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ORIGINAL PAPER

Genetic features of *Pseudomonas aeruginosa* isolates associated with eye infections referred to Farabi Hospital, Tehran, Iran

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Abstract

Background Pseudomonas is the most common cause of microbial keratitis especially in people who use contact lens. The virulence of *Pseudomonas aeruginosa* in different eye infections is associated with different virulence factors.

Methods In this study, 54 *P. aeruginosa* isolates including 39 isolates from keratitis and 15 isolates from conjunctivitis were evaluated for their ability to form biofilm, production of protease, elastase, alkaline protease and their antibiotic-resistant patterns. The distribution of the exoS and exoU genes in the test strains were determined using PCR assays.

Results Most of the eye infections (90.74%) were seen in people who used contact lenses, and in most of patients (72.22%), the infection was presented as keratitis. None of the isolates were resistant to a single antibiotic as tested. Multidrug resistance (MDR) was detected in two isolates (3.5%) which were resistant to more than one category of antibiotics. The $exoU^+$/$exoS^+$ isolates were in majority although in total, compared to exoS, there were more exoU in a greater number of samples. Most of the strains produce elastase but among all of ocular isolates, only 5.8% of the strains showed alkaline protease activity. Most of the ocular isolates were not capable of producing biofilm.

Conclusions In our study, a high prevalence of virulence factors was observed in *P. aeruginosa* isolates from contact lens wearer with keratitis. As the *P. aeruginosa* isolates from different infection origins and different geographic region may have different virulence factors, having a better perception of these differences could help to improve development of clinical instructions for the control of keratitis.

Keywords *Pseudomonas aeruginosa* · Eye infections · Virulence factors · Exotoxins · Contact lens

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Introduction

Pseudomonas aeruginosa keratitis is an aggressive infection, which can cause severe injury and damage to the cornea through breakdown of collagen [1, 2]. *Pseudomonas aeruginosa* keratitis is much more frequent in people who use contact lens than in people who do not use them [3–5]. Therefore, it is a major

reason of contact lens–associated bacterial keratitis in many countries including Iran [3, 6, 7]. On the other hand, previous studies have shown that *P. aeruginosa* is one of the most frequent bacteria isolated from non-contact lens-related injuries of the eye that results from keratitis [3, 8, 9]. The most important virulence factors of *P. aeruginosa* associated with ocular infections are the exoenzymes U and S, alkaline protease, elastase, and protease IV [3, 10]. The alkaline protease, elastase, and protease IV relate to wide tissue destruction, invasion, and distribution of bacteria. Hence, they could cause damage to the cornea. In addition, the elastase and alkaline protease of the bacterium could cause permeation through the corneal epithelium [1, 11, 12].

Pseudomonas aeruginosa strains can be classified as either cytotoxic or invasive [13]. These phenotypes are associated with the presence of exoS or exoU respectively [14]. The exoS plays an important role in attacking non-phagocytic cells, regulating bacterial phagocytosis by phagocytes, and inducing apoptosis of fibroblasts, lymphocytes, and epithelial cells. The exoU causes quick lysis of a series of mammalian cell types including fibroblasts, macrophages, and epithelial cells in vitro [15]. The occupation of exoU gene is one of several virulence factors, which exist in the clinical isolates of *P. aeruginosa* as obtained from patients with bacterial keratitis [16]. Previous studies have shown that infections simultaneously containing both cytotoxic and invasive strains, cause severe keratitis. However, pathologically, these strains respond differently; cytotoxic strains mainly cause corneal edema, while invasive strains are mainly related with corneal ulceration. The strains that are neither cytotoxic nor invasive produce minimal keratitis [17].

Pseudomonas aeruginosa produces a biofilm that is a mucoid layer. This layer provides a physical barrier to clearance and can help the organism evade the host defense mechanisms. It is thought that the biofilm of *P. aeruginosa* is a major cause of continuous ocular infections in affected contact lens wearers since the bacteria attach to the contact lens and the surface of contact lens storage case. The biofilms cause increased resistance to antimicrobial therapy of contact lens-related keratitis [3]. Since having a better awareness of virulence factors as related to various infections could help to improve the control of infections, this study was designed to evaluate the phenotype (antibiotic-resistant pattern, protease profile, and biofilm formation) and genotype (exoS and exoU) of *P. aeruginosa* isolates as implicated for eye infection cases in Iran.

