

Occurrence of *Pseudomonas aeruginosa* in Kuwait environment

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Abstract

Deliberate and accidental releases of opportunistic pathogens such as *Pseudomonas aeruginosa* into the environment increase the likelihood of spreading infectious diseases. Also, *P. aeruginosa* is responsible for nosocomial infections and usually harbour several antibiotic resistance genes. In the present study total of 76 strains of *P. aeruginosa* were characterized (54 strains were isolated from soil samples collected from a crude oil contaminated site located north of Kuwait and 22 strains were isolated from medical samples). Whereas the phenotypic characterization such as the biochemical tests using VITEK 2 system (bioMérieux) and antibiotics susceptibility testing (AST) showed the high similarity between the medical and the environmental strains, the molecular analyses based on multilocus sequence typing (MLST) and restriction fragment length polymorphism (RFLP) demonstrated the heterogeneity of isolated *P. aeruginosa* indicating the presence of different clusters. Comparison of fingerprints obtained, confirmed the negative correlation between the environmental and the medical strains. In addition, results demonstrated that environmental strains are better adapted to crude oil contaminated sites. However, environmental strains may represent hidden reservoirs for pathogenic traits that may disseminate to other bacteria in the environment.

Keywords: Pseudomonas aeruginosa; pathogenicity; MLST; RFLP; phenotype; antibiotic.

1 Introduction

The versatile and ubiquitous bacterium *Pseudomonas aeruginosa* is one of the most important opportunistic pathogens that can affect a wide variety of



organisms, such as insects, nematodes, plants, birds, and mammals including humans (Pukatzki et al., [1]; Jander et al., [2]). *Pseudomonas aeruginosa* can cause infections in immuno-compromised patients suffering from burns, or with underlying diseases such as cystic fibrosis, cancer or AIDS (Doring, [3]). Nevertheless, *P. aeruginosa* also affects healthy individuals and is an etiological agent to consider in the differential diagnosis of rapidly progressing diseases such as community-acquired pneumonia (McCallum, [4]). Furthermore, *P. aeruginosa* has low antibiotic susceptibility attributed to the presence of antibiotic resistance. Acquired resistance to antibiotics can also be developed by either mutation or the horizontal gene transfer of antibiotic resistance determinants (Livermore, [5]).

Environmental strains of *P. aeruginosa* can degrade environmental pollutants and can be bioaugmented in contaminated soils as a strategy in bioremediation programs to treat crude oil contaminated sites. The presence of *P. aeruginosa* in large numbers in the environment may represent a putative risk to wild life (Garnica and Vives-Flórez, [6]) and to people who might unsuspectingly be in direct contact with the environment.

In 1991 after the Gulf War over 300 oil lakes formed in the Kuwait desert. The bulk volume of the oil-contaminated soils has been estimated as 18 million cubic meter (Readman et al., [7]) that created selection pressure for crude oil-degrading bacteria in contaminated soils resulting in high occurrence of crude oil-degraders such as *P. aeruginosa*. Consequently, the high spread of such opportunistic bacteria in the environment of Kuwait increases the probability of spreading infectious diseases.

In this study, differences between the environmental and medical *P. aeruginosa* strains were determined using biochemical tests and antibiotic susceptibility testing (AST). Furthermore, genotyping of isolated bacteria using multilocus sequence typing (MLST) and restriction fragment length polymorphism (RFLP) were used to distinguish between different strains of *P. aeruginosa*.

2 Materials and methods

2.1 Isolation of *P. aeruginosa*

Some *P. aeruginosa* isolates were collected from microbiology laboratories in hospitals in Kuwait. Environmental *P. aeruginosa* strains were isolated from crude oil contaminated soil samples by standard plate dilution technique. Soil samples (1g) were mixed with of Hutners media (50 ml), shaken for 1 hour and the resulting suspension was allowed to settle for 30 min. The supernatant was serially diluted and spread (0.1 ml) on Hutners minimal agar plates, incubated in presence of crude oil at 25°C for 14–21 days. Grown colonies were re-streaked on nutrient agar plates (NA). Pure cultures grown on NA plates were used for biochemical tests, antibiotic susceptibility testing (AST) and DNA extraction. The biochemical tests and AST were conducted using the VitechII (bioMérieux) following the manufacturer's instructions.



