Journal of Microbiology and Infectious Diseases / JMID
2013; 3 (3): 116-122
doi: 10.5799/ahinjs.02.2013.03.0093

ORIGINAL ARTICLE

An investigation of type 3 secretion toxins encoding-genes of *Pseudomonas aeruginosa* isolates in a University Hospital in Egypt

Awny A. Gawish, Nahla A. Mohammed, Gehan A. El-Shennawy, Heba A. Mohammed

Department of Microbiology and Immunology, Faculty of Medicine, Zagazig University, Egypt

ABSTRACT

Objective: *Pseudomonas aeruginosa* uses type 3 secretion system (T3SS) to directly inject four toxins into host cells. The aim of the study was to determine the prevalence of the toxins-encoding genes among *P. aeruginosa* isolates collected from nosocomial infections and environmental sources.

Methods: Clonal relatedness of the studied *P. aeruginosa* isolates (n=85) was studied by RAPD typing. Multiplex PCR was performed on the 85 *P. aeruginosa* isolates (68 nosocomial isolates obtained from hospitalized patients present in different wards and departments, and 17 environmental isolates including 2 isolates from health-care worker hands from ICU) to detect the secretion toxins-encoding genes.

Results: RAPD fingerprinting demonstrated that most strains were of distinct genotypes and determined the presence of 54 RAPD patterns. The prevalence of the genes among all isolates was as follows; exoT-100%, exoS- 40%, exoY- 83.5% and exoU- 62.4%. No significant differences in the prevalence of these genes were observed between nosocomial and environmental isolates or between isolates cultured from different sites of infection. The part of *P. aeruginosa* strains harboring either exoS (37.6%) or exoU (60%) gene was significantly higher (P<0.001) than that contained both genes (2.4%).

Conclusion: ExoT was present in all isolates and exoS, exoY and exoU are variable traits with exoU gene was the third in prevalence after exoT and exoY. No significant differences in exoS, exoY and exoU prevalence were observed between nosocomial and environmental isolates or between isolates from different sites of infection. ExoS and exoU genes were mutually exclusive that almost all isolates contain either exoS without exoU or exoU without exoS. *J Microbiol Infect Dis 2013; 3(3): 116-122*

Key words: Pseudomonas aeruginosa, type 3 secretion toxins, multiplex PCR

Mısır'da bir üniversite hastanesinde Pseudomonas aeruginosa izolatlarında tip 3 sekretuar toksin kodlayan genlerin araştırılması

ÖZET

Amaç: *Pseudomonas aeruginosa* tip 3 sekresyon sistemiyle (T3SS) direk dokuya salınan 4 sekretuar toksin kullanır. Bu çalışmanın amacı hastane enfeksiyonlarından ve çevresel kaynaklardan izole edilen *P. aeruginosa* izolatlarında sekretuar toksin kodlayan üç genin prevalansını saptamaktır.

Yöntemler: Çalışılan *P. aeruginosa* izolatlarının klonal yakınlığı RAPD tiplendirme metodu ile bakılmıştır. Sekretuar toksin kodlayan genleri saptamak için 85 *P. aeruginosa* izolatında (farklı klinik ve bölümlerde yatan hastalardan izole edilen 68 hastane kaynaklı izolatı ve ikisi yoğun bakım ünitesindeki sağlık çalışanının ellerinden izole edilen 17 çevresel izolat) multiplex PCR çalışıldı.

Bulgular: RAPD fingerprint analizi ile varlığı tespit edilen 54 RAPD paterninin varlığı suşların çoğunun uzak genotiplere sahip olduğunu gösterdi. Tüm izolatlarda saptanan genlerin prevalansı sırasıyla exoT (% 100), exoS (% 40), exoY (% 83,5) ve exoU (% 62,4) şeklindeydi. Farklı enfeksiyon bölgelerinden üretilen izolatlar arasında yada hastane ve çevresel kaynaklardan elde edilen izolatlar arasında bu genlerin prevalansı açısından anlamlı bir fark yoktu. *P. aeruginosa* suşlarından exoS (% 37,6) veya exoU (% 60) genlerinden birini taşıyanlar her iki geni taşıyanlardan (% 2,4) anlamlı olarak daha fazlaydı (p<0.001).

