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Molecular Characterization, Multiple Drug Resistance, and Virulence Determinants of *Pseudomonas aeruginosa* Isolated from Lebanon

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Authors' contributions

This work was carried out in collaboration between all authors. Author ST supervised, did all the coordination and analyses and wrote the manuscript, author RT did the PFGE and author NI did the toxin profiling and the antibiotic testing and author GA supervised and provided the clinical samples. All authors read and approved the final manuscript.

Short Communication

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ABSTRACT

Aims: Typing and characterization of 100 *P. aeruginosa* clinical isolates by pulse field gel electrophoresis (PFGE) to detect changes in the clonal composition of local strains and to correlate banding patterns with site of infection, drug resistance and Type III secretion system effectors.
Methodology: A total of 100 *P. aeruginosa* isolates obtained from clinical specimens were used to study resistance profiles, PFGE banding patterns and virulence determinants.
Results: Results from antimicrobial susceptibility testing yielded showed that 77 of the strains were multi drug resistant (MDR). Grouping isolates as non-susceptible when tested intermediate and resistant accordingly showed that resistance was 25% each for imipenem and piperacillin-tazobactam, while it was 29% for ceftazidime these drugs are among the ones most commonly used in treating infections caused by *P. aeruginosa*. Studying effectors released by the type III secretion system including *exoU* and *exoS* revealed that 48% of the isolates harbored *exoS* toxin gene and 46% the *exoU*, with 3% having both and 8% having none. When the different pulsotypes were compared on a dendogram, 45

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groups emerged showing vast differences among the isolates.
Conclusion: This study showed the emergence of drug resistance in *P. aeruginosa* against the antimicrobial agents being routinely used for treatment and revealed the likely presence of co-selected traits that result in highly virulent and resistant strains. Further clinical investigations are warranted to combat infections caused by this important human pathogen in Lebanon.

Keywords: P. aeruginosa; typing; toxins; PFGE; antibiotic resistance.

1. INTRODUCTION

P. aeruginosa is a ubiquitous Gram-negative opportunistic pathogen that has the ability to survive in a wide range of natural reservoirs [1]. It is involved in many types of human infections in the community as well as health care settings [2-3]. Eighty percent of cystic fibrosis (CF) patients are infected with *P. aeruginosa*. CF and immunocompromised patients are more vulnerable to sepsis and pneumonia caused by this pathogen [2,4], which can also cause contact lens associated infections [5].

An important characteristic of *P. aeruginosa* is its capability to confer resistance to several categories of antibiotics [2,6]. Intrinsic mechanisms of antimicrobial tolerance include the expression of β-lactamases and efflux pumps, and decreased permeability of the outer membrane. It also has certain characteristics enabling it to invade its host causing serious infections. The type III secretion system allows the organism to inject secreted toxins directly into the eukaryotic cytoplasm [7]. The *exs* genes encode four effector proteins: ExoS, ExoT, ExoU and ExoY [8]. ExoU is considered a cytotoxin needed for full virulence of *P. aeruginosa*. Both ExoS and ExoU cause cytotoxicity in epithelial cells [9].

Molecular typing of *P. aeruginosa* has become important in identifying cross-infections in hospitals and specifically in CF centers, thus helping to control transmission means. Pulsed-field gel electrophoresis (PFGE) has a high discriminatory power making it a reliable standard for DNA fingerprinting of isolates to identify or confirm outbreak situations.

This work aims at the typing and characterization of 100 *P. aeruginosa* isolates collected and looking at correlations between banding patterns, drug resistance and exotoxin production.