Material and methods

Target population

A set of 60 *P. aeruginosa* clinical isolates were collected between September 2015 and February 2016 from the patients attending Farabi Hospital in Tehran, but six isolates were excluded due to lack of response to some experiments. The remaining 54 isolates included 39 isolates from keratitis cases and 15 isolates from conjunctivitis cases. From the keratitis samples, 34 isolates were obtained from contact lens user cases and five isolates were obtained from non-contact lens user cases; all the conjunctiva samples were from contact lens user cases. Therefore, overall 49 from 54 isolates (90/74%) were isolated from contact lens wearers. Most of the patients were female (i.e., 36 from 54, or 66.0%) and in the age range of 20–29 years. None of patients used antibiotics before referring to the hospital.

Based on the colony morphology, Gram's stain, positive oxidase test, growth at 41 °C, and production of pyocyanin, all the isolates were confirmed as *P. aeruginosa*. The isolates were preserved in tryptic soy broth (TSB) containing 30% glycerol at − 70 °C. The reference strain (ATCC 27853) was used as a control.

Antibiotic susceptibility tests

Antibiotics including b-lactams (ceftazidime, cefazolin and imipenem), aminoglycosides (gentamicin and amikacin), fluoroquinolones (ofloxacin, ciprofloxacin and norfloxacin), chloramphenicol, vancomycin and trimethoprim–sulfamethoxazole were used in the antibiotic susceptibility tests. The antibiotic susceptibility pattern of the bacterial strains was analyzed using the disk diffusion method based on Clinical and Laboratory Standards Institute (CLSI) guidelines [18].

Analysis of type III secretion toxins-encoding genes

The presence of exoU and exoS genes in the test strains was determined using PCR assay. The

chromosomal DNA was extracted as reported previously [19]. The exoU (134 bp) and exoS (118 bp) genes were amplified using primers and a PCR condition reported previously [3].

Gelatin zymography

The gelatin zymography for the protease activity was performed by a modification of the method described by Zhu et al. [15]. In this method, the 25 µl aliquot of each 50-fold concentrated culture supernatant was separated using SDS polyacrylamide gel 7.5% (w/v) which was co-polymerized with gelatin 0.1% (w/v) with 120 Vat 4 °C. The gels were washed for 1 h in 100 ml Triton X-100 (2.5%) and then were incubated in the gelatin gel substrate buffer for 20 h at 37 °C. The gels were stained in the Coomassie Brilliant Blue R-250 (0.25%) in acetic acid–methanol-dH_2O (1:4:5) for 30 min and then destained in 100 ml acetic acid, methanol, distilled water (1:4:5) for 1 h (2).

Biofilm assay

The biofilm assay was performed using the method described by O'Toole and Kolter [20]. Briefly, *P. aeruginosa* strains were cultured overnight in a LB medium and then diluted 1:20 in a fresh medium. Further, 200 ml of this medium was loaded to each well of a flat bottom 96-well polyvinyl chloride microtitre plate. After incubating for 24 h at 37 °C, the wells were washed using phosphate-buffered saline and then placed in an inverted position to dry. Later, the wells were stained with crystal violet 1% for 15 min and were rinsed again. The crystal violet color was solubilized in 200 mL of ethanol–acetone (80:20), and by using an ELISA reader, the optical density was determined at 570 nm (OD570). The following values were considered for determining of the biofilm production: OD570 $\leq$ 1 (no biofilm production), 1 $<$ OD570 $\leq$ 2 (weak biofilm production), 2 $<$ OD570 $\leq$ 3 (medium biofilm production) and OD570 $>$ 3 (strong biofilm production) [3].

The age range of the patients was from 18 to 58 years, while the mean age was 27 years. The male to female ratio was 21:22.