Sonuç: ExoT tüm izolatlarda bulunurken exoS, exoY ve exoU genlerinin varlığı değişkenlik gösteriyordu. ExoU geni exoT ve exoY geninden sonra üçüncü sıklıkta görülmekteydi. exoS, ExoY ve exoU görülme prevalansı hastane kaynaklı ve çevre kaynaklı suşlar arasında veya değişik bölge veya enfeksiyonlar arasında anlamlı bir farklılık göstermedi. ExoS ve exoU genleri hemen tüm izolatlarda ya birlikte bulunmakta veya iki de bulunmamakta idiler.

Anahtar kelimeler: Pseudomonas aeruginosa, type-3 sekresyon toksinleri, multiplex PCR

Correspondence: Nahla Abdelhamid Mohammed,
Microbiology and Immunology Dept., Faculty of Medicine, Zagazig University, Zagazig, Egypt Email: nahla_abdelhamid@yahoo.com
Received: 04.02.2013, Accepted: 19.06.2013

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram negative pathogen widespread thought the environment. In normal hosts, with intact epithelial barrier, *P. aeruginosa* rarely causes disease. However in immunocompromised and / or hospitalized patients, *P. aeruginosa* is a leading cause of nosocomial infections. Most of these are acute infections, including sepsis, ventilator-associated pneumonia (VAP), and infections in post-operative wound and burn patients. Despite treatment with appropriate antibiotics, mortality remains as high as 40% in acute infections.[1]

To cause such severe diseases, *P. aeruginosa* utilizes a large number of virulence factors, which are described as belonging to adhesins and other secreted toxins. Exotoxins are either passively secreted from the cell or actively secreted via type I secretion system (T1SS), type II secretion system (T2SS) or the type III secretion system (T3SS).[2]

The complex T3SS is important and recently recognized virulence determinant of *P. aeruginosa* capable of injecting certain effector proteins (secretion toxins) into the host cell.[3] The genes encoding the type III secretion toxins appear to be scattered through the chromosome.[4] To date, four secretion toxins have been identified; exoS (exoenzyme S), exoU (exoenzyme U), exoT (exoenzyme T), and exoY (exoenzyme Y).[1]

The first identified *P. aeruginosa* T3SS toxins, exoT and exoS, share 75% homology at the amino acid level.[5] It has found that exoS is the major cytotoxin involved in colonization, invasion and dissemination during infection.[6] ExoS also targets small ras-like proteins, inhibiting bacterial internalization by eukaryotic cells and DNA synthesis, and inducing apoptosis.[5,7] ExoT targets host kinases involved in focal adhesion and phagocytosis,[7] and has been associated with dissemination of disease from the lung to the liver in mice[8] and induction of apoptosis in HeLa cells.[9] ExoY is an adenylate cyclase that cleaves the intracellular cAMP in eukaryotic cells causing rounding of certain cell types. It shows little pathology in mouse pneumonia[6], but has significant cytotoxicity in MDCK cells.[10]

In contrast, the fourth and most recently described toxin of *P. aeruginosa* T3SS toxins, exoU shows marked cytotoxic capabilities with remarkably rapid and fulminant cytotoxic effects in many cell types.[7,10] Deletion of exoU severely limits the toxicity of *P. aeruginosa* strain in lung, and the enzyme has been implicated as an agent associated with septic shock and increased disease severity and mortality in pneumonia.[7,11]

Interestingly, the genes encoding *P. aeruginosa* T3SS toxins are found in some isolates but not in others.[12] The distribution of these genes among isolates of *P. aeruginosa* and the frequency in populations of isolates from different disease sites has not been thoroughly examined in our country

The study was done to determine the prevalence of type 3 secretion toxins- encoding genes among nosocomial as well as hospital environmental and health- care worker hand *P. aeruginosa* isolates, and to analyze the values in respect to the infection localization.

METHODS

This study was done in Microbiology and Immunology Department, Faculty of Medicine, Zagazig University in the period from February 2009 to December 2011. Ethical considerations: informed consent was obtained from all participants, and the study was re-viewed and approved by the review boards of the participating institutes.