2. MATERIALS AND METHODS

2.1 Clinical Isolates

A total of 100 *P. aeruginosa* isolates were collected from the American University Hospital which provides medical care for patients across Lebanon, in the period between March and November of 2007. The isolates were mainly recovered from tracheal aspirates (28%), urine (21%), sputum (16%), pus (11%), and wound (7%). The remaining isolates were distributed among blood (3%), bronchial washing (3%), ear (3%), bile (2%), pleural fluid (1%), catheter (1%), urethral discharge (1%), and other tissues (3%). The 100 isolates were recovered from 51 female patients and 49 males of all age categories including 41 seniors, 34 adults, 8 youth, 11 children, and 6 neonates. The samples were streaked on Tryptone Soy Agar (TSA) and stored in Cryobanks at -80ºC.

2.2 Antibiotic Susceptibility Test

All *P. aeruginosa* isolates and a standard culture ATCC 27853 were tested by the agar disc diffusion method against 6 out of 8 antimicrobial categories used to define multidrug resistance in *P. aeruginosa* according to Magiorakos et al. [10]. The following are the categories along with the representative antimicrobial agents: Carbapenems (Imipenem; IPM), Aminoglycosides (Gentamicin; GEN, Tobramycin; TOB, Amikacin; AMK), Monobactams (Aztreonam ATM), Penicillins + β-lactamase inhibitors (Pipercillin/Tazobactam; TZP), Cephalosporins (Ceftazidime; CAZ), Fluoroquinolones (Ciprofloxacin; CIP) [11].

2.3 Detection of *exoS* and *exoU* Genes

Reference strains used in this study were the ATCC BAA-47 and ATCC 29260 carrying the *exoS* and *exoU* genes respectively. PCR assays were performed using the following primers: *exoU* 2998 (5'-GCTAAGGCTTGGCGGAATA-3') and *exoU* 3182 (5'-AGATCACACCCAGCGGTAAC-3') amplifying a 250-bp fragment of the *exoU* gene, and *exoS* 1783 (5'-GGAGCTGGATGCGGGACA-3') and *exoS* 2135 (5'-GGCCGCCTCTTCGAGAAC3') amplifying a 370-bp fragment of the *exoS* gene (Berthelot *et al.*, 2003). PCR mixture contained 2µl of template DNA, 20µM of each pair of primers, 25 mM $MgCl_2$, 2mM dNTPs and PCR-grade sterile water (final volume, 20µl). The same parameters were used for amplification of both genes: an initial denaturation step of 12 min, followed by 30 cycles of denaturation (30 s at 94ºC), annealing (30 s at 63ºC), extension (1 min at 72ºC), and final extension at 72ºC for 10 min.

2.4 Molecular Typing of *P. aeruginosa* by PFGE

Preparation of genomic DNA of the 100 isolates was performed as described in the ARPAC PFGE protocol (http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1194947324361). All isolates were digested with *Spe*I (Roche), and the resulting fragments were separated by electrophoresis on 1.2% agarose gels with a CHEF DR III apparatus (Bio-Rad) at 14ºC, 5.9 V/cm, for 22 h, with a time switch of 5 to 50 s in 0.5× Tris-borate EDTA (TBE) buffer containing 50µM thiourea. The 48.5 kb lambda ladder (Bio-Rad) was used as the molecular weight marker. Gel densitometric analysis was performed by GelCompar II software (Applied Maths) where the band patterns are compared by the use of the Dice coefficient and the unweighted pair group method (optimization of 0.5% and band tolerance of 1.25%) to determine band similarity according to criteria established by Tenover et al. [12]. A Dice coefficient of ≥ 80% was considered suggestive of possible clonal relatedness.

3. RESULTS AND DISCUSSION

3.1 Antimicrobial Susceptability Testing and ExoS/ExoU Detection

Antimicrobial susceptibility testing yielded 77 MDR strains according to criteria reported by Magiorakos et al. [10]. Grouping isolates as non-susceptible when tested intermediate and resistant according to CLSI [11] guidelines, showed that resistance was 25% each for imipenem and piperacillin-tazobactam, while it was 29% for ceftazidime; these drugs are among the ones most commonly used in treating infections caused by *P. aeruginosa* in Lebanon.

