Molecular Detection of the Virulent *ExoU* Genotype of *Pseudomonas aeruginosa* Isolated from Infected Surgical Incisions

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Abstract

Background: *Pseudomonas aeruginosa* is one of the major pathogens responsible for hospital-acquired infections, which harbor a wide array of virulence factors. The main aim of this study was to determine the frequency of the virulent *ExoU* genotype in relation to the *ExoS* genotype among isolated *P. aeruginosa* from infected surgical incisions, followed by phylogenetic analysis.

Methods: A total of 66 *P. aeruginosa* isolates were identified by cultural and biochemical characteristics. All isolates were tested for antimicrobial susceptibility against the following antimicrobial agents: imipenem, amikacin, gentamicin, amoxycillin, cefotaxime, cefepime, and levofloxacin. Molecular detection of the *ExoS* and *ExoU* as well as two other virulence genes was done by polymerase chain reaction (PCR). Sequencing of *ExoU* gene and phylogenetic analysis was performed.

Results: Approximately 81% of the isolated *P. aeruginosa* were multi-drug resistant. The *ExoS* genotype was more prevalent (63%) among the isolates than the *ExoU* genotype (18%), with 9% of the isolates possessing both toxins. *LasB* and *AprA* were detected in 63.6% and 27.2% of the isolates, respectively. An association was observed between the number of virulence genes and the presence of multi-drug resistance. All the *ExoU* were multi-drug resistant (MDR), whereas 71% of the *ExoS* were MDR. Phylogenetic analysis of *ExoU* gene showed a 99% similarity with four different strains.

Conclusion: Despite the greater frequency of the *ExoS* genotype, the presence of the virulent MDR *ExoU* genotype isolates from surgical site infections is an alarming sign requiring further intervention and investigations.

PSEUDOMONAS AERUGINOSA is a common opportunistic micro-organism that causes various infections in human beings. It is often associated with the health-care setting in the form of nosocomial infection and is responsible for urinary tract infections (UTIs), respiratory infections, soft tissue infections, and many others. *Pseudomonas aeruginosa* may also cause systemic infection especially in patients with severe burns, bed sores, and in immune compromised patients [1]. Because it possesses a battery of virulence genes, *P. aeruginosa* can cause both acute and chronic diseases [2]. Type III secretion system (TTSS) has been identified as a major virulence determinant in the pathogenesis of acute *P. aeruginosa* infections. Type III secretion system allows the delivery of various effector toxins as ExoS, ExoU, ExoY, and ExoT into host cells, which can facilitate the pathogen cellular invasion. *ExoS* and *ExoU* are present as variable traits, which are mutually exclusive,

with the *ExoS* genotype being more prevalent than *ExoU* [3]. The impact of the two toxins on the pathogen virulence is distinct because various studies suggest that *ExoU*-producing strains are associated with poor outcomes and high mortality rates [4].

In addition, *P. aeruginosa* secretes other virulence factors that facilitate invasion such as elastases (*LasB*) and alkaline protease (*AprA*); such invasiveness is usually associated with high mortality [5]. The elastase breaks down host proteins such as collagen, immunoglobulins, and complement proteins [6]. Alkaline protease is an enzyme that cleaves various components, such as complement C1q and C3 [7], and prevents TLR5 activation through flagellin destruction [8].

Despite the fact that the proportion of infections caused by *P. aeruginosa* remains constant, the proportion of resistant isolates is increasing, with high resistance rates to imipenem, quinolones, and third-generation cephalosporins [9]. The

emergence of multi-drug-resistant strains of *P. aeruginosa* has been attributed partially to the selective use of antibiotics [10]. Higher rates of multi-drug resistance are often associated with hospital-acquired strains rather than community-acquired ones [11].