Results

Antibiotic resistance

All the strains were resistant to vancomycin, cefazolin, chloramphenicol, and trimethoprim–sulfamethoxazole. Moreover, all the isolates were susceptible to aminoglycosides and fluoroquinolones, except for two strains, that showed resistance to gentamycin. Therefore, among the aminoglycosides, amikacin was more efficient than gentamicin. None of the isolates were resistant to ciprofloxacin, ofloxacin and norfloxacin (Table 1). All the isolates showed resistance to more than one of the antibiotics that were assayed. MDR was found in only two isolates (3.5%) which were resistant to more than one category of the antibiotics including aminoglycoside (gentamicin) and β-lactam cefazolin.

TTSS genotyping

The PCR assay was used to analyze the distribution of the TTSS effector genes in the test strains. According to the exoS and exoU genes amplification, the strains were split into three groups exoS$^+$/exoU$^-$, exoS$^-$/exoU$^+$ and exoS$^+$/exoU$^+$. Of the total 39 keratitis isolates, 51.28% (20/39) possessed the exoS$^+$/exoU$^+$ genotype, and 41.02% (16/39) were exoS$^-$/exoU$^+$, whereas 7.69% (3/39) was exoS$^+$/exoU$^-$. In the conjunctivitis isolates, 80% (12/15) were exoS$^+$/exoU$^+$ and 20% (3/15) possessed the exoS$^-$/exoU$^+$

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

Antibiotic	No. clinical isolates (%)	
	Resistant	Sensitive
Vancomycin	57 (100%)	0
Cefazolin	57 (100%)	0
Chloramphenicol	57 (100%)	0
Trimethoprim–sulfamethoxazole	57 (100%)	0
Gentamycin	2 (3.5%)	55 (96.5%)
Amikacin	0 (0%)	57 (100%)
Imipenem	0 (0%)	57 (100%)
Ciprofloxacin	0 (0%)	57 (100%)
Ofloxacin	0 (0%)	57 (100%)
Norfloxacin	0 (0%)	57 (100%)

genotype. In both contact lens-related and non-contact lens-related cases, three genotypes were seen (Table 2).

Exoproteases production by strains

The gelatin zymography of the culture supernatants revealed that all the *P. aeruginosa* isolates were able to produce protease IV. As shown previously, under none reducing conditions, protease IV aggregates under none reducing conditions into a high molecular mass complex (> 200 kDa) that appears near the top of the separating gel. However, contrary to protease IV production, the alkaline protease and elastase production were variable among the *P. aeruginosa* isolates. Most of the isolates (93.3% of conjunctivitis isolates, 94.1% contact lens-related keratitis, and all the non-contact lens-related keratitis) produced elastase. Among all the ocular isolates, only 5.8% of contact lens-related keratitis strains showed alkaline protease activity by zymography (Table 2). The molecular weight of alkaline protease and elastase are 48.4 and 33 kDa, respectively, but the visible sizes of alkaline protease and elastase in the polyacrylamide gel are associated with the concentration of gelatin and polyacrylamide in the gel. In this study, the 150 and 50 kDa bands showed elastase activity and alkaline protease activity, respectively (Fig. 1).

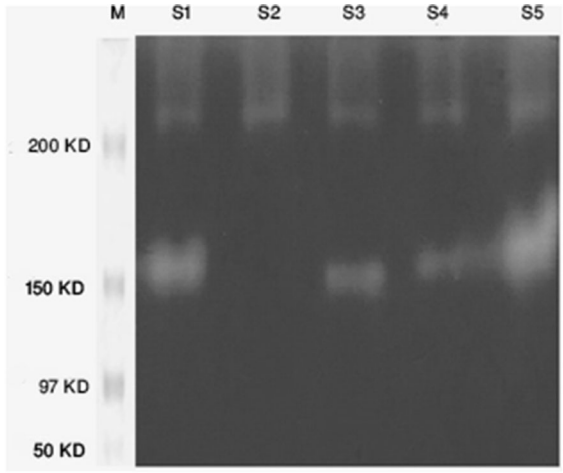

Fig. 1 Protease profiles produced by *P. aeruginosa* strains. Bands > 200 kDa: protease IV, 150 kDa: elastase B, 50 kDa: alkaline protease

Biofilm assay

Table 2 exhibits the number of *P. aeruginosa* isolates that have ability to form biofilms. In this regard, 37% of total ocular isolates were not capable of producing biofilm and among the remaining cases 17% (11% CLK, 4% NCLK, and 2% CON) showed strong biofilm formation, 17% (7.5% CLK, 2% NCLK, 7.5% CON) of isolates formed moderate biofilms and weak biofilm formation was detected in 29% (22% CLK, 2% NCLK, and 5% CON) of isolates. The ATCC 27853 showed medium biofilm production.

