Molecular characterization of the pilS2 gene and its association with the frequency of Pseudomonas aeruginosa plasmid pKLC102 and PAPI-1 pathogenicity island

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Introduction: Pseudomonas aeruginosa is the most common opportunistic pathogen associated with a broad range of infections, including cystic fibrosis, ocular, otitis media, and burn infections. The aim of this study was to show the frequency of the pilS2 gene, and its association with P. aeruginosa plasmid pKLC102 and PAPI-1 pathogenicity island among P. aeruginosa strains.

Methods: The samples were collected from patients with cystic fibrosis, ocular, otitis media, and burn infections between January 2016 and November 2017. DNA was extracted using the DNA extraction kit and was used for PCR assay. PCR with 4 primer-pairs including 976 F/PAPI-1R, 4542 F/intF, SojR/4541 F, and intF/sojR was performed to identify PAPI-1. pKLC102 was detected using three other primer-pairs including cp10F/cp10R, cp44F/cp44R, and cp97F/cp97R.

Results: A total of 112 P. aeruginosa isolates were collected from patients with cystic fibrosis (36), burn (20), otitis media (26), and ocular (30) infections. The results of PCR showed that pilS2 gene was identified in 96 (85%) strains. PAPI-1–attB integration was detected among 38 (33.9%) isolates and the circular form of PAPI-1 detected among 17 (14%) isolates. In addition, 79 (70.5%) strains were found to be positive for pKLC102.

Conclusion: We found that the majority of the isolates may be susceptible to transfer this significant island and the related element pKLC102 into recipient isolates lacking the island owing to high association of the PilS2 pilus with the islands in the studied strains. It is anticipated that strains isolated from burn and eye with the highest rate of PilS2, PAPI-1, and pKLC102 association have a high level of antibiotic resistance.

Keywords: Pseudomonas aeruginosa, pilS2, cystic fibrosis, pKLC102, PAPI-1

Introduction

Pseudomonas aeruginosa (P. aeruginosa) is the most common bacterial pathogen associated with persistent bronchial infections among cystic fibrosis patients. This pathogen is capable of causing several infections including nosocomial pneumonia, nosocomial urinary tract infections, acute diffuse otitis media, conjunctivitis, and keratitis. The pathogenesis of P. aeruginosa is due to the production of extracellular virulence factors (such as pili, hemolysins, pyocyanin, proteases, alginate, lipopolysaccharide, and the Type III secretion system) and effector proteins (such as ExoU, ExoT, ExoY, and ExoS).

P. aeruginosa isolates have shown the ability of adherence to different receptors and production of biofilm by Type IV pili. Type IV pili have two major subfamilies including Type IVa (T4aP) and IVb (T4bP). T4bP are a more heterogeneous group,
whereas T4aP are a somewhat homogeneous and Tad pili (a monophyletic class of T4bP) were seen in a broad variety of environmental species.8

Although T4aP- and Tad-encoding genes are found in all the P. aeruginosa isolates, the gene encoding major subunit of the Type IVb pili (pilS2 gene) is seen in the strains that have related elements such as P. aeruginosa plasmid pKLC102 or PAPI-1 pathogenicity island.8,9 Carter et al showed that PAPI-1 can be transferred to other P. aeruginosa strains following excision from the chromosome of the donor. Also, they demonstrated that PAPI-1 is transferred into recipient P. aeruginosa by a conjugal mechanism, via a Type IV pilus, encoded in PAPI-1 by a ten-gene cluster (8).

There are two trnALys genes located in the P. aeruginosa genome, known as PA0976.1 and PA4541.1. pKLC102 is a 103,532 bp integrative and conjugative element that integrates into one of these positions.10 However, PAPI-1 can integrate into the attB sites of the both PA0976.1 and PA4541.1 positions.11 In addition to this integrated form of PAPI-1, the island can also exist in some populations of bacteria as an extrachromosomal circular form.12

Interestingly, the 10 PAPI-1-encoded pilus proteins are well conserved in several P. aeruginosa strains that carry this island, including PA2192, PA7, C3710, PACS2, and PSE9 (PAGI-5), and in P. aeruginosa clone C strain that carry a pKLC102-like element.