Data on the effect of multidrug resistance on bacterial virulence are controversial, with some studies suggesting that increased antibiotic resistance comes at the cost of bacterial virulence, while others reported no effect on bacterial fitness or virulence [12–14]. Understanding the distribution of virulence genes in clinical strains of *P. aeruginosa* is crucial for understanding the infection epidemiology. Accordingly, the aim of this study is to determine the prevalent TTSS effector genotype as well as two more toxic protein exoproducts, *LasB* and *AprA*, among patients with surgical site infections and its correlation with the presence of multiple drug resistance.

Patients and Methods

This is a cross-sectional study carried out in the Microbiology and Immunology Department at the Faculty of Medicine, Minia University between June 2013 and May 2015. The study was conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from patients before enrollment in the study.

Bacterial isolation and antibiotic susceptibility testing

A total of 66 *P. aeruginosa* isolates were obtained from 230 infected surgical sites from three main hospitals. The isolates were identified by colony morphology, oxidase test, and specific *Pseudomonas* biochemical reactions.

Antibiotic susceptibility testing was performed by the disk diffusion method on Muller Hinton agar, with the following antimicrobial agents: Imipenem (IPM; 10 mcg), amikacin (AMK; 30 mcg), gentamicin (CN; 10 mcg), cefotaxime (CTX; 30 mcg), ceftazidime (FEP; 30 mcg), levofloxacin (LEV; 10 mcg) (Bioanalyse, Ankara, Turkey). The results were interpreted according to guidelines recommended by the Clinical and Laboratory Standards Institute [15]. Multi-drug-resistant (MDR) *P. aeruginosa* isolates were defined as resistant to at least three of the following antibiotics: Cefotaxime or ceftazidime, imipenem, gentamicin, or levofloxacin [16].

DNA extraction and amplification of the virulence genes

Bacterial DNA was extracted using a Thermo Scientific GeneJET Genomic DNA Purification Kit (Thermo Scientific, Tewksbury, MA) according to the manufacturer's protocols. Deoxyribonucleic acid was used immediately or stored at -20°C .

LasB, *AprA*, *ExoS*, and *ExoU* genes were amplified using specific primers as described previously [2,17]. The PCR amplification was carried out in 50 μL reactions and each consisted of 10 pmol of each primer, 1 μL DNA sample (2.5 mcg/ μL), and 25 μL of PCR master mix (Thermo Scientific). The amplification conditions for *LasB* and *AprA* included initial denaturation at 94°C for four minutes, 25 cycles each included: One minute at 94°C , 40 sec at 46°C , one minute at 72°C , and a final extension at 72°C for two minutes. For the *ExoS* and *ExoU* genes, initial denaturation

was carried at 95°C for five minutes followed by 35 cycles of denaturation at 95°C for one minute, annealing at 55°C for one minute, extension at 72°C for one minute followed by final extension at 72°C for 10 min.

DNA sequencing

Dideoxy sequencing was performed using BigDyeTM Terminator Chemistry v. 3.1 (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. Forward and reverse primers for *ExoU* were used as sequencing primers using the 3500 Genetic Analyzer (Applied Biosystems).

Similarity search was conducted using the BLAST software by NCBI (National Center Biotechnology Information, www.ncbi.nlm.nih.gov). Phylogenetic analysis of the *ExoU* sequences was conducted with MEGA software version 4.0 (MEGA4; www.megasoftware.net). Tree was generated by neighbor joining using the maximum composite likelihood model [18].

Statistical analysis

The results were analyzed statistically χ^2 tests and $p < 0.05$ were considered significant.

Results

Antibiotic resistance profile

A total of 66 *P. aeruginosa* isolates were obtained from infected surgical incisions. All isolates were resistant to cefotaxime and ceftazidime. The percentage of strains resistant to ofloxacin, gentamicin, and amikacin was 72.7%, 63.6%, and 54.5%, respectively. Only 27.3% of the strains showed resistance to imipenem (Fig. 1). Fifty-four (81%) isolates were multi-drug resistant showing resistance to three or more of the following drugs: Cefotaxime or ceftazidime, imipenem, gentamicin, or levofloxacin.