Bacterial isolates and growth conditions

Sixty-eight nosocomial isolates of *P. aeruginosa* were obtained from hospitalized patients present in different wards and departments at Zagazig University Hospitals. To prevent over presentation of certain strains, only one isolate per patient was included in the analysis. The clinical isolates of *P. aeruginosa* were identified to be nosocomial if patients were admitted in the hospital for at least 48 h with the previous cultures at date of admission were negative for *P. aeruginosa*. Seventeen environmental isolates of *P. aeruginosa* including 2 isolates from health-care worker hands were isolated from 70 samples taken from ICU unit. The origin of nosocomial and environmental isolates were described In Table 2. All isolates were identified as *P. aeruginosa* according to colony morphology, Gram stain appearance, oxidase reaction, growth at 42°C and reaction patterns in API 20 NE (Bio-Merieux). The isolated *P. aeruginosa* were stored in 25% glycerol in Luria-Bertani (LB) broth at -70°C.

DNA extraction

Bacterial DNA was extracted from *P. aeruginosa* isolates subcultured on nutrient agar plates using QIAamp DNA Mini kit (QIAGEN, USA) according to manufacturer's instructions. The extracted DNA was checked by measuring the optical density (OD) at 260 nm and 280 nm using spectrophotometer.

DNA concentration in each sample was calculated according to the following equation given.[13]

DNA Concentration (ug ml^{-1}) =OD at 260 nm × 50 ug ml^{-1} × dilution factor. The DNA extract samples were stored at -20°C until being used.

Random amplified polymorphic DNA (RAPD) PCR

It was performed with PureTaq Ready-To-Go PCR beads (Amersham Biosciences, UK). The amplification was carried out with 25 ng template DNA and 0.2 uM primer (RAPD-4 5´-AAGAGCCCGT-3´), 2.5 U Taq DNA polymerase, 200 uM of each dNTPs in a total volume 25 ul. A negative control without template DNA was included in each experiment.[14]

The DNA was amplified as follows: initial denaturation at 95°C for 5 minutes; followed by 45 cycles consists of denaturation (95°C for 1 min), annealing (36°C for 1 min) and extension (72°C for 2 minutes); with a single final extension step at 72°C for 10 minutes. The amplification products were compared by electrophoresis in 2% agarose gel in Tris-acetate buffer stained with Ethidium bromide (0.5 ug ml^{-1}) then detected by UV transilluminator.

The presence or absence of bands in gel lane was determined by visual inspection of the patterns independently by two observers. Fingerprint patterns were considered similar (indistinguishable) if all bands had the same migration distance or if there were no more than two band differences for patterns comprising 6-11 products or three band differences for patterns comprising 12-18 products.[15]

PCR amplification of type III secretion toxins-encoding genes

The type III secretion toxins- encoding genes were amplified by multiplex PCR[16] with specific primers (Table 1). PCR was carried out with 2 ul DNA template (100-200 ng), 1 ul equivalent to 100 pM each primer (total 8 ul PCR primers), 2.5 U Taq DNA polymerase, 200 uM of each dNTPs in a total volume 25 ul using PureTaq Ready-To-Go PCR beads (Amersham Biosciences, UK). For negative control, all components of PCR reaction were added to the bead except for DNA. The DNA was amplified using a DNA thermal circler as follows: initial denaturation at 94°C for 3 minutes; followed by 36 cycles consists of denaturation (94°C for 40 seconds), annealing (58°C for 40 seconds) and extention (68°C for 1 minute); with a single final extension step at 68°C for 7 minutes. PCR products were separated in 2.5% agarose gel stained with ethidium bromide (0.5 ug ml^{-1}), then detected by UV transilluminator. Amplified genes were identified on basis of fragment size compared with the DNA MW marker (Figure 1).

Table 1. PCR primers used for genes amplification and multiplex PCR.[16]

Amplified Gene	Primer, DNA sequence
exo S (118-bp fragment)	*exo S* -F, (5´-GCG AGG TCA GCA GAG TAT CG-3´)
	exo S -R, (5´-TTC GGC GTC ACT GTG GAT GC-3´)
exo T (152-bp fragment)	*exo T* -F, (5´-AAT CGC CGT CCA ACT GCA TGC G-3´)
	exo T-R, (5´-TGT TCG CCG AGG TAC TGC TC-3´)
exo U (134- bp fragment)	*exo U* -F, (5´-CCG TTG TGG TGC CGT TGA AG-3´)
	exo U -R, (5´-CCA GAT GTT CAC CGA CTC GC-3´)
exo Y (289-bp fragment)	*exo Y* -F, (5´-CGG ATT CTA TGG CAG GGA GG-3´)
	exo Y -R, (5´-GCC CTT GAT GCA CTC GAC CA-3´)

Statistical analysis

The prevalence of T3 secretion toxins- encoding genes was compared using the chi-squared test or Fisher´s exact test when appropriate. P. value below 0.05 was considered to be statistically significant.