Table 2 Genotype and phenotype of the test strains

Source of strains[a]	Genotype			Enzyme			Biofilm density			
	$exoS^+$	$exoS^+$/ $exoU^+$	$exoU^+$	Protease IV	Alk.p	Elastase	Strong	Moderate	Weak	No biofilm
CL-Keratitis (34)	2	17	15	34	2	32	6	4	12	12
NCL-Keratitis (5)	1	3	1	5	0	5	2	1	1	1
CL-Conjunctivitis (15)	0	12	3	15	0	14	1	4	3	7
Total (54)	3	32	19	54	2	51	9	9	16	20
ATCC27853	+	±	−	+	+	+	−	+	−	−

Pseudomonas aeruginosa ATCC 27853 were used as control

CLK Contact lens-related Keratitis, *NCLK* non-contact lens-related Keratitis, *CL-CON* Contact lens-related conjunctivitis

[a]Number of isolates are in parentheses

Discussion

Pseudomonas aeruginosa is one of the most common bacteria isolated from infections caused by the use of contact lens. In contact lens users contact lens owing to the risk of contamination of the lens, lens care solution and lens case, the bacteria can reach the eyes and cause keratitis, while non-CL-keratitis is usually developed by trauma, severe ocular surface disease, and Dacryocystitis [19, 21, 22].

Pseudomonas aeruginosa is a rare agent of conjunctivitis and usually cause this infection in infants, elderly people, or immunocompromised hosts [21–23]. In our study, most of the eye infections (90.74%) were seen in people who used contact lenses and in most of the patients (72.22%), the infection was presented as keratitis, which is in accordance with other studies as well [21–23]. In recent years, the usage of contact lens has increased remarkably in Iran, and mostly in youngsters [24]. Previously, it was shown that using contact lens could select for certain virulence factors in *P. aeruginosa* and contact lens-related keratitis showed various clinical manifestations [11].

Recent studies have indicated that the presence of the exoU and exoS genes is related to the cytotoxic and invasive phenotypes. On the other hand, a meaningful relationship is seen between the *P. aeruginosa* phenotype and the patient age, so that the cytotoxic strains is mostly observed in patients aged less than 50 years and the invasive strains is mostly observed in patients over 50 years [5, 17, 25]. It is proposed that decline in immune function with aging reduces the host's skill to respond to infections and as a result, the prevalence of the invasive strains increases in older individuals (6). In this research, the mean age is 27 years, which is within the age range where use of contact lens is most common. Previous studies have shown that in the non-ocular clinical isolates of *P. aeruginosa* as well as the non-contact lens-related keratitis, exoS is reported more frequently than exoU; however, both the exoS and exoU toxins could share in the formation of the corneal infection [26–28]. In the present work, we found the $exoU^+/exoS^+$ isolates to be in the majority, however, in total; compared to the exoS, there were more exoU in a greater number of samples. Consequently, these results are consistent with the findings of previous researches, which showed a greater representation of strains containing the exoU gene in contact lens-related infections [21, 29, 30].

The predominance of the exoU isolates in contact lens-related keratitis indicates that cytotoxicity is a more important virulence factor in these infections than other *P. aeruginosa* infections [31]. It can be suggested that because of its ability to induce apoptosis of macrophages and epithelial cells, exoU is an important factor in the pathogenesis of experimental keratitis [3, 21]. Furthermore, it is shown that patients infected with exoU expressing strains have a worse outcome in terms of visual acuity after treatment than patients infected with the exoS-expressing strains [32].