The PAPI-1 pilS2 gene encodes for a major pilin subunit, which is a 176-amino-acid protein containing a conserved PilS superfamily domain. Horizontal gene transfer is known as a main evolutionary mechanism and contributes to the virulence properties of many bacterial pathogens (V).

There is no specific study on the prevalence of the pilS2 gene and its association with P. aeruginosa plasmid pKLC102 and PAPI-1 pathogenicity island among P. aeruginosa in Iran. This study could be the basis for further research in the future.

So, the aim of this study was to evaluate the prevalence of the pilS2 gene, and its association with P. aeruginosa plasmid pKLC102 and PAPI-1 pathogenicity island among P. aeruginosa strains isolated from patients with cystic fibrosis, ocular, otitis media, and burn infections.

Methods
Study design, data, and specimens collection
A prospective cross-sectional study was conducted between January 2016 and November 2017. The research was approved by the ethical committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran with ethics number: IR.SBMU.RETECH.REC.1395.460. As a part of the Shahid Beheshti University of Medical Sciences policy, written informed consent was obtained from all patients. The study was conducted in accordance with the Declaration of Helsinki. The clinical samples were collected from inpatients with otitis media, burn wound infections, keratitis, and cystic fibrosis who were hospitalized in Shahid Motahari Hospital (level I burn care center). The sputum samples of the patients with cystic fibrosis were examined macroscopically for the presence of salivary contamination. Following this, briefly, sputum was incubated with an equal volume of sputolyisin for 15 minutes at room temperature and plated onto chocolate agar. The deep oropharyngeal swab samples were collected and cultured on blood agar and MacConkey agar media (Merck, Germany).13

For bacterial isolation from the burn patients, surface culture swabs were collected from them and were inoculated onto blood agar, MacConkey agar, and tryptic soy broth media (Merck, Germany).14 The ocular specimens from the patients with conjunctivitis and keratitis were collected by an ophthalmic surgeon and ophthalmologist. These specimens were transferred into 2 mL of brain-heart infusion broth (Oxoid, Hampshire, UK).15

The outer ear of the patients with otitis media was cleaned with normal saline and the pus discharges were collected with sterile cotton swab sticks. Pus swabs were added to stuart transport medium (Merck, Germany) and cultured on blood agar, chocolate agar, and MacConkey agar media (Merck, Germany).16

The isolated colonies from the positive cultures were investigated for biochemical characteristics of P. aeruginosa. Briefly, the biochemical tests include gram staining, pigment production, colony morphology, growth at 42°C on Mueller–Hinton agar, the ability of oxidase and catalase production, and oxidative-fermentative test.1

Antimicrobial susceptibility testing
Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method according to Clinical & Laboratory Standards Institute17 recommendations. The susceptibilities of all isolates to different antibiotics were determined using cefepime (30 µg), amikacin (30 µg), ceftazidime (30 µg), tobramycin (10 µg), ciprofloxacin (5 µg), meropenem (10 µg), cefoperazone (75 µg), piperacillin (100 µg), imipenem (10 µg), gentamicin (10 µg), ceftazidime (30 µg), piperacillin-tazobactam (110/10 µg), colistin (10 µg), cefoperazone-sulbactam (100/10 µg), and cotrimoxazole (10 µg).
DNA extraction and PCR
The clinical samples were cultured on chocolate agar medium and the isolated single colonies of *P. aeruginosa* were incubated in 5 mL Luria–Bertani medium at 37°C overnight. Then, DNA templates were prepared using the DNA extraction kit (Genet Bio Company, Korea, Cat. No, K-3000) as previously described.18

PCR assay with four primer-pairs including 976F/PAPI-1R, 4542F/intF, SojR/4541F, and intF/sojR was performed to identify the *P. aeruginosa* PAPI-1 pathogenicity island.

Likewise, *P. aeruginosa* plasmid pKLC102 was detected using three other primer-pairs including cp10F/cp10R, cp44F/cp44R, and cp97F/cp97R by PCR method. Primer sequence used in the genetic characterization of *P. aeruginosa* clinical strains and for detecting the pilS2 gene is shown in Table 1. Amplified products were analyzed by electrophoresis on 1% agarose gel containing safe stain, and finally in the case of DNA replication and seeing the sharp band, it is considered as positive.

