION**

\textit{P. aeruginosa} has emerged as an important eye pathogen, responsible for serious ophthalmic infections such as keratitis [21, 22]. Pseudomonal infections have increased due to growing usage of medical and beauty contact lenses [23]. In the present study, sex-wise prevalence of isolates shows that infections caused by \textit{P. aeruginosa} are more common in females than males. The greater use of contact lenses and cosmetics in women could be the reason. Also, the age-wise prevalence of clinical isolates in our study shows that most of the patients were aged between 20 and 39 years, which is comparable with other studies [24, 25]. The higher rates of pseudomonal infections in patients aged 20–39 years may be due to higher use of cosmetics and contact lenses in this age group. Ciprofloxacin has been reported as effective empiric treatment for bacterial keratitis [26]. However, there are reports of ciprofloxacin-resistant \textit{P. aeruginosa} which is threatening for treatment of \textit{P. aeruginosa} ocular infections [27, 28]. The rate of MDR \textit{P. aeruginosa} in our isolates was 6.52\%, which is similar to other studies in Iran [4], but significantly lower than that reported in India [29].

None of our isolates showed resistance to fluoroquinolones. This suggests that fluoroquinolone resistance is still relatively rare among \textit{P. aeruginosa} isolates from ocular infections in Iran. Our results are in accordance with many of the previous studies from different countries which show very low rates of resistance to fluoroquinolones in non-nosocomially acquired \textit{P. aeruginosa} infections [4, 30, 31].

In \textit{P. aeruginosa} infections, biofilm production has been measured as an important determinant of pathogenicity [32]. All our studied \textit{P. aeruginosa} isolates had the ability of biofilm production. Furthermore, assessment of the association between biofilm formation and antibiotic resistance revealed that biofilm production was significantly higher among MDR \textit{P. aeruginosa} isolates in comparison to susceptible isolates. This probably could be due to the delayed penetration of antimicrobial agents inside the biofilm cells. Our results are consistent with reports from Abidi et al. [33].

Finally, according to PCR tests the genes \textit{ndvB} and \textit{tssC1} were found in 96.7 and 90.2\% of the \textit{P. aeruginosa} isolates, and there was no correlation between the distribution of

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**Table 2.** Isolation of \textit{P. aeruginosa} from different ocular specimens

<table>
<thead>
<tr>
<th>Ocular specimens</th>
<th>No. of \textit{P. aeruginosa} isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornea</td>
<td>77 (83.7)</td>
</tr>
<tr>
<td>Vitreous chamber</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td>Aqueous humour</td>
<td>6 (6.5)</td>
</tr>
<tr>
<td>Nasolacrimal duct</td>
<td>2 (2.2)</td>
</tr>
<tr>
<td>Conjunctiva</td>
<td>6 (6.5)</td>
</tr>
<tr>
<td>Total</td>
<td>92 (100)</td>
</tr>
</tbody>
</table>

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**Table 3.** Antibiotic susceptibility patterns of strains of \textit{P. aeruginosa} isolated from ocular infections in Farabi Hospital in Tehran, Iran

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Sensitive</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td>89 (96.7)</td>
<td>0 (0)</td>
<td>3 (3.3)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>41 (44.6)</td>
<td>5 (5.4)</td>
<td>46 (50)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>69 (75)</td>
<td>18 (19.6)</td>
<td>5 (5.4)</td>
</tr>
<tr>
<td>Cefepime</td>
<td>88 (95.7)</td>
<td>0 (0)</td>
<td>4 (4.3)</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>59 (64.1)</td>
<td>29 (31.5)</td>
<td>4 (4.3)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>88 (95.7)</td>
<td>0 (0)</td>
<td>4 (4.3)</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>55 (59.8)</td>
<td>25 (27.2)</td>
<td>12 (13)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>61 (66.3)</td>
<td>15 (16.3)</td>
<td>16 (17.4)</td>
</tr>
<tr>
<td>Piperacillin-tazobactam</td>
<td>85 (92.4)</td>
<td>3 (3.3)</td>
<td>4 (4.3)</td>
</tr>
<tr>
<td>Colistin</td>
<td>39 (42.4)</td>
<td>0 (0)</td>
<td>39 (57.6)</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Gel image of product of \textit{ndvB} PCR with a size of 157 bp. Lane 1, negative control; lane 2, 100 bp DNA ladder; lane 3, positive control; lanes 4–9, \textit{ndvB}-positive \textit{P. aeruginosa} isolates.
$ndvB$, $tssC1$ and antibiotic resistance. This difference could be due to low levels of gene expression.

In conclusion, our results showed a considerable ability of biofilm production, as well as the occurrence of biofilm-specific antimicrobial resistance genes ($ndvB$ and $tssC1$), in $P.\ aeruginosa$ isolates from ocular infections in Farabi Hospital.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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