Expression Levels of Four pil Genes Encoding Type 4 Fimbrial Biogenesis Proteins in Pseudomonas aeruginosa Strains Prevalent in Nosocomial Infections at a Tertiary Care Center

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ABSTRACT

Background

The polar type IV pili of *Pseudomonas aeruginosa* consisting of fimbrial biogenesis proteins are virulence factors that play a critical role in mediating bacterial adherence and colonization of mucosal surfaces. In this study, we compared the expression levels of a set of pil genes encoding type IV fimbrial biogenesis proteins in selected nosocomial isolates of *P. aeruginosa* representing genotypes with various prevalence rates recovered at a tertiary-care center in Lebanon.

Findings

PCR-based detection of *pilG, pilH, pilI*, *pilK* present as a single gene cluster, was performed on 90 consecutive isolates of *P. aeruginosa* previously genotyped by RAPD analysis. Real-Time RT-PCR with SybrGreenI detection of the *pil* genes was performed on 10 selected isolates representing the various genotypes and expression ratios were measured in comparison to the housekeeping gene *rpoD* using in-house developed assays. All pil genes were detected in all 90 isolates by PCR. Among 10 selected isolates of the various genotypes, *pil K, H, I* and *G* were over-expressed relative to *rpoD* and that these *pil* proteins were encoded on a single polycistronic mRNA

Conclusion

This study demonstrated the over-expression of the *pil K, H, I* and *G* relative to *rpoD* by determining their transcription levels using quantitative real-time RT-PCR and we found that pil genes are encoded by a single polycistronic mRNA. In addition, relative gene expression showed the absence of significant relationship between the prevalence of a particular genotype and expression levels of these genes

BACKGROUND

Despite the advances in hospital care and the introduction of a wide variety of antimicrobial agents, *Pseudomonas aeruginosa* continues to be a major nosocomial pathogen particularly in patients who suffer from immunosuppression [1]. *P.aeruginosa* is a ubiquitous pathogen prevalent in the hospital environments, and can cause severe nosocomial infections[2]. The latter involve a broad spectrum of infections including the respiratory, gastrointestinal, and urinary tracts as well as wound infections, sepsis and others[3]. Various possible sources of *P. aeruginosa* infection in hospitals have been identified; such as tap water, medical equipment, and hospital personnel and other patients[2, 4]. In the Sentry study, accounts for 10% of all hospital acquired infections[5]. During the past three years, the average prevalence of *P. aeruginosa* nosocomial infections in our medical center was increasing reaching the highest last year with 18 % prevalence rate. Such a high rate prompted us to study the *P. aeruginosa* genotypes circulating in the various units to reveal the clonal relationship between clinical and environmental isolates and to allow elucidating the source and mode of transmission of this important bacterium at this medical center.

Our data demonstrated the predominant prevalence of a potentially virulent *P. aeruginosa* genotype, circulating in the most majority of the medical center units and emphasize the need to reinforce infection control measures[6, 7]. One possible mechanism that enhances colonization and spread of particular genotypes is the presence of adhesins on the surface of these bacterial cells. Adhesins are surface proteins that enhance attachment and motility of the bacterial cells on surfaces. Major filamentous structured adhesins, Type IV pili are retractile and mediate a mode of surface translocation termed twitching motility. The *P. aeruginosa* twitching motility proteins or the type 4 fimbrial biogenesis proteins such *PilI, PilG*, and *PilH* and the methyl transferase *PilK* are encoded by a set of *Pil* gene cluster[8]. They are composed primarily of a single small protein subunit, usually termed *PilA* or pilin, which is arranged in helical conformation with 5 subunits per turn and which may be glycosylated and/or phosphorylated in different species. These proteins demonstrate remarkable similarity to the chemotaxis proteins of enterics and the gliding bacterium Myxococcus xanthus and are thought to be part of a signal transduction system that controls *P. aeruginosa* pilus biosynthesis that has an adhesive nature and twitching motility[9]. Apart from cell movement, functional type IV *pili* are also required for a wide variety of other processes, including transformation, conjugation and bacteriophage infection[10]. Detection of these genes and determination of their level of expression in the most prevalent genotype, in comparison to other less prevalent ones, will shed light on their possible role in enhancing the spread of the prevalent genotype in the medical center.