Data analysis
The data were analyzed using the SPSS statistical package version 22 (IBM SPSS Statistics, USA) and Microsoft excel 2016 (Microsoft Corporation, USA) statistical software. The results are presented as descriptive statistics in terms of relative frequency.

**Results**
A total of 112 *P. aeruginosa* isolates were collected from different sources, of which 36 were isolated from patients with cystic fibrosis, 20 from burn wound infections, 26 from otitis media, and 30 from ocular infections.

Antibiotic susceptibility tests using the Kirby–Bauer method revealed that the highest resistance percentage was related to cefepime, gentamicin, and ciprofloxacin 99 (88%), followed by meropenem and imipenem 98 (87%). The results showed that colistin and cefoperazone-sulbactam were the best antibiotics against *P. aeruginosa* isolates. Antibiotic resistance patterns are detailed in Table 2.

**Table 1** Primer sequence used in the genetic characterization of *Pseudomonas aeruginosa* clinical strains

| Gene/genomic islands | Forward primer sequence (5′–3′) | Reverse primer sequence (5′–3′) | Size of PCR product (bp) | Tm (°C) |
|----------------------|---------------------------------|---------------------------------|--------------------------|---------|
| pilS2                | GCGTTTCTCGTTCCATCGAG            | GGTCACCTTCGCGTGATCG            | 428                      | 60      |
| pKLC102              | GGGTCCGCAAAAATTCTCGCC           | GCTGATTGGGCCAACATCG           | 272                      | 61      |
| cp10                 | GGGATCTCATGTACCTCCCCGCGC       | CTTTTATCCCCGGCAGTGCGG         | 337                      | 61      |
| cp44                 | GGGTCCGCAAAAATTCTCGCC           | GCTGATTGGGCCAACATCG           | 272                      | 61      |
| cp97                 | GGGTCCGCAAAAATTCTCGCC           | GCTGATTGGGCCAACATCG           | 272                      | 61      |
| PaPI-1               | GCCCTGACGGGTGTCCTGGATTT         | GCTGACGGGTGTCCTGGATTT         | 2,600                    | 58      |
| 976F/PAPI-1R         | GTGTTGATGACCTCCAACCT           | AGCTACATCGGGCAGCTTGGA         | 1,600                    | 58      |
| 4542F/intF           | CGCACAGGAAAGGGGTCTGGGA         | GCAAGACATCGGCGACACTA          | 1,600                    | 58      |
| SojR/4541F           | AGCTACATCGGGCAGCTTGGA         | GCAAGACATCGGCGACACTA          | 1,600                    | 58      |

**Table 2** Antibiotic resistance patterns in *Pseudomonas aeruginosa* isolates

| Antibiotic                  | Cystic fibrosis N (%) | Burn wound infections N (%) | Otitis media N (%) | Ocular infections N (%) | Total N (%) |
|-----------------------------|-----------------------|----------------------------|--------------------|-------------------------|-------------|
| Cotrimoxazole               | 27 (75)               | 17 (85)                   | 22 (84)            | 22 (73)                 | 88 (79)     |
| Cefoperazone-sulbactam      | 17 (47)               | 9 (45)                    | 12 (46)            | 14 (46)                 | 52 (46)     |
| Amikacin                    | 24 (66)               | 16 (80)                   | 19 (73)            | 25 (83)                 | 84 (75)     |
| Piperacillin–tazobactam     | 28 (77)               | 16 (80)                   | 20 (76)            | 23 (76)                 | 87 (77)     |
| Piperacillin                | 32 (88)               | 14 (70)                   | 21 (80)            | 24 (80)                 | 91 (79)     |
| Cefepime                    | 31 (86)               | 17 (85)                   | 22 (84)            | 29 (96)                 | 99 (88)     |
| Tobramycin                  | 23 (63)               | 10 (50)                   | 11 (42)            | 17 (56)                 | 61 (54)     |
| Meropenem                   | 33 (91)               | 17 (85)                   | 22 (84)            | 26 (86)                 | 98 (87)     |
| Imipenem                    | 33 (91)               | 17 (85)                   | 22 (84)            | 26 (86)                 | 98 (87)     |
| Gentamicin                  | 34 (94)               | 16 (80)                   | 23 (88)            | 26 (86)                 | 99 (88)     |
| Ceftazidime                 | 22 (61)               | 14 (70)                   | 22 (84)            | 24 (80)                 | 82 (73)     |
| Colistin                    | 33 (91)               | 18 (90)                   | 21 (80)            | 27 (90)                 | 99 (88)     |
| Ciprofloxacin               | 30 (83)               | 16 (80)                   | 20 (76)            | 27 (90)                 | 93 (83)     |
| Aztreonam                   | 30 (83)               | 16 (80)                   | 20 (76)            | 27 (90)                 | 93 (83)     |
Frequency of the pilS2 gene