2.2 Identification of bacteria using 16S rDNA sequencing

Genomic DNA was purified from pure bacterial cultures using the Wizard Genomic DNA purification kit as recommended by the manufacturer's (Promega). Then, the 16S rRNA gene was amplified using polymerase chain reaction (PCR). Amplification was performed using puReTaq Ready to Go PCR beads (Amersham Biosciences) according to manufacturer's recommendations, 200 pmol of mixture of the two primers used 27F (5'-CUACUACUACUAAAGGAGGTGTCCARCC-3') and 1492R (5'-GAATTCTACGGYTACCTTGTACGACTT-3') was added. The reactions were amplified using (GeneAmp PCR system 9700, Applied Biosystem). The PCR program used was a touchdown protocol that started with an initial denaturation step at 94°C for 5 min, followed by a denaturation step at 94°C 45s, annealing step at 56°C for 45s, and an extension step at 72°C for 1 min. Annealing temperature was decreased by 2°C each 6 cycles for a total of 30 cycles. A final extension step was carried at 72°C for 7 min. Products were analyzed by gel electrophoresis where 5µl of each samples was run on 0.5% (w/v) agarose gel along with 1kb DNA ladder.

The PCR products obtained were purified using a Nucleospin Extract II kit (Macherey–Nagel, USA). The purified products were amplified using big dye terminator cycle sequencing kit according to manufacturer's instructions. The mixtures were incubated in the thermocycler at 96°C for 1 minute followed by 25 cycles of denaturation at 96°C for 1 minute, annealing at 50°C for 5s and extension at 60°C for 4 minutes. The final products were further purified, using 3µl of 3M sodium acetate pH4.6, 62.5 µl of nondenatured 95% ethanol and 14.5 µl of deionized water. Samples were incubated in the dark at room temperature for 20 minutes before being centrifuged at 15,000xg in room temperature for 20 minutes. The pellet formed was washed with 250 µl of 70% ethanol and re-centrifuged at 15,000xg in room temperature for 5 minutes. The pellets were air-dried for 10–15 minutes, re-suspended in 20 µl of Hi-Di-Formamide and loaded directly in the 3130xl genetic analyzer (Applied Biosystems). Sequence analysis and GeneBank database searches were performed by using the BLAST program (Altschul *et al.*, [8]).

2.3 Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) was performed as proposed by (Curran *et al.*, [9]). Seven internal fragments of the genes *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE* were amplified, and the resulting PCR products were purified with the Nucleospin[®] Extract II kit (Macherey–Nagel, USA). PCR Sequencing was carried out with sequencing primers by the 3130xl genetic analyzer (Applied Biosystems, USA). The database at <http://pubmlst.org> was used to assign numbers to particular alleles and to identify sequence types (STs).



2.4 RFLP analysis of 16S rDNA

16S rDNA sequences of cultured isolates were amplified directly from glycerol stock using M13 vector-specific primers. Each 50- μ l reaction mixture contained 30 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, each deoxynucleoside triphosphate will be at a concentration of 50 μ M, with 50 pmol of each primer, and 1.9 U of *Taq* polymerase (AmpliTaq; Applied Biosystems). Following PCR amplification of 16S sequences, 10 μ l of PCR products were digested separately with 2.5 U of *Rsa*I or 2.5 U of *Bst*UI (New England Biolabs) in 12- μ l reaction mixtures as recommended by the manufacturer. *Rsa*I and *Bst*UI digests were electrophoresed in 2% agarose gels, with TBE buffer. The gels were stained with ethidium bromide and photographed under UV light using InGenius gel documentation system (Syngene, Inc.). DNA fragment sizes were determined using GeneTools analysis software (Syngene, Inc.). The quality of the numerical data was checked by comparing the sum of fragment sizes in each restriction pattern with the original product size (approximately 1,499 bp). Cluster analysis of band patterns was carried out using GelComparII software (Applied Maths). Each phylotype was defined as a group of sequences that have indistinguishable *Rsa*I and *Bst*UI restriction patterns.

2.5 Mineralization of crude oil by bacteria

The amounts of carbon dioxide evolved were measured using a Micro-oxymax respirometer (Columbus instruments, USA). The reaction bottles contain: 49 ml sterile Hunter's minimal medium, crude oil (200 μ l) and 1ml of overnight bacterial suspension (OD = 1 at λ 600nm). The reaction bottles were incubated shaking at 30°C. The amount of carbon dioxide evolved was plotted against time and the rate of carbon dioxide evolution was calculated and used as indication for crude oil mineralization.

3 Results and discussion

The biochemical characterization of isolated bacteria showed the high similarity between the environmental and the medical strains. In addition, the AST data revealed the high similarity in the reaction of bacteria to antibiotics used. However, the medical strains showed wider range of resistance to three antibiotics (Table 1). While all environmental strains were sensitive to cefepime, 65% of medical strains were resistant to this antibiotic. Furthermore, 85% of medical strains were resistant to ceftazidime compared to 6% of that of environmental strains. Also, 50% of the medical strains were resistant to piperacillin/Tazobactam in contrast to 4% resistance demonstrated by the environmental strains. The resistance to wider range of antibiotics demonstrated by the medical strains is expected and can be attributed to the potential of bacteria to acquire resistance to antibiotics especially under frequent encounter with antibiotics in hospitals (Okeke et al., [10]).