Virulence genes pattern

The prevalence of the TTSS-encoded toxins, *ExoS* and *ExoU*, was 63% and 18%, respectively. Only 9% of the isolates harbored both *ExoS* and *ExoU* genes. The frequency of *LasB* and *AprA* (invasion-mediating) genes was evaluated in the isolated *P. aeruginosa*. *LasB* was detected in 63.6% of isolates, while *AprA* was found in 27.2% of isolated *P. aeruginosa*. A significant proportional association was observed between the number of detected virulence gene and the presence of multiple drug resistance, as all the non-multi-drug-resistant strains had either one (50%) or two virulence genes (50%). On the other hand, more than 66% of MDR strains had two or more virulence genes (Table 1).

As shown in Figure 2, all the isolated *ExoU*-positive *P. aeruginosa* were MDR, whereas 71.4% of *ExoS*-positive isolates were MDR. Regarding fluoroquinolone resistance, all *ExoU* isolates were resistant to fluoroquinolones and approximately 71% of *ExoS* were resistant to this antimicrobial.

BLAST was used to determine the species identity of *P. aeruginosa* isolate and the phylogenetic tree obtained by sequence analysis of *ExoU* of the isolates and the sequences of 10 other *P. aeruginosa*; the current isolate showed 99% similarity with various strains as shown in Figure 3.

FIG. 1. Resistance profile of isolated *Pseudomonas aeruginosa* strains isolated from surgical site infections.

Discussion

Pseudomonas aeruginosa is a common opportunistic pathogen responsible for 10%–22% of hospital-acquired infections often causing surgical site infections [19], which results in increased morbidity and prolonged hospital stay. The presence of MDR strains in surgical site infections is usually associated with adverse effects caused by the limited effective antimicrobial agents. In the current study, approximately 72% of the isolates were resistant to levofloxacin followed by 63% resistance to gentamicin and 54% to amikacin. The relatively high frequency of resistance to fluoroquinolones is caused by the intrinsic low susceptibility of *P. aeruginosa* to the fluoroquinolones [20,21]. An alarming finding is the development of strains resistant to imipenem with a rate of 27%. A high frequency of MDR *P. aeruginosa* (81%) was observed in this study, which is greater than previous studies that reported lower frequencies varying between 20% and 52% [22,23]. The most likely explanations of the higher rate of multi-drug resistance in this study could be because of the difference in the antibiotics used and the various definitions of multi-drug resistance. The high frequency of MDR isolates in this study could shift the treatment to the use of polymyxin as a last choice with its marked adverse effects [24,25]. One of the virulence mechanisms possessed by *P. aeruginosa* is known as TTSS, which is a syringe-like structure that allows the introduction of toxins (e.g., *ExoS* and *ExoU*) into target cytoplasm [26]. A substantial association between TTSS-associated virulence and poor clinical outcome for *P. aeruginosa*-infected patients has been reported [27], with higher incidence of massive tissue destruction and spread of blood infection

and increased mortality, which was found to be higher with genotypes expressing various exotoxins rather than those with no toxin-expression [28,29]. For that reason, it was important to evaluate the association of the TTSS genotype related to surgical site infection.

A higher prevalence of *ExoS* toxin than *ExoU* was observed in the current study. The frequency of *ExoS* toxin (63%) was in agreement with other studies done on various clinical and environmental isolates [2,30,31]. The frequency of the *ExoU* gene (18%) was similar to that obtained by Feltman et al. [31] and Bradbury et al. [32], however, it was relatively lower than that reported by Mitov et al. [30] with a frequency of 30%. Moreover, the proportion of isolates (9%) having both genes was in agreement with Finnan et al. [33] and Mitov et al. [30], suggesting that the isolates with dual expression of both genes are increasing compared with earlier studies that showed a low number of such isolates (down to one isolate from a total of 115 isolates) [31]. *ExoS* is known to modify bacterial invasion while *ExoU* is recognized for inducing cytotoxicity and is more detrimental than *ExoS* toxin [31,34], thus, the presence of strains producing both toxins found in this study could indicate the emergence of highly virulent strains with mixed invasive-cytotoxic genotype.

