Antimicrobial Susceptibility Testing and Identification of *exoS* and
exoU Toxin Genes of *Pseudomonas aeruginosa* Isolated From Clinical
Samples in Lebanon J

Rt
00595
c.1

By
Nahla G. ISSA

**A thesis submitted in partial fulfillment of the
requirements for the degree of**

Master of Science
in
Molecular Biology
Lebanese American University
2008

Under the supervision of Dr. Sima Tokajian

LEBANESE AMERICAN UNIVERSITY

Thesis approval Form (Annex III)

Student Name: Nahla Issa

I.D. #: 200503385

Thesis Title :

Antimicrobial Susceptibility Testing and Identification of *exoS* and *exoU* Toxin Genes of *Pseudomonas aeruginosa* Isolated from Clinical Samples in Lebanon

Program : MS in Molecular Biology

Division/Dept : Natural Sciences Division

School : School of Arts and Sciences

Approved by:

Thesis Advisor:

Sima Tokujian

Member :

Roy Khalaf

Member :

ROAD HADHWA

Member :

Date

20/06/05

(This document will constitute the first page of the Thesis)

Plagiarism Policy Compliance Statement

I certify that I have read and understood LAU's Plagiarism Policy. I understand that failure to comply with this Policy can lead to academic and disciplinary actions against me.

This work is substantially my own, and to the extent that any part of this work is not my own I have indicated that by acknowledging its sources.

Name: Nahla Issa

Signature:

Date: June 23, 2008

I grant to the **LEBANESE AMERICAN UNIVERSITY** the right to use this work, irrespective of any copyright, for the University's own purpose without cost to the University or to its students, agents and employees. I further agree that the University may reproduce and provide single copies of the work, in any format other than in or from microfilms, to the public for the cost of reproduction

Nahla G. Issa

LEBANESE AMERICAN UNIVERSITY

ABSTRACT

Antimicrobial Susceptibility Testing and Identification of *exoS* and *exoU* Toxin Genes of *Pseudomonas aeruginosa* Isolated From Clinical Samples in Lebanon

by Nahla G. Issa

Pseudomonas aeruginosa is a major cause of nosocomial infections. It can secrete toxins directly into the eukaryotic host through the type III secretion system. ExoS and ExoU are two major exotoxins that contribute to the virulence and pathogenicity of *P. aeruginosa*. The emergence of antibiotic resistance strains is increasing due to the high consumption of antimicrobial agents. PCR assays were used in this study to determine the distribution of *exoS* and *exoU* toxin genes among 100 *P. aeruginosa* clinical samples. Additionally, E-test was used to determine the antibiotic resistant profiles using five different antibiotics ciprofloxacin (CI), tobramycin (TM), ceftriaxone (TX), gentamycin (Gm), amikacin (Ak) that represent different classes of antimicrobial agents including aminoglycosides, fluoroquinolones, and third generation cephalosporins. The most effective antimicrobial agent was amikacin with 91% of the isolates being susceptible while the least effective drug was ceftriaxone with 45% of samples being susceptible. Multiple drug resistance with *P. aeruginosa* was rarely detected with 4% of the isolates exhibiting resistance to all of the five tested antibiotics, 8% of the sampled showed multiple drug resistance to 3 and 4 drugs.

Almost all samples contained either *exoS* or *exoU* gene but not both, with 48% harboring *exoS*, 47% harboring *exoU*, and only 4% having both. The prevalence of the genes encoding for their toxins was independent of gender and age. The toxin gene encoding *exoS* was more common among isolates recovered from urine and tracheal aspirates, while that encoding *exoU* was mainly detected from isolates taken from wound, blood, and pus. Isolates having the *exoS* gene were more resistant than those having the *exoU*. The majority of the *exoS* positive isolates (78%) showed resistance to amikacin, tobramycin (75%), and ciprofloxacin (72%), while 63% were resistant to gentamicin and 48% to ceftriaxone.

This study which was conducted for the first time in Lebanon revealed the resistance profiles and virulence potential within *P. aeruginosa* clinical isolates. These however, are important findings for epidemiologic and infection control aspects.