This was in harmony with the finding that the occurrence of carbapenem-resistant strains is gradually increasing over time. Deficiency of the outer membrane protein OprD confers resistance in *P. aeruginosa* especially to imipenem [13]. Additionally, plasmid mediated efflux pumps could be the factor contributing to fluoroquinolone resistance observed in this study, while tolerance to tetracycline could be correlated to the low permeability of bacterial outer membranes [14-15].

Studying effectors released by the type III secretion system including *exoU* and *exoS* revealed that 48% of the isolates harbored *exoS* toxin gene and 46% the *exoU*, with 3% having both and 8% having none. Thirty six and 38% of the isolates harboring *exoU* and *exoS* respectively were additionally MDR (Fig. 1). Twenty eight of the isolates were recovered from tracheal aspirates out of which 2 were MDR and positive for both toxin genes. It is noteworthy that a high percentage of the isolates showing resistance to each of the tested antimicrobial agents, were also found to be positive for ExoS and/or ExoU and at the same time MDR (Fig. 1).

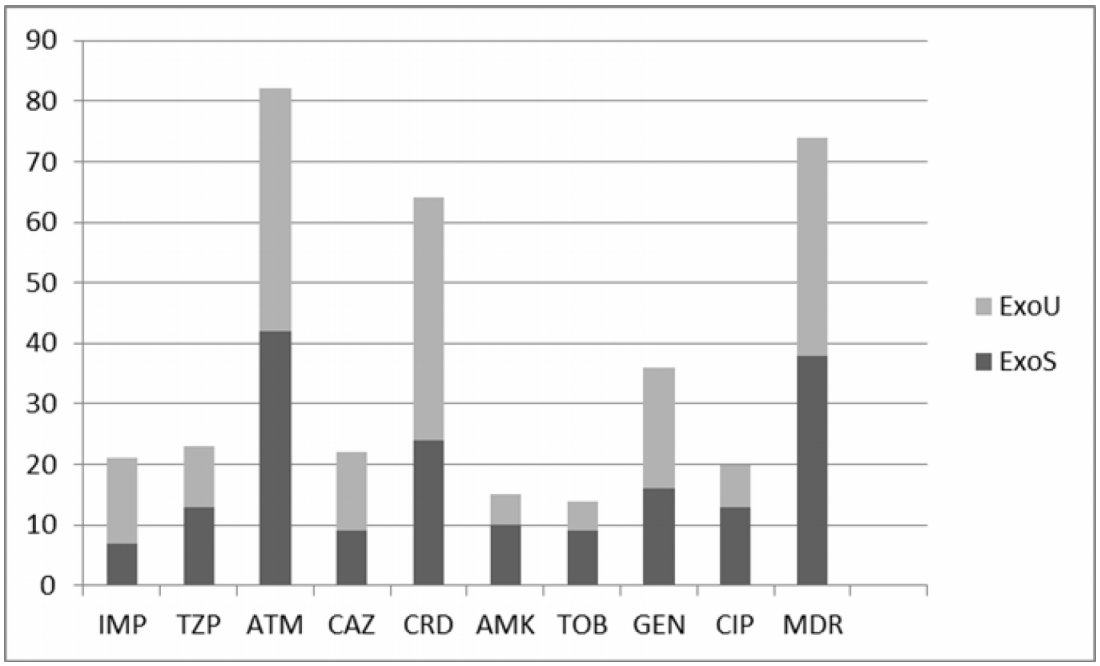

Fig. 1. Percentage of *P. aeruginosa* isolates that are non-susceptible to the tested antimicrobial agents and positive for ExoS and ExoU, along with the percentage of strains that were MDR and positive for ExoS or ExoU (Resistant or Intermediate according to CLSI). (MDR). Imipenem (IPM), Piperacillin/tazobactam (TZP), Aztreonam (ATM), Ceftazidime (CAZ), Ceftriaxone (CRD), Amikacin (AMK), Tobramycin (TOB), Gentamicin (GEN) and Ciprofloxacin (CIP)