To that purpose the present study aimed at 1) Detection of the *Pil* gene cluster encoding the above stated adhesins in the most prevalent genotype 1 by Polymerase Chain Reaction (PCR) in comparison to those encountered in other genotypes that are less prevalent in the medical center and 2) Determination of the expression levels of the *pil* genes in both categories by quantitative real-time RT-PCR.

MATERIAL AND METHODS

Source of isolates

In the previous study[7], consecutive (90) isolates were recovered from different patients specimens (one per patient) submitted for bacteriological investigations at the Clinical

Table 1: *Expression levels of the 4 pil genes and the in-house designed bracket gene as compared to the housekeeping rpoD gen*

	Pil K/rpoD	Pil H/rpoD	Pil I/rpoD	Pil G/ rpoD	Pil G-H-1/ rpoD	RAPD Genotype
Pseudo 20	1.45	2.23	1.99	1.65	0.22	26
Pseudo 23	1.46	2.7	3.59	0.56	0.25	3
Pseudo 36	2.39	3.91	5.52	1.86	0.19	2
Pseudo 40	2.32	14.99	7.73	5	0.59	3
Pseudo 43	2.69	12.3	7.38	3.38	0.54	1
Pseudo 47	2.14	5.72	7.13	3.61	0.61	1
Pseudo 69	0.38	1.16	0.64	0.5	0.27	1
Pseudo 75	4.28	3.51	5.44	0.24	0.33	7
Pseudo 81	1.54	10.97	7.58	0.87	0.18	1
Pseudo 96	0.21	3.09	1.15	0.42	0.28	1

Microbiology Laboratory between September 2003 and May 2004. Isolates from patients who have acquired a nosocomial infection due to as determined by clinical and laboratory testing[6], and indicated in their medical records, were only considered in this study.

DNA extraction

DNA was extracted from *P. aeruginosa* ATCC strain and from all isolates of by the GFXTM Genomic Blood DNA Purification Kit (Amersham PharmaciaBiotech,Uppsala, Sweden) according to the manufacturers' specifications.

PCR amplification of 4 Pil genes

PCR amplification of fragments on the *Pil* genes were done separately using specific primers designed in our laboratory with amplicon sizes as follows: *PilI*: 519 bp; *PilG*: 288 bp; *PilK*: 422 bp; *PilH*: 204 bp. PCR using standardized conditions was done for all reactions using the PTC-100TM Programmable Thermal Controller (MJ Research, Inc., Watertown, Mass., USA)[7]. Amplicons were detected on agarose gels on UV transilluminator (UVP, Uppland, CA) and photographed by Olympus 3.0 camera (Japan) and the Doc-it program (UVP, Upland, CA).