Table 1: Antibiotic susceptibility test of isolated *P. aeruginosa*.

Antibiotic	AST
Amikasin, Piperacillin, Tobramycin, Ciprofloxacin, Gentamicin	S
Ampicillin, Amoxicillin\ clavulanic acid, Cefalotin, Cefotaxime, Cefoxitin, Cefpodoxime, Nitrofurantoin, Trimethoprim\Sulfamethoxazole	R
Cefepime	65% of medical strains are R, all environmental strains are S
Ceftazidime	85% of medical strains are R, 6% of environmental strains are R
Piperacillin\Tazobactam	50% of medical strains are R, 4% of environmental strains are R

AST= antibiotic susceptibility testing; S= sensitive; R=resistant.

Whereas the phenotypic characterization using biochemical tests and AST demonstrated the high similarity between the medical and the environmental strains, the molecular analyses based on MLST and RFLP revealed the heterogeneity of isolated *P. aeruginosa* as demonstrated by the presence of different sequence types and clusters. MLST fingerprints showed that the medical and the environmental strains were assigned to different sequence types and thus probably belong to different phylotypes (Table 2). The environmental strains were grouped into two phylotypes (with ST of 408 and 219) and the medical strains were grouped into twelve phylotypes. This indicated the distinct evolutionary origins of the two groups of tested bacteria and the higher heterogeneity of the medical strains compared to the environmental strains. Other studies have demonstrated the potential of MLST approach to determined genetic relatedness among different clones of the same bacterium (Curran et al., [9]). However, 16S-RFLP fingerprints showed that isolated bacteria were grouped into five distinctive phylotypes (Table 3). Except for group four which comprised of environmental strains, the majority (88–77%) of other groups (group 1, 2, 3 and 5) was formed by either environmental (group 2 and 5) or medical (group 1 and 3) strains. Thus, MLST and RFLP methods suggest that the environmental and the medical strains were not closely related. This may suggest that infections caused by *P. aeruginosa* were probably due to bacterial strains acquired by patients subsequent to hospital admission but not from the environment as confirmed from patient's history that indicated the infections



Table 2: Multilocus sequence typing of isolated *P. aeruginosa*.

<i>P. aeruginosa</i> strain	Source of strains	STs
SK27, SK 31, SK 39, SK 44, SK 52, SK 60, SK 61, SK 90,	Crude oil contaminated soil	408
SK 4, SK 8, SK17, SK 63, SK 64, SK 66, SK 68, SK 69		219
Ps 1	Medical samples from hospitals	250
Ps 4		320
Ps 5		506
Ps 6		106
Ps 7		270
Ps 9		387
Ps17		357
Ps 18		447
Ps 11, Ps 2		313
Ps 22, Ps 15		316
Ps 20		511
Ps 24		325

Table 3: Restriction fragment length polymorphism analysis of isolated *P. aeruginosa*.

Phylotype	Environmental strains	Medical strains
1	(1) SK2	(5) Ps2, Ps3, Ps8, Ps9, Ps12
	(8) SK1, SK24, SK27, SK29, SK68, SK70, SK71, SK75	(1) Ps14
3	(3) SK72, SK89, SK90	(15) Ps15, Ps16, Ps17, Ps20, Ps21, Ps1, Ps11, Ps10, Ps2, Ps4, Ps5, Ps6, Ps7
	(35) SK39, SK47, SK44, SK41, SK52, SK50, SK53, SK58, SK60, SK48, SK61, SK22, SK64, SK35, SK38, SK6, SK34, SK21, SK88, SK91, SK69, SK76, SK78, SK66, SK82, SK84, SK86, SK67, SK87, SK79, SK63, SK73, SK65, SK31, SK77	0
5	(7) SK19, SK17, SK20, SK8, SK11, SK12, SK4	(1) Ps19

Numbers between brackets indicate the number of strains included in a particular group.



were nosocomial. Nonetheless, more bacteria must be tested with several techniques to reach such conclusion.

The crude oil mineralization studies demonstrated that all the environmental strains were able to utilize crude oil as sole carbon and energy source. Other studies indicated that, pathogenic bacteria such as *P. aeruginosa* able to utilize crude oil can be isolated from the environment (Chaerun et al., [11]; Hasanuzzaman et al., [12]). However, none of the medical strains grew in the presence of crude oil. Hence, results demonstrated that environmental strains are better adapted to crude oil contaminated sites.

The high virulence of *P. aeruginosa* warrant more investigations to elucidate the relation between the environmental and the medical strains in order to reveal the points of release of pathogenic bacteria into the environment. The environmental strains may be better adapted to stress conditions such as crude oil pollution but may represent hidden reservoirs for pathogenic traits that may disseminate to other bacteria in the environment.

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