The prevalence of another virulence gene, *LasB*, was 63%, which is in agreement with the findings of Wolska et al. [35,36] who found a rate of 76%. The low incidence of the multi-substrate protease (27%) in this study was in agreement with Finnan et al. [33].

TABLE 1. PREVALENCE OF VIRULENCE GENES AMONG ISOLATES OF *PSEUDOMONAS AERUGINOSA*

Number of virulence gene	MDR n = 54	Non-MDR n = 12	p
0	18 (33.3%)	0	0.009
1	0	6 (50%)	0.002
2	18 (33.3%)	6 (50%)	0.1
3	12 (22.2%)	0	0.03
4	6 (11.1%)	0	0.1

MDR = multi-drug-resistant.

FIG. 2. Frequency of multi-drug resistance among *ExoS*- and *ExoU*-positive isolates.

FIG. 3. Neighbor-joining tree showing the estimated phylogenetic relationships of *ExoU* from the current study (as presented with a black dot) and nearest members of *Pseudomonas aeruginosa*.

There is a controversy regarding the relation between the degree of virulence and multi-drug resistance [37]. All *ExoU* isolates were MDR, which is consistent with Mitov et al. [30], who found a higher frequency of MDR among *ExoU* isolates compared with *ExoS* isolates. Consistent with the current finding regarding the high prevalence of fluoroquinolone resistance in *ExoU* isolates (100%), Wong-Beringer et al. [38] found that 92% of *ExoU* were fluoroquinolone-resistant compared with 61% resistance in *ExoS*. The association between MDR and the presence of the *ExoU* genotype has been associated with poor clinical outcome in patients with pneumonia in two different studies, particularly in immunocompromised patients [39,40], with no clear genetic explanation. Proper surveillance for the emergence of the cytotoxic and MDR *P. aeruginosa* isolates would be essential for preventing serious *P. aeruginosa* outbreaks in surgical wards. In fact, further studies are needed to evaluate the clinical outcome of surgical site infections with isolates showing cytotoxic and MDR traits [41].

It is well known that the first-recognized *P. aeruginosa* strain, PAO1, lacks the *ExoU* gene [42] and the gene was first cloned from the PA103 strain [43]. Accordingly, the *ExoU* gene identified in this work was sequenced and underwent BLAST to identify the similarity with the closest strains. A 99% similarity was observed between our strain and four other strains including X13273 and PA14.

In conclusion, *P. aeruginosa* isolated from surgical site infections exhibited a high incidence of multi-drug resistance, showing an alarming increase in resistance to imipenem. Furthermore, the *ExoS* genotype was more frequent than the highly virulent *ExoU* genotype, although the latter showed a higher degree of MDR.

Author Disclosure Statement

No competing financial interests exist.