RESULTS

Clonal relatedness of the studied *P. aeruginosa* isolates by RAPD typing

A total of 85 (68 nosocomial and 17 environmental) *P. aeruginosa* isolates were diagnosed during the

study period. All isolates were analyzed by RAPD typing. RAPD fingerprinting demonstrated that most strains were of distinct genotypes and determined the presence of 54 RAPD patterns. A large number of bands (with size from 130 to 2,200 bp) were generated by the RAPD-4 primer. In summary, the RAPD analysis confirmed the significant diversity of strains included in the present study (Data not shown).

Prevalence of type 3 secretion toxins-encoding genes

The frequencies of occurrence of exoenzymes' genes detected by PCR (Figure 1) in all studied strains (n=85) were as follows; exoT- 100%, exoS- 40%, exoY-83.5% and exoU- 62.4% (Table 2).

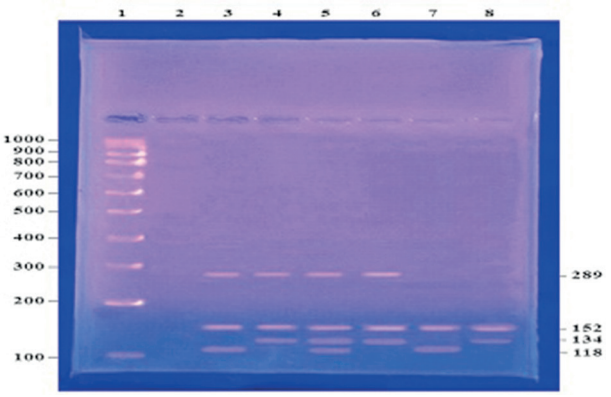

Figure 1. Agarose gel electrophoresis shows bands representing amplified DNA fragments of exoenzymes' genes (lane 3- 8). All isolates were positive for exoT, while others (exoS, exo Y and exoU) are variable traits. Lane 1: MW marker (100-200-300-1000). Lane 2:control.

Table 2. Prevalence of type 3 secretion toxins-encoding genes among nosocomial and environmental (including health-care worker hands) *P. aeruginosa* isolates

Nosocomial *P. aeruginosa* isolates, (n)	Exo T No (%)	Exo Y No (%)	Exo U No (%)	Exo S No (%)
Source of P. aeruginosa isolates				
Corneal swabs (15)	15 (100)	11 (73.3)	11 (73.3)	4 (26.7)
LRTIs (13)	13 (100)	10 (76.9)	8 (61.5)	6 (46.2)
Blood (27)	27 (100)	22 (81.5)	17 (63.0)	11 (40.7)
Urine (5)	5 (100)	5 (100)	3 (60.0)	2 (40)
Wound (5)	5 (100)	5 (100)	3 (60.0)	2 (40)
Burn (3)	3 (100)	3 (100)	2 (66.7)	1 (33.3)
Subtotal (nosocomial isolates) (68)	68 (100)	56 (82.4)	44 (64.7)	26 (38.2)
Hospital environmental samples				
Umbo bags (2)	2 (100)	2 (100)	2 (100)	0 (0.0)
Air condition outlets (2)	2 (100)	2 (100)	0 (0.0)	2 (100)
Ventilator connection (1)	1 (100)	1 (100)	1 (100)	1 (100)
Mops (3)	3 (100)	3 (100)	2 (66.7)	1 (33.3)
Tap handles (1)	1 (100)	1 (100)	1 (100)	1 (100)
Basins (6)	6 (100)	5 (83.3)	4 (66.7)	2 (33.3)
Health-care worker hands (2)	2 (100)	1 (50)	0 (0.0)	1 (50)
Subtotal (environmental isolates) (17)	17 (100)	15 (88.2)	9 (52.9)	8 (47.1)
Total (85)	85 (100)	71 (83.5)	53 (62.4)	34 (40)

LRTIs; Lower respiratory tract infections

Unlike exoT, exoS was not found in all examined isolates. ExoY gene was present in the majority of isolates and exoU was the 3rd in prevalence after exoT and exoY (Table 2). The fraction of isolates harboring each of the variable genes; exoS, exoY and exoU gene did not differ significantly (P >0.05) between nosocomial and environmental isolates and between different sites of infection (Table 2).