Considering the increasing prevalence of antibiotic-resistant isolates of *Pseudomonas,* the antibiotics to treat ocular *Pseudomonas* infections must be chosen more carefully [33]. Previous researches have shown a relationship between the exoU expression and resistance of *P. aeruginosa* to CL disinfectants and the multiple antimicrobials [7, 11, 21, 30]. Thus, the exoU-positive *P. aeruginosa* strains could survive the CL disinfectants and cause keratitis.

Pinna et al. indicate that it is a relation between MDR and the exoU expression in the ocular isolates of *P. aeruginosa* such that the isolates containing $exoU^+$ have shown greater fluoroquinolone resistance [19]. In this study, all the strains are resistant to vancomycin, cefazolin, chloramphenicol, and trimethoprim–sulfamethoxazole. All the isolates are susceptible to aminoglycosides and fluoroquinolones, except for two strains (3.84%) which showed resistance to gentamycin, and both strains harbor exoU. It could be concluded that since isolates containing exoU are more virulent and cause greater disease signs, they tend to be exposed to large amount of antimicrobial agents, which could lead to the emergence of resistance [19]. The present study in accordance with previous studies [19, 34] indicates that the exoU isolates are simultaneously competitively superior in terms of antibiotic-resistant and cytotoxicity.

The bacterial biofilm formation is important for the initial adhesion and colonization of *P. aeruginosa* in the host [25, 35]. The *Pseudomonas* biofilm plays a role in a variety of ocular infections and may be related to the development of contact lens-associated keratitis by facilitating the persistence of bacteria on contact lens surfaces [28, 31, 35]. Previous investigations have shown different results regarding the prevalence of

biofilm formation among *pseudomonas* isolates from the eye infections [28, 35]. Priya et al. show that 47% of endophthalmitis isolates have the ability to form a strong biofilm [25]. Choy et al. report a relationship between the exoU$^+$ genotype and strong biofilm formation and concludes that the contact lens and their storage cases could cause the selection of ocular isolates, which contain exoU$^+$, and isolates with the potential of strong biofilm formation [3]. However, Pinna et al. displays that the *P. aeruginosa* isolates from contact lens-associated infections have restricted power of biofilm formation [19]. In our research, most of the ocular isolates were not capable of producing biofilm and the remaining cases were either weak, moderate, or strong biofilm formers. These results are somewhat in agreement with other studies, which indicate that there is no uniform biofilm formation pattern for *P. aeruginosa* isolates in keratitis.

Prior studies on keratitis have demonstrated that the *P. aeruginosa* enzymes including alkaline protease, elastase, proteases, and staphylolysin have a role in the pathogenesis of this disease [15, 36]. Fukuda et al. propose that in infectious keratitis, not only live bacteria but the components of *P. aeruginosa* too, such as enzymes, could contribute to subsequent corneal damage [37]. In the present work, all the *P. aeruginosa* isolates were able to produce protease IV; however, alkaline protease and elastase production were variable among the *P. aeruginosa* isolates. Therefore, most of the isolates (93.3% of conjunctivitis isolates, 94.1% contact lens-related keratitis isolates, and all the non-contact lens-related keratitis isolates) produced elastase but among all of the ocular isolates only 5.8% of contact lens-related keratitis isolates showed alkaline protease activity. Our results are in accordance with other studies that suggest that protease IV has an important role in the life of ocular *P. aeruginosa* isolates suggesting that in certain conditions, such as using soft contact lens while sustaining a corneal injury, the isolates containing protease activity could cause keratitis [36]. Pillar et al. [38] show that the loss of alkaline protease and elastase activity is not related to a substantial decrease in corneal virulence which is in agreement with our results.

In conclusion, in our study, a high prevalence of virulence factors was observed in *P. aeruginosa* isolates from contact lens wearer with keratitis. As the *P. aeruginosa* isolates from different infection origins and different geographic region may have different characteristics, the identification of different bacterial genotypes and its relation with clinical outcomes could greatly help both the microbiologists and clinicians in determining prognosis and better treatment modifications [39].

Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest and have no proprietary interest in any of the materials mentioned in this article.

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