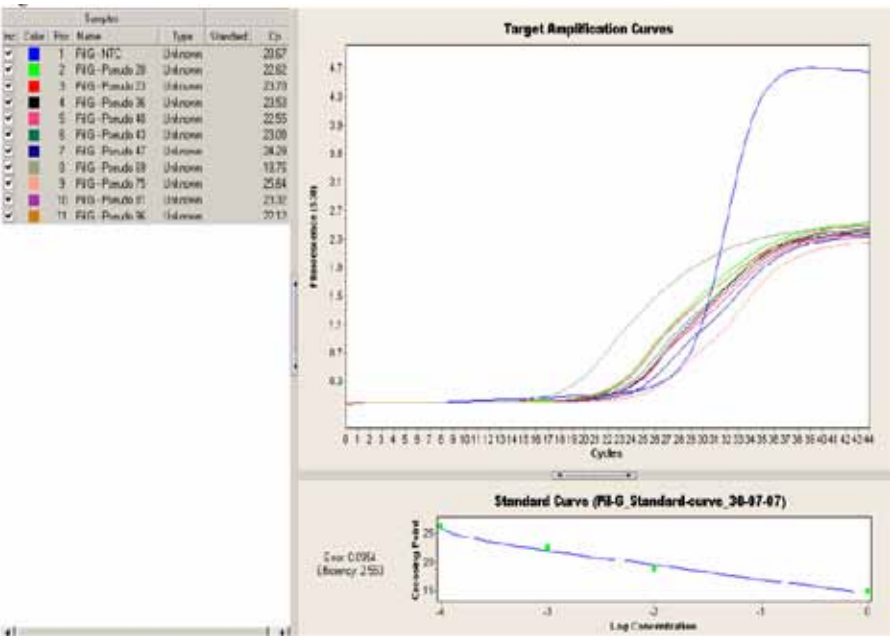
RNA Extraction

RNA was extracted from 10 selected isolates representing the various genotypes, using the RNeasy MiniKit (QIAGEN, Hilden, Germany) according to the manufacturers' specifications. The Ready-To-Go Kit (Amersham Biosciences) for c-DNA synthesis was used according to the manufacturers' instructions.

Real Time RT-PCR

Transcription levels of the *Pil* genes were performed by real–time RT- PCR. The primers were re-designed, using ClustalX1 and LightCycler Probe Design Software (Roche Applied Science, Germany), to suite the SYBR Green protocols for the LightCycler 2.0 (Roche Diagnostics GmbH, Mannheim, Germany). Since these pil genes are located on the same gene cluster, we also designed primers (*PilG-H 1* and *PilG-H 2*) targeting the 2 neighboring bracket genes. Real-time PCR of the c-DNAs was performed using primers yielding amplicon sizes as follows: *pilI*: 209 bp; *pilG*: 208 bp; *pilK*: 218 bp; *pilH*: 204 bp; *pilG-H-1*: 201 bp; *pilG-H-2*: 190 bp; *rpoD*: 200 bp. Relative expression ratios of the *pil* genes were measured in comparison to the housekeeping gene RNA polymerase D subunit (rpoD) using an in-house developed assays on 10 selected isolates. The cycling parameters followed standard protocols. Amplification curves, melting curves, and relative quantification were generated using the LightCycler soft-

Figure 1: *Relative quantification by Real time RT-PCR of the pilG gene for P. aeruginosa isolates*



ware version 4.05. The software was used to calculate the relative quantification ratio of target versus reference gene on extracted RNA, according to Morrison et. al[11].

RESULTS

Antimicrobial Susceptibility and PCR amplification of *Pil* genes.

Most of the clinical isolates were susceptible to the tested antimicrobial agents[7]. PCR amplification of genomic DNA extracted from all the 90 isolates showed the presence of all tested pil genes. Previously performed RAPD analysis determined the presence of 31 genotypes, of which genotype 1 was the most prevalent encompassing 42% of the isolates[7].