In this study there was statistical significant (P<0.001) disagreement in the distribution of exoS and exoU genes in *P. aeruginosa* isolates, where of 85 isolates examined, 32 (37.6%) contained exoS but not exoU, 51 (60%) contained the exoU but not the exoS gene and no isolates contained neither of these genes. However, 2 isolates (2.4%) (one from endotracheal aspirate and one from blood) contained both genes.

DISCUSSION

Type III secretion system in *P. aeruginosa* is known to be a very important virulence factor in acute human infections.[6] This system facilitates the secretion and translocation of exotoxins into target host cells and is triggered by contact with host cells.[17]

In our study, exoT was found in all the 85 clinical and environmental isolates examined, suggesting that this gene is not a variable trait. The absolute presence of exoT in all *P. aureginosa* isolates was not surprising as it was reported by several studies.[2,12,14,18] Interestingly, the presence of this gene in all examined environmental isolates suggests that there may be selection for the exoT gene in hospital environments' *P. aeruginosa* isolates.

Our result regarding the prevalence of exoS gene was in agreement with previous reports, which had suggested that the exoS gene unlike exoT was not found in all *P. aeruginosa* isolates. However, estimates of the actual prevalence of this gene differed markedly. While in our work exoS gene was 40% in all isolates (38.2% of clinical isolates and 47.1% of environmental isolates) which was nearest to that reported in a study on nosocomial isolates in Bulgaria[14], it was characteristically lower than that reported by others.[2,12,19] The conflicting results of these studies may be due to differences in the source of isolates, number of samples taken or to inability of the assay to differentiate exoS gene from exoT gene.

ExoS gene was found in 4 of 15 (26.7%) examined corneal *P. aeruginosa* isolates and this was nearly in agreement with Fleiszing and coworkers[20] who noted that only 2 of 10 (20%) examined corneal isolates contained exoS gene.

There was non-significant difference in the prevalence of exoS gene among LRTIs (46.2%) and blood, urine and wound isolates (each was 40%). This was in agreement with that study in USA[12] and was in contrast with, another study in Bulgaria[14] who reported a significant higher prevalence of exoS among *P. aeruginosa* isolates from blood (87.5%) than those obtained from LRTIs (53.8%).

The prevalence of exoY gene in nosocomial and environmental isolates was more or less similar to that ascertained in studies made on different clinical and environmental isolates in different countries.[12,14,21] These results indicate that exoY gene is present in the vast majority of *P. aeruginosa* regardless it is clinical or environmental isolates.

ExoY gene was disseminated in all studied isolates from urine, wound, and burn which was non-significantly higher than that in corneal isolates (73.3%), LRTIs isolates (76.9%) and blood isolates (81.5%). Contrasting with this result, the prevalence of exoY gene in *P. aeruginosa* isolates in USA[12] was statistically significantly higher in endotracheal isolates (95%) than urine isolates (70%), and in Bulgaria[14] exoY gene was disseminated among all studied isolates from blood , which was significantly higher than that urine (85.1%), LRTIs (82.7%) and wound (82.8%) isolates. The distribution of exoY gene in different clinical samples in our study and the previous studies are not fixed and this indicates no clinical implications.

The prevalence of exoU was 62.4% in all examined *P. aeruginosa* isolates which was third in prevalence to exoT and exoY in the T3SS genotypes of isolates tested, contrasting with a number of studies [11,12,18] that reported exoU prevalence to be the lower one in *P. aeruginosa* isolates compared to the other secretion toxins genes. It was stated that exoU gene was acquired via horizontal transmission on a plasmid, and then integrated in the *P. aeruginosa* genome.[22] Given this, it points to the high rate of plasmid transmission among our isolates.