The results showed that the pilS2 gene was identified in 85% (n=96) of the P. aeruginosa strains. This major subunit of Type IVb pili was detected among 24% (n=23) of the P. aeruginosa strains isolated from cystic fibrosis patients, 21% (n=20) isolated from burn patients, 31% (n=30) isolated from patients with ocular infections, and 24% (n=23) isolated from patients with otitis media. Agarose gel electrophoresis of PCR products after amplification of the pilS2 gene is shown in Figure 1.

Frequency of the P. aeruginosa PAPI-1 pathogenicity island

Overall, the integration of the PAPI-1 to the attB site of positions PA0976.1 and PA4541.1 was identified among 33.9% (n=38) of P. aeruginosa isolates. Approximately 18.5% (n=7) of the strains were isolated from cystic fibrosis patients, 39.5% (n=15) isolated from burn patients, 21% (n=8) isolated from patients with ocular infections, and 21% (n=8) isolated from patients with otitis media.

In addition, the PCR assay using primer-pair intF/sojR has detected the circular form of PAPI-1 among 14% (n=17) of P. aeruginosa strains isolated from hospitalized patients (including six cystic fibrosis patients, six burn patients, and five patients with ocular infections).

Frequency of the P. aeruginosa plasmid pKLC102

Overall, 70.5% (n=79) of the P. aeruginosa strains were found to be positive for pKLC102. The highest frequency of this element was detected among patients with ocular (34%) and burn (25%) infections, followed by cystic fibrosis (22%) and otitis media (19%). The results of PCR method for identification of the pKLC102 demonstrated that CP10, CP44, and CP97 genes were present in 49%, 51%, and 17% of the P. aeruginosa strains, respectively (Table 2). Agarose gel electrophoresis of PCR products after amplification of the CP44 and CP10 genes is shown in Figures 2 and 3.

The association of the pilS2 gene with pKLC102

The pilS2 gene was simultaneously associated with pKLC102 in 75 (70%) isolates, of which 27 (36%), 19 (25.4%), 15 (20%), and 14 (18.6%) were detected from ocular, burn, otitis media, and cystic fibrosis infections, respectively (Table 3).

The association of the pilS2 gene with PAPI-1 and pKLC102

In this study, 36 (32.1%) P. aeruginosa strains encoded all pKLC102, chromosomal PAPI1, plasmid PAPI1, and pilS2 genes. Moreover, the pKLC102+, chromosomal PAPI1+, plas-

Table 3 Frequency of the specific genes CP10, CP44, and CP97 of pKLC102

| Genotype                  | CF | Eye | Ear | Burn | Total (%) |
|---------------------------|----|-----|-----|------|-----------|
| cp10− cp44− cp97−         | 18 | 3   | 11  | 1    | 33 (29.4) |
| cp10− cp44− cp97−         | 6  | 9   | 2   | 4    | 21 (18.7) |
| cp10− cp44− cp97−         | 9  | 0   | 0   | 2    | 11 (9.8)  |
| cp10− cp44− cp97−         | 0  | 0   | 0   | 0    | 0         |
| cp10− cp44− cp97−         | 3  | 16  | 0   | 9    | 28 (25)   |
| cp10− cp44− cp97−         | 0  | 0   | 0   | 1    | 1 (0.9)   |
| cp10− cp44− cp97−         | 0  | 1   | 12  | 0    | 13 (11.6) |
| cp10− cp44− cp97−         | 0  | 1   | 1   | 3    | 5 (4.4)   |

Abbreviation: CF, Cystic fibrosis.
mid PAPI1+, pilS2+ genotype was the most frequent among the isolates (Table 4).