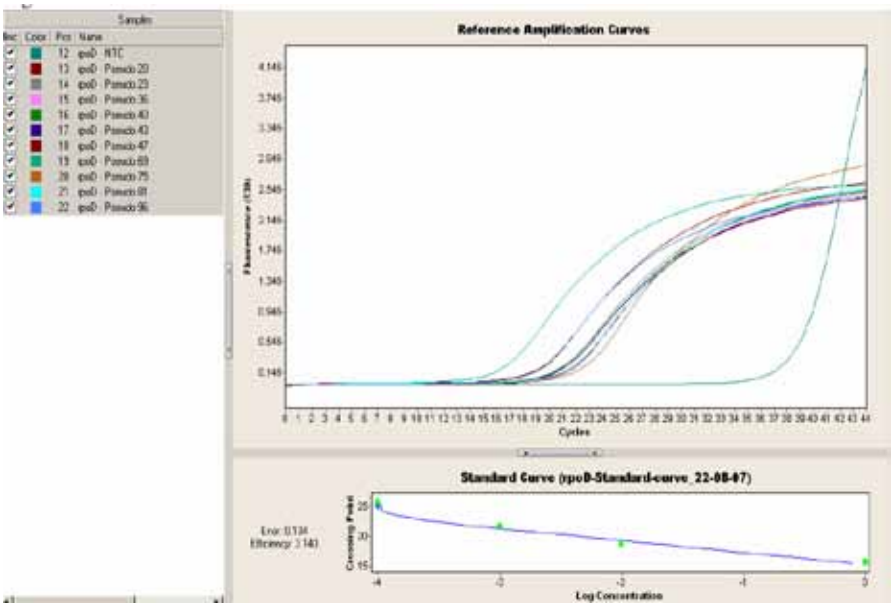
Gene Expression by Real Time RT-PCR

Relative quantification by real-time RT-PCR was performed to determine the expression levels of the 4 *pil* genes and the in-house designed bracket gene compared to the housekeeping rpoD gene. Among the 10 selected clinical isolates representing the various genotypes, the polar type 4 *pili* encoding genes *pilK, H, I* and *G* were significantly over-expressed relative to housekeeping *rpoD* gene (Table 1). The bracket gene *pil G-H-1*, neighboring *pil G* and *pil H*, was also detected in all 10 selected clinical isolates by real-time RT-PCR. There were no significant differences in expression levels among the tested genotypes.

DISCUSSION

Previously, we demonstrated the high prevalence of a *P. aeruginosa* genotype (genotype 1), circulating in the majority of the medical center units[7]. PCR amplification of genomic DNA extracted from 90 clinical isolates showed that all harbored the 4 studied *pil* genes. This is in concordance with many previously conducted studies, such carried out by Darzin's et. al, who succeeded in the detection of *pilG, -H, -I, -J* and *-K* gene cluster, which resides on the SpeI-H fragment (approx. 20 min on the PAO1 genetic map). This cluster encodes proteins that have significant sequence similarity to chemotaxis proteins. The sequence similarities between the *pilG, -H, -I, -J* and *-K* gene products and several chemotactic proteins impart strong evidence that they are part of a signal transduction network that controls *P.*

Figure 2: *Relative quantification by Real time RT-PCR of the rpoD gene for P. aeruginosa isolates*



aeruginosa pilus production and twitching motility[8]. Also, Mattick et. al reviewed the involvement of twitching motility in various aspects of bacterial biology, based around a core process of assembly and retraction of type IV *pili. PilA* is also essential for optimal protein secretion in *P. aeruginosa* and may interact with the pseudopilin proteins involved[9].

Relative expression by real-time RT-PCR of the *pil* genes is novel. The level of expression of polar type IV *pili* encoding genes in *P. aeruginosa* has not been previously tested by this approach. New primers were designed to determine the expression levels of the detected 4 *pil* genes and the in-house designed bracket gene in comparison to the *rpoD* gene that was selected as a housekeeping gene. Selection of *rpoD* is in concordance with Savli et. al who showed in a study conducted in 2003 that *rpoD*, along with proC, form the most stable pair in a set of clonally unrelated *P. aeruginosa* strains with various resistance phenotypes. Thus, this pair may be used as internal controls in relative comparison studies of resistance genes[12].

Our data demonstrated that the polar type IV *pili* encoding genes *pilK, H, I* and *G* were significantly over-expressed relative to housekeeping *rpoD* gene among the 10 randomly selected *P. aeruginosa* isolates. The data confirm the presence of these adhesins on the surface of the bacterial strains; however, there were no significant differences in their expression levels among the tested genotypes. This observation denotes that though type IV fimbrial proteins encoded by the *pil* genes are important in the adhesive process of *P. aeruginosa* to surfaces, there is no significant relationship between the prevalence of a genotype in the medical center and expression levels of these pil genes. The prevalence of a particular genotype in the medical center is due to additional factor(s) facilitating its spread. This requires further investigations. The bracket gene *pil G-H-1*, neighboring *pil G* and *pil H*, was also detected in all 10 selected clinical isolates by real-time RT-PCR. This result shows that pil proteins detected in our *P. aeruginosa* strains were encoded by a single polycistronic m-RNA, denoting the presence of a single promoter region for the expression of the cluster genes.

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