The distribution of exoU was homogenous among different samples ranging from 60% in urine and wound isolates to 73.3% in corneal isolates The prevalence of exoU gene in corneal isolates was characteristically high (73.3%) and this value lies between the result of Fleiszing and his colleagues[20] (80%) and Winstanely and co-workers[23] (59%). The blood isolates showed a prevalence of 63% of exoU gene and this is in agreement with a study[24] reported a similar value among bacteraemic isolates. In contrast, other investigators [12,13] have reported significant different values for the prevalence of exoU gene in blood, ranging from %25 to 40%. The prevalence of exoU was 60% in wound isolates and this is more or less similar to that ascertained in USA,[12] although it was absent in a small series of 11 wound *P. aeruginosa* isolates.[18] These variations of exoU in different clinical isolates may due to the isolates of the same source were from patients with different clinical conditions and different duration of hospitalization and different sources of infection

and this confirms that exoU gene is a variable trait like exoS gene and present in different prevalence among *P. aeuginosa* strains.

The virulence genes in genome of *P. aeruginosa* strains regardless of their origin whether clinical or environmental were conserved, as detected by whole-genome DNA microarray, and this lead to the suggestion that the environmental strains possess the ability to cause human infections despite the low probability of encountering a human host.[26] In our study, no statistical significant differences were detected between the environmental isolates and the nosocomial isolates with respect to the prevalence of all type 3 secretion toxin genes.

Interestingly, exoU and exoS genes were not randomly distributed among isolates. Rather, a trend towards mutual exclusivity between these two genes was observed, a nearly every isolate that contained exoS gene did not contain exoU gene and vice versa as previously reported in different studies.[2,11,12,18,22] This phenomenon is surprising, as no easily identifiable linkages between these two genes nor their products in the virulence pathway.[12,22] However, it is conceivable that possession of both genes in some way reduces organism fitness, encouraging the loss of one or the other from an individual organism's genome. It may also be possible that simultaneous production of both exoenzyme products results in an increased or up regulated host immune response. Therefore, isolates of this genotype would be less capable of establishing infection in a new host due to their higher immunogenicity and resultant increased clearance from that host. In this study there were only two cases of $exoS^{+}exoU^{+}$ in nosocomial *P. aeruginosa* isolates from ICU but not in environmental isolates in the same unit. One case is from a terminal patient with bacteremia and the second from a patient with severe associated pneumonia. This provides a tentative basis for this argument, as the genotype of these isolates would not be influenced by host immune response.[12]

In conclusion, our results suggested that all *P. aeruginosa* isolates harbor at least some of type III secretion genes. Where exoT gene was found in all examined isolates, the other 3 genes were variable traits. Contrasting with previous results, the exoU gene was the third in prevalence after exoT and exoY in the type III secretion genes of isolates tested. No significant differences in exoS, exoY and exoU prevalence were observed between nosocomial and environmental isolates or between isolates cultured from different sites of infection. Our results confirmed also the previous reports that noted exoS and exoU genes are almost mutually exclusive.

Conflict of interest: Authors declare no conflict of interest.