A variety of factors have been described to contribute to the virulence detected in *P. aeruginosa* including secreted exotoxins. The type III secretion system in *P. aeruginosa* facilitates the translocation of effector proteins including ExoY, ExoS, ExoT and ExoU into the eukaryotic cytoplasm. In the current study out of the 100 isolates, 41% possessed the $exoS^{+/}exoU^{-}$ genotype versus 42% having the $exoS^{-/}exoU^{+}$. It was previously shown that

invasive strains possess *exoS* while cytotoxic strains appear to have *exoU* [16-17]; ExoU is over 100 fold more cytotoxic than ExoS [18]. Multiple drug resistance was additionally detected among those being *exoS* and *exoU* positive. Isolates that produce ExoU protein are able to evade host immune response at the beginning of the infection period while those producing ExoS protein can decrease DNA synthesis. Strains having virulence factors, such as the toxin genes, cause more disease symptoms and hence are exposed to more antimicrobial agents, thereby giving rise to the emergence of resistance [19]. High percentage of the isolates showing resistance to each of the tested antimicrobial agents, were also found to be positive for ExoS and/or ExoU and at the same time MDR. On the other hand, 28 of the isolates were recovered from tracheal aspirates out of which 17 (61%) were positive for *exoS*. ExoS, an extracellular ADP-ribosyltransferase enzyme, was shown to play a role in chronic lung infections with strains possessing this genotype eliciting greater degree of lung damage [7,20].

3.2 PFGE

DNA fingerprinting by PFGE of genomic DNA after digestion with *Spe*I resulted in distinct genomic fingerprints among the 100 examined isolates. None of the isolates were genetically identical with 100% similarity index. Eighty-three isolates were found to be genetically different where less than 80% band similarity was detected [12]. The remaining 16 isolates were classified in clones that belonged to different groups and only one of the isolates could not be typed.

When the different fingerprints were compared on a dendogram (Fig. 2), 45 groups emerged; majority of these groups consisted of strains with pattern similarity greater than 60%. No significant correlation could be seen between the pulse field pattern, resistance and secreted toxins.

PFGE revealed that most of the isolates had distinct PFGE patterns where the similarity was below 80%. This however, was consistent with other studies; such heterogeneity indicates endogenous origin of *Pseudomonas* infections of unrecognized molecular clusters [21-22] and the existence of a non-clonal population structure [23]. Very closely related isolates in this study, having more than 90% genetic similarity index were shown to be recovered from the same patient. This indicated also that patients being admitted were not harboring or acquiring other clones of *P. aeruginosa*, a result also reported in a previous study by Heo et al. [24].

Finding a correlation between PFGE patterns and site of infection could be of interest especially with outbreak cases. However, in this study, where molecular typing showed vast differences among the isolates, it was unlikely to find any significant correlation between PFGE groups and site of infection. Nevertheless, group A17 consisted of 5 isolates all recovered from tracheal aspirates with all being multi drug resistant. The association between this group and respiratory tract infections was noteworthy, specially that this type of infections in the hospital settings, were previously reported as being the most common type in intensive care units (ICU) [25]. Other sites of infection, such as wounds, ear, blood, and catheter were sporadic among the groups. However, those isolates were few; hence data may not be sufficient to correlate with their corresponding PFGE profiles.