The association of the pKLC102 with PAPI-1

The results showed that all the *P. aeruginosa* strains that are pKLC102 positive encoded also the PAPI-1 element. Although among 37 (33%) strains, pKLC102 was associated with both the chromosomal and plasmid PAPI-1, in 41 (36.6%) strains, pKLC102 was present along with only the plasmid form of the PAPI-1 (Table 5).

Discussion

*P. aeruginosa* is an opportunistic human pathogen capable of causing a broad range of human life-threatening acute and chronic infections.19 The bacterium is capable of inhabiting a wide range of environmental niches, in part, owing to have a diverse genomic repertoire that encodes multiple virulence factors such as Type IVb pilus, which is encoded by the island PAPI-1.6 Also, this pathogen can adhere to different receptors and produce biofilm by its extracellular virulence factor, Type IV pili.20

The current study showed the frequency of the gene encoding major subunit of the Type IVb pili, the pilS2 gene, and its association with *P. aeruginosa* plasmid pKLC102 and PAPI-1 pathogenicity island among *P. aeruginosa* strains isolated from patients with cystic fibrosis, keratitis, otitis media, and burn infection. The results demonstrated that 85% of the *P. aeruginosa* strains encoded the pilS2 gene. Indeed, 21% (n=20) and 31% (n=30) of the *P. aeruginosa* strains isolated from patients with burn and ocular infections were pilS2-positive. Furthermore, patients with otitis media (88.4) and cystic fibrosis (63.8) also had a high prevalence of the pilS2 gene.

These findings are comparable with the study performed by Dabiri et al,14 in Iran. They evaluate the Type IV pilin subgroups in *P. aeruginosa* strains and showed that pilS2 gene was found among 67.5% of cystic fibrosis and 73.3% of burn

| Genotype                          | Cystic fibrosis N (%) | Eye N (%) | Ear N (%) | Burn N (%) | Total N (%) |
|-----------------------------------|-----------------------|-----------|-----------|------------|-------------|
| pKLC102+ chromosomal PAPI1+ plasmid PAPI1+ pilS2+ | 6 (16.6)               | 0         | 3 (11.5)  | 0          | 9 (8)       |
| pKLC102+ chromosomal PAPI1+ plasmid PAPI1+ pilS2- | 0                      | 0         | 0          | 0          | 0           |
| pKLC102- chromosomal PAPI1+ plasmid PAPI1+ pilS2+ | 3 (8.3)                | 0         | 4 (15.3)  | 0          | 7 (6.9)     |
| pKLC102- chromosomal PAPI1+ plasmid PAPI1+ pilS2- | 0                      | 0         | 0          | 0          | 0           |
| pKLC102+ chromosomal PAPI1+ plasmid PAPI1+ pilS2+ | 3 (8.3)                | 0         | 0          | 0          | 3 (2.6)     |
| pKLC102+ chromosomal PAPI1+ plasmid PAPI1+ pilS2- | 0                      | 0         | 0          | 0          | 0           |
| pKLC102- chromosomal PAPI1+ plasmid PAPI1+ pilS2+ | 0                      | 0         | 1 (5)      | 0          | 1 (0.8)     |
| pKLC102- chromosomal PAPI1+ plasmid PAPI1+ pilS2- | 0                      | 0         | 0          | 0          | 0           |
| pKLC102+ chromosomal PAPI1+ plasmid PAPI1+ pilS2+ | 11 (30.5)              | 17 (56.6) | 6 (23)    | 5 (25)    | 42 (38.8)   |
| pKLC102+ chromosomal PAPI1+ plasmid PAPI1+ pilS2- | 3 (8.3)                | 10 (33.3) | 9 (34.6)  | 14 (70)   | 36 (32)     |