TABLE OF CONTENTS

List of Figures	iv
List of Tables	v
Acknowledgments	vi
1. INTRODUCTION	1
2. LITERATURE REVIEW	3
2.1. Overview on <i>Pseudomonas aeruginosa</i>.....	3
2.2. Pathogenesis of <i>P. aeruginosa</i> infections	4
2.3. Virulence Factors	4
2.3.1. Extracellular Toxins.....	4
2.3.2. Flagellum, pilus, and alginate.....	4
2.3.3. Type II and Type III secreted toxins.....	5
2.3.3.1. ExoS and ExoT	6
2.3.3.2. ExoU and ExoY	7
2.3.4. Biofilms.....	8
2.3.5. Quorum Sensing.....	9
2.4. Regulation of type III secretion system.....	9
2.5. Antibiotic resistance and antibiotic susceptibility testing.....	10
2.5.1. Targets for the main classes of antibacterial drugs.....	10
2.5.2. Molecular mechanisms that confer antimicrobial drug resistance.....	10
2.5.3. Factors contributing to <i>P. aeruginosa</i> resistance.....	11
2.5.4. Treatment of multidrug resistant <i>P. aeruginosa</i>.....	12

3. Materials and Methods.....	13
3.1. Reference strains.....	13
3.2. Clinical isolates and storage conditions.....	13
3.3. Identification using API 20NE strips.....	13
3.4. DNA isolation and extraction.....	13
3.5. PCR amplifications.....	14
3.5.1. 16S rDNA amplification.....	14
3.5.2. Detection of <i>exoS</i> and <i>exoU</i> genes.....	14
3.6. Determination of antibiotic resistance profiles and minimum inhibitory concentration by the E-test.....	15
4. Results.....	16
4.1. Identification using API 20NE.....	16
4.2. Biotypes.....	16
4.3. Antimicrobial susceptibility testing (E test).....	16
4.3.1 Antibiotic resistance.....	16
4.3.1.1 Antibiotics and percentage of resistance isolates.....	16
4.3.1.2 Multiple drug resistance.....	17
4.3.1.3 Antibiotic resistance versus age, gender, and site of infection.....	17
4.3.1.3.1. Age.....	17
4.3.1.3.2. Gender.....	17
4.3.1.3.3. Site of infection.....	17
4.4. PCR assays.....	18
4.4.1 16S rDNA amplification.....	18
4.4.2 PCR for the detection of <i>exoS</i> and <i>exoU</i> toxin genes.....	18

5. Discussion.....	29
6. Conclusion.....	35
7. Bibliography.....	37
8. ANNEX.....	52

LIST OF FIGURES

Figure	Page
1. Summary of the mode of action of <i>Pseudomonas aeruginosa</i> cytotoxins.....	9
2. <i>Pseudomonas aeruginosa</i> biotypes 1 to 10 classified based on differences in the morphological characteristics of the colonies	19
3. Antimicrobial Susceptibility test (E-test) for <i>P. aeruginosa</i> susceptible to Ak, TM, TX, CI, and Gm.....	19
4. Resistance and/or intermediate resistance detected within <i>P. aeruginosa</i> clinical isolates using five different antimicrobial agents	21
5. Distribution of multidrug resistance among <i>P. aeruginosa</i> clinical isolate.....	21
6. <i>P. aeruginosa</i> infections versus age group.....	22
7. Multiple drug resistance versus age.....	22
8. <i>P. aeruginosa</i> infection in females versus males.....	23
9. Average multiple drug resistance in males versus females.....	23
10. The distribution of <i>P. aeruginosa</i> isolates undertaken in this study within different sites of infection.....	24
11. Average multiple drug resistance versus site of infection	24
12. 16S rRNA gene amplification.....	25
13. PCR assay for the detection of <i>exoU</i> gene using PAO1 reference strain as a positive control	26
14. PCR assay for the detection of <i>exoS</i> gene using PA103 reference strain as a positive control.....	26
15. <i>exoS</i> and <i>exoU</i> distribution within <i>P. aeruginosa</i> clinical isolates versus sites of infection.....	27
16. Isolates harboring <i>exoS</i> and <i>exoU</i> genes at different sites of infection.....	27
17. <i>exoS</i> and <i>exoU</i> genes versus antimicrobial resistance	28

18. Average multiple drug resistance among isolates harboring either *exoS* or *exoU*.....28

LIST OF TABLES

Table	Page
1. The characteristic features of colonies representing the different assigned biotypes.....	20
2. Isolates showing multiple drugs resistance.....	20
3. Resistance versus toxigenicity within the studied isolates.....	25

ACKNOWLEDGEMENTS

My deepest appreciation goes to Dr. Sima Tokajian with whom I spent two years of research. She always impressed me with her kindness, her loyalty, and her dedication to her students and her work. I want to thank you for your advice, your care, your encouragement, and your trust. May God almighty give you the strength, patience, and wisdom to fulfill your responsibilities for years and years to come.