REFERENCES

1. Engel J, Balachandran P. Role of *Pseudomonas aeruginosa* type III effectors in disease. Current Opin Microbiol 2009;12:61-66.
2. Bradbury RS, Roddam LF, Merritt A, et al. Virulence gene distribution in clinical, nosocomial and environmental isolates of Pseudomonas aeruginosa. J Med Microbiol 2010;59:881-890.
3. Haucer A. The type III secretion system of pseudomonas aeruginosa: infection by injection. Nat Rev Microbiol 2009;7:654-665.
4. Stover CK, Pham XQ, Ewin AL, et al. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 2000;406:956-964.
5. Engel JN. Molecular pathogenesis of acute *Pseudomonas aeruginosa* infections. In: Hauser AR, Rello J, eds. Severe infections caused by Pseudomonas aeruginosa. Dordrech, Kluwer Academic Publishers, 2003:201-229.
6. Lee VT, Smith RS, Tummler B, Lory D. Activities of *Pseudomonas aeruginosa* effectors secreted by the type III secretion system in vitro and during infection. Infect Immun 2005;73:1695-1705.
7. Vance RE, Rietsch A, mekalanos JJ. Role of the type III secreted exoenzymes S, T and Y in systemic spread of *Pseudomonas aeruginosa* Pa01 in vivo. Infect Immun 2005;73:1706-1713.
8. Shaver CM, Haucer AR. Relative contribution of *Pseudomonas aeruginosa* exoU, exoS and exoT to virulence in the lung. Infect Immun 2004;72:6969-6977.
9. Shafikhani SH, Morales C, Engel J. The *Pseudomonas aeruginosa* type III secreted toxin exoT is necessary and sufficient to induce apoptosis in epithelial cells. Cell Microbiol 2008;10:994-1007.
10. Lin HH, Huang SP, Teng HC, et al. Presence of exoU gene of *Pseudomonas aeruginosa* is correlated with cytotoxicity in MDCK cells but not with colonization in BALB/c mice. J Clin Microbiol 2006;44:4596-4597.
11. Wong-Beringer R, Wiener-Kronish J, Lynch S, Flanagan J. Comparison of type III secretion system virulence among fluoroquinolon-susceptible and –resistant clinical isolates of Pseudomonas aeruginosa. Clin Microbiol Infect 2008;14:330-336.
12. Feltman H, Schulert G, Khan S, et al. Prevalence of type III secretion genes in clinical and environmental isolates of Pseudomonas aeruginosa. Microbiol 2001;147:2659-2669.
13. Wilfinger WW, Mackey M, Chomcynski P. Effect of pH and ionic strength on the spectrophotometeric assessment of nucleic acid purity. Bio Techniques 1997;22:474.
14. Strateva T, Markova B, Ivanova D, Mitove I. Distribution of the type III effector proteins-encoding genes among nosocomial *Pseudomonas aeruginosa* isolates from Bulgaria. Ann Microbiol 2010;60:503-509.
15. Woods CR, Versalovic J, Koeuth T, Lupski JR. Analysis of relationships among isolates of Citrobacter diversus by using DNA fingerprints generated by repetitive sequence-based primers in the polymerase chain reaction. J Clin Microbiol 1992;30:2921-2929.

16. Ajayi T, Allmond LR, Sawa T, Wiener-Kronish JP. Single-nucleotide-polymorphism mapping of the *Pseudomonas aeruginosa* type III secretion toxins for development of a diagnostic multiplex PCR system. J Clin Microbiol 2003;41:3526-3531.
17. Verove J, Bernarde C, Bohn YS, et al. Injection of *Pseudomonas aeruginosa* exo toxins into host cells can be modulated by host factors at the level of translocon assembly and/or activity. Biol Cancer Infect 2012;7:e30488.
18. Lomholt JA, Poulson 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.
19. Rumbaugh KP, Hamood AN, Griswold JA. Analysis of *Pseudomonas aeruginosa* clinical isolates for possible variations within the virulence genes exotoxin A and exotoxin S. J Surg Res 1999;82:95-105.
20. Fleiszig SMj, Zaidi TS, Preston MJ, et al. Relationship between cytotoxicity and corneal epithelial cell invasion by clinical isolates of Pseudomonas aeruginosa. Infect Immun 1996; 64:2288-2294.
21. Dacheux D, Toussaint B, Richard M, et al. *Pseudomonas aeruginosa* cystic fibrosis isolates induce rapid , type III secretion- dependant, but exoU-independent, oncosis of macrophages and polymorphonuclear neutrophils. Infect Immun 2000;68:2916-24.
22. Kulasekara BR, Kulasekara HD, Wolfgang MC, et al. Acquisition and evolution of the exoU locus in Pseudomonas aeruginosa. J Bacteriol 2006;188:4037-4050.
23. Winstanley C, Kaye SB, Neal TJ et al. Genotypic and phenotypic characteristics of *Pseudomonas aeruginosa* isolates associated with ulcerative keratitis. J Med Microbiol 2005;54(Pt6):519-260.
24. Allewelt M, Coleman FT, Grout M, et al. Acquisition of expression of the *Pseudomonas aeruginosa* ExoU cytotoxin leads to increased bacterial virulence in a murine model of acute pneumonia and systemic spread. Infect Immun 2000;68:3998–4004.
25. Hirakata Y, Finlay BB, Simpson DA, et al. Penetration of clinical isolates of *Pseudomonas aeruginosa* through MDCK epithelial cell monolayers. J Infect Dis 2000;181:765-769.
26. Wolfgang MC, Kulasekara BR, Liang X, et al. Conservation of genome content and virulence determinants among clinical and environmental isolates of Pseudomonas aeruginosa. PNAS 2003;100:8484-8489.