References

- Aloush V, Navon-Venezia S, Seigman-Igra Y, et al. Multidrug-resistant *Pseudomonas aeruginosa*: Risk factors and clinical impact. *Antimicrob Agents Chemother* 2006;50:43–48.
- Agnello M, Wong-Beringer A. Differentiation in quinolone resistance by virulence genotype in *Pseudomonas aeruginosa*. *PLoS One* 2012;7:e42973.
- Veesenmeyer JL, Hauser AR, Lisboa T, Rello J. *Pseudomonas aeruginosa* virulence and therapy: Evolving translational strategies. *Crit Care Med* 2009;37:1777–1786.
- El Solh AA, Akinnusi ME, Wiener-Kronish JP, et al. Persistent infection with *Pseudomonas aeruginosa* in ventilator-associated pneumonia. *Am J Respir Crit Care Med* 2008;178:513–519.
- Makedou KG, Tsiakiri EP, Bisiklis AG, et al. Changes in antibiotic resistance of the most common Gram-negative bacteria isolated in intensive care units. *J Hosp Infect* 2005;60:245–248.
- Matsumoto K. Role of bacterial proteases in pseudomonal and serratia keratitis. *Biol Chem* 2004;385:1007–1016.
- Hong YQ, Ghebrehewet B. Effect of *Pseudomonas aeruginosa* elastase and alkaline protease on serum complement and isolated components C1q and C3. *Clin Immunol Immunopathol* 1992;62:133–138.
- Bardol BW, van der Ent S, Pel MJ, et al. *Pseudomonas* evades immune recognition of flagellin in both mammals and plants. *PLoS Pathog* 2011;7:e1002206.
- National Nosocomial Infections Surveillance S. National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2004, issued October 2004. *Am J Infect Control* 2004;32:470–485.
- Tsukayama DT, van Loon HJ, Cartwright C, et al. The evolution of *Pseudomonas aeruginosa* during antibiotic rotation in a medical intensive care unit: The RADAR-trial. *Int J Antimicrob Agents* 2004;24:339–345.
- Queenan AM, Bush K. Carbapenemases: The versatile beta-lactamases. *Clin Microbiol Rev* 2007;20:440–458.
- Andersson DI. The biological cost of mutational antibiotic resistance: Any practical conclusions? *Curr Opin Microbiol* 2006;9:461–465.
- Ramadhan AA, Hegedus E. Survivability of vancomycin resistant enterococci and fitness cost of vancomycin resistance acquisition. *J Clin Pathol* 2005;58:744–746.
- Deptula A, Gospodarek E. Reduced expression of virulence factors in multidrug-resistant *Pseudomonas aeruginosa* strains. *Arch Microbiol* 2010;192:79–84.