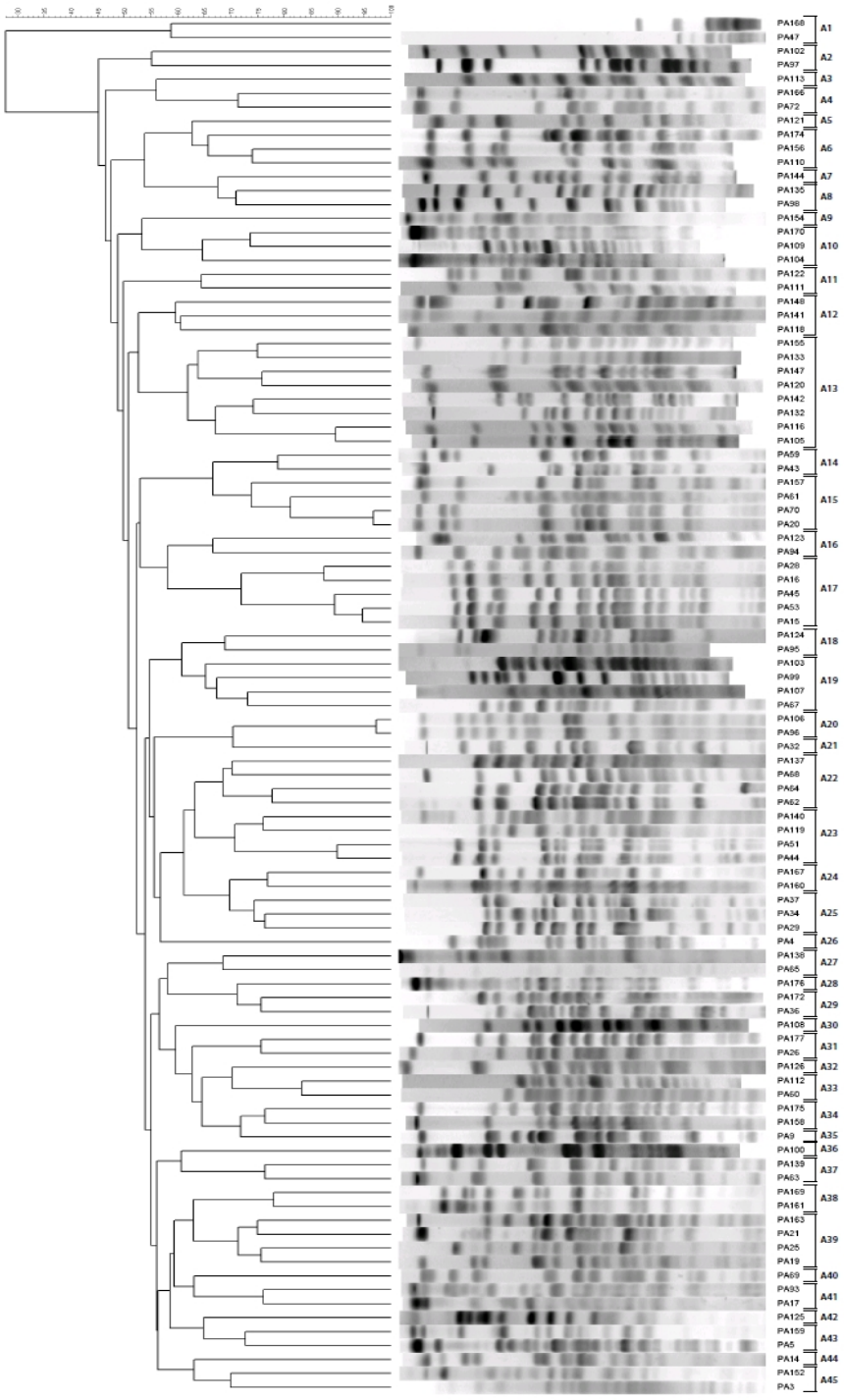

Fig. 2. Dendrogram of PFGE macrorestriction patterns of *P. aeruginosa* isolates generated with the Gel Compar II software. The scale indicates percent similarity.

4. CONCLUSION

This study showed the emergence of drug resistance in *P. aeruginosa* against the antimicrobial agents being routinely used for treatment, and suggested the likely presence of co-selected traits that result in highly virulent and resistant strains. It also revealed the existence of a non-clonal population structure within the studied population. Routine drug susceptibility testing and molecular fingerprinting are recommended to monitor routes of infection and changes in drug resistance patterns. Further clinical investigations however, are warranted to combat infections caused by this important human pathogen.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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