Table 5 The association of pKLC102 with chromosomal PAPI-1 and plasmid PAPI-1

| Genotype                          | Cystic fibrosis N (%) | Eye N (%) | Ear N (%) | Burn N (%) | Total N (%) |
|-----------------------------------|-----------------------|-----------|-----------|------------|-------------|
| pKLC102+ chromosomal PAPI1+ plasmid PAPI1+ pilS2+ | 9 (25)                | 3 (10)    | 7 (26.9)  | 0          | 19 (16.9)   |
| pKLC102+ chromosomal PAPI1+ plasmid PAPI1+ pilS2- | 0                      | 0         | 0          | 0          | 0           |
| pKLC102- chromosomal PAPI1+ plasmid PAPI1+ pilS2+ | 9 (25)                | 0         | 4 (15.3)  | 1 (5)      | 14 (12.5)   |
| pKLC102+ chromosomal PAPI1+ plasmid PAPI1+ pilS2- | 0                      | 0         | 0          | 0          | 0           |
| pKLC102+ chromosomal PAPI1+ plasmid PAPI1+ pilS2+ | 14 (38.8)             | 17 (56.6) | 6 (23)    | 5 (25)    | 42 (37.5)   |
| pKLC102- chromosomal PAPI1+ plasmid PAPI1+ pilS2+ | 4 (11.1)              | 10 (33.3) | 9 (34.6)  | 14 (70)   | 37 (33)     |
patients. In this manner, regarding the importance of Type IV pili in virulence and pathogenesis of *P. aeruginosa*, the results highlight the need for a main program for identification of this pilin and urgently interventions to prevent the development of *P. aeruginosa* strains isolated from hospitalized patients.

In a study by Sadeghifard et al in 2012, the existence of PAPI-1 in clinical *P. aeruginosa* isolates was evaluated; in that study, 35% of isolates were positive for this genomic island, so that most of the PAPI-1-positive isolates showed high levels of antibiotic resistance. In another study done by Morales-Espinosa et al in Mexico, the genetic and phenotypic features of 100 *P. aeruginosa* isolates were studied. They have detected PAPI-1 among 81% of the isolates. This island was reported to be integrated into the chromosome at locus PA0976.1 and PA4541.1 among 65% and 16% of the strains, respectively. However, the circular form of PAPI-1 was not found in any of the strains.

In addition, in Morales-Espinosa et al’s work, 87% of isolates carried pKLC102, so that CP10, CP44, and CP97 were positive in 87, 88, and 70 strains, respectively, and more than half of the isolates carried all three genes. Also, Klockgether et al found 50 isolates of *P. aeruginosa* with pKLC102-like islands in their study.

Similarly, we reported that the prevalence of integrated form of PAPI-1 was 33.9%. This rate was the highest among patients with burn infection (39.5%), followed by ocular (21%), otitis media (21%), and cystic fibrosis (18.5%) infections. Interestingly, all isolates positive for pKLC102 in the current study were also shown to carry PAPI-1 (in 70.5% of the isolates) and this association was more frequently detected among eye and burn isolates (Table 4).

Furthermore, as our results revealed, the association of pilS2 gene with PAPI-1 and pKLC102 was found predominantly in *P. aeruginosa* isolates from burn (pKLC102+, chromosomal PAPI-I*, plasmid PAPI-I*, and pilS2+ genotype) and eye infection (pKLC102*, chromosomal PAPI, plasmid PAPI-I, and pilS2+ genotype) (Table 4); taken together, in 70.8% of the isolates, the association was established and in 9.8% of the isolates pilS2 gene was only associated with PAPI-1 (chromosomal PAPI-I).

**Conclusion**

In this study, we found that the majority of the isolates may be susceptible to transfer this significant island and the related element pKLC102 into recipient isolates lacking the island owing to high association of the PilS2 pilus with the islands in the studied strains, which may affect the pathogenesis of these isolates.

Moreover, based on this data, it is anticipated that strains isolated from burn and eye with the highest rate of PilS2, PAPI-1, and pKLC102 association have a high level of antibiotic resistance and a higher potential of biofilm formation. Regarding this, more investigations are needed to evaluate the frequency of PilS2, PAPI-1, and pKLC102 and its association with antibiotic-resistance properties and biofilm synthesis in *P. aeruginosa* isolates from Iran.

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**Disclosure**

The authors report no conflicts of interest in this work.

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