15. Clinical and Laboratory Standards Institute. CLSI Performance Standards for Antimicrobial Susceptibility Testing—22nd Informational Supplement, M100-S23. Wayne, PA: Clinical and Laboratory Standards Institute, 2013.
16. Karlowsky JA, Draghi DC, Jones ME, et al. Surveillance for antimicrobial susceptibility among clinical isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* from hospitalized patients in the United States, 1998 to 2001. *Antimicrob Agents Chemother* 2003;47:1681–1688.
17. Andrejko M, Zdybicka-Barabas A, Janczarek M, Cytrynska M. Three *Pseudomonas aeruginosa* strains with different protease profiles. *Acta Biochim Pol* 2013;60:83–90.
18. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007;24:1596–1599.
19. Simonetti A, Ottaiano E, Diana MV, et al. Epidemiology of hospital-acquired infections in an adult intensive care unit: Results of a prospective cohort study. *Ann Ig* 2013;25:281–289.
20. Ledizet M, Murray TS, Puttagunta S, et al. The ability of virulence factor expression by *Pseudomonas aeruginosa* to predict clinical disease in hospitalized patients. *PLoS One* 2012;7:e49578.
21. Araque M, Velazco E. In vitro activity of fleroxacin against multiresistant gram-negative bacilli isolated from patients with nosocomial infections. *Intensive Care Med* 1998;24:839–844.
22. Hassuna NA, Mohamed AHI, Abo-Eluoon SM, Rizk HA-WA. High prevalence of multidrug resistant *Pseudomonas aeruginosa* recovered from infected burn wounds in children. *Arch Clin Microbiol* 2015;6:1–7.
23. Wassef MA, Hussein A, Abdul Rahman EM, El-Sherif RH. A prospective surveillance of surgical site infections: Study for efficacy of preoperative antibiotic prophylaxis. *Afr J Microbiol Res* 2012;6:3072–3078.
24. Kim J, Lee KH, Yoo S, Pai H. Clinical characteristics and risk factors of colistin-induced nephrotoxicity. *Int J Antimicrob Agents* 2009;34:434–438.
25. Hartzell JD, Neff R, Ake J, et al. Nephrotoxicity associated with intravenous colistin (colistimethate sodium) treatment at a tertiary care medical center. *Clin Infect Dis* 2009;48:1724–1728.
26. Engel J, Balachandran P. Role of *Pseudomonas aeruginosa* type III effectors in disease. *Curr Opin Microbiol* 2009;12: 61–66.
27. El-Solh AA, Hattemer A, Hauser AR, et al. Clinical outcomes of type III *Pseudomonas aeruginosa* bacteremia. *Crit Care Med* 2012;40:1157–1163.
28. Breidenstein EB, de la Fuente-Nunez C, Hancock RE. *Pseudomonas aeruginosa*: All roads lead to resistance. *Trends Microbiol* 2011;19:419–426.
29. Roy-Burman A, Savel RH, Racine S, et al. Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. *J Infect Dis* 2001;183:1767–1774.
30. Mitov I, Strateva T, Markova B. Prevalence of virulence genes among bulgarian nosocomial and cystic fibrosis isolates of *Pseudomonas aeruginosa*. *Braz J Microbiol* 2010;41:588–595.
31. Feltman H, Schulert G, Khan S, et al. Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. *Microbiology* 2001;147(Pt 10): 2659–2669.
32. Bradbury RS, Roddam LF, Merritt A, et al. Virulence gene distribution in clinical, nosocomial and environmental isolates of *Pseudomonas aeruginosa*. *J Med Microbiol* 2010; 59(Pt 8):881–890.
33. Finnan S, Morrissey JP, O’Gara F, Boyd EF. Genome diversity of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients and the hospital environment. *J Clin Microbiol* 2004;42:5783–5792.
34. Rabin SD, Hauser AR. *Pseudomonas aeruginosa* ExoU, a toxin transported by the type III secretion system, kills *Saccharomyces cerevisiae*. *Infect Immun*. 2003;71:4144–4150.
35. Wolska K, Szveda P. Genetic features of clinical *Pseudomonas aeruginosa* strains. *Pol J Microbiol* 2009;58:255–260.
36. Lomholt JA, Poulsen K, Kilian M. Epidemic population structure of *Pseudomonas aeruginosa*: Evidence for a clone that is pathogenic to the eye and that has a distinct combination of virulence factors. *Infect Immun* 2001;69:6284–6295.
37. Di Martino P, Gagniere H, Berry H, Bret L. Antibiotic resistance and virulence properties of *Pseudomonas aeruginosa* strains from mechanically ventilated patients with pneumonia in intensive care units: Comparison with imipenem-resistant extra-respiratory tract isolates from uninfected patients. *Microbes Infect* 2002;4:613–620.
38. Wong-Beringer A, Wiener-Kronish J, Lynch S, Flanagan J. Comparison of type III secretion system virulence among fluoroquinolone-susceptible and -resistant clinical isolates of *Pseudomonas aeruginosa*. *Clin Microbiol Infect* 2008; 14:330–336.
39. Sullivan E, Bensman J, Lou M, et al. Risk of developing pneumonia is enhanced by the combined traits of fluoroquinolone resistance and type III secretion virulence in respiratory isolates of *Pseudomonas aeruginosa*. *Crit Care Med* 2014;42:48–56.
40. Cho HH, Kwon KC, Kim S, Koo SH. Correlation between virulence genotype and fluoroquinolone resistance in carbapenem-resistant *Pseudomonas aeruginosa*. *Ann Lab Med* 2014;34:286–292.
41. Maatallah M, Cheriaa J, Backhrouf A, et al. Population structure of *Pseudomonas aeruginosa* from five Mediterranean countries: Evidence for frequent recombination and epidemic occurrence of CC235. *PLoS One* 2011;6:e25617.
42. Stover CK, Pham XQ, Erwin AL, et al. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 2000;406:959–964.
43. Finck-Barbancon V, Goranson J, Zhu L, et al. ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. *Mol Microbiol* 1997;25: 547–557.

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