I should also give my sincere appreciation and thanks to Dr. Roy Khalaf, for his support, care, encouragement, and for always being around for help; Dr. Fouad Hashwa, for his great support, opportunities he provided, and for being part of the committee; Dr. Costantine Daher, for the great help he offered to me; Dr. Brifgette Wex who always impressed me with her hard work; Miss Helena Abou Farah for her patience and great help. I would also like to thank Dr. George Araj for supplying us with the samples we needed all through our study.

My great thanks and gratitude go to my friends who helped and supported me throughout my journey in LAU, I mean: Maya Farah, for being a sister, friend, and great colleague, Pascal Yazbeck for his everlasting support, Dominik Haddad for his care and sincere friendship, and Rami Abou Zeinab for his advice and support. Thanks for being beside me always. You are not only friends but much more, I will never forget you.

My gratefulness I give to Lara Semaan who always made things easier on me through her sincere, genuine and true friendship.

I want also to give thanks to the lovely and kind labmates: Najwan Fares, Mazen Rizk, Rana Indrawos, Nina Najjar, and Farah Ghaziri.

Especially, I would like to give my special thanks to my parents and brothers whose patient love enabled me to complete this work.

Chapter 1

INTRODUCTION

The genus *Pseudomonas* includes more than 140 species with *P. aeruginosa* being the most common species associated with serious infections in immuno-compromised hosts, especially those suffering from AIDS, cancer and burns (Murray et al., 2005).

P. aeruginosa is a Gram negative opportunistic human pathogen. The bacterial pathogenesis is associated with several virulent mechanisms including colonizing and injuring epithelial surfaces (Kang et al., 1997). It produces a variety of virulence factors with strains ranging from weakly virulent to highly virulent (He et al., 2004). It is a common pathogen of surgical wounds, respirator and urinary tract especially in intensive care units (Bertrand et al., 2001).

P. aeruginosa has certain characteristics that enable it to invade its host causing serious infections. These virulent features include adhesiveness, motility, ability to form biofilm, and the presence of specific type III secretion genes as well as other secreted and cell associated virulence factors including exotoxin A, phospholipase, alkaline protease, elastase, pyocyanin, pili, flagella and lipopolysaccharide. The secretion, translocation and regulatory machinery of type III secretion system are encoded by genes located in the 55 min region of the *P. aeruginosa* chromosome, designated as *pse*, *prf*, *exs* and *pop* genes (Zaborina et al., 2006).

The *exs* genes encode four effector proteins: ExoS, ExoT, ExoU (also called PepA) and ExoY (Feltman et al., 2001). ExoS and ExoT are two ADP-ribosylating proteins having 76% homology, although ExoT has only 0.2% of the in vitro activity of ExoS. ExoU (also called PepA) is considered a necessary cytotoxin for full virulence of *P. aeruginosa*. Both ExoS and ExoU cause cytotoxicity in epithelial cells (Hauser et al., 1999).

Another important clinical aspect related to *P. aeruginosa* is its high resistance to antimicrobial agents including β -lactam antibiotics (Okamoto et al., 2001). Such resistance further increases through chromosomal mutations leading to overexpression of multidrug efflux pumps, reduced expression of porin pathways, and derepression of the chromosomal AmpC β -lactamase. *P. aeruginosa* acquires its antimicrobial resistance from altered gene expression affecting indirectly antimicrobial targets (Reinhardt et al., 2007). The resistance of *P. aeruginosa* is rising dramatically, increasing the rates of mortality and morbidity in infected patients. Thus, the task of choosing the appropriate antimicrobial therapy has become extremely difficult requiring a progressive supervision of emerging resistance trends (Obritsch et al., 2004).

The major objectives of this study, conducted for the first time in Lebanon, to the best of our knowledge, are:

- Testing the antimicrobial susceptibility of *Pseudomonas aeruginosa* clinical isolates to five different antibiotics using the E-test.
- Determining the prevalence of the *exoS* and *exoU* toxin genes in *P. aeruginosa* from clinical samples.
- Correlating toxigenicity to antibiotic resistance, site of infection, age, and gender.

Chapter 2

LITERATURE REVIEW

2.1 Overview on *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a motile, straight or slightly curved, aerobic Gram negative rod usually arranged in pairs. Some strains appear mucoid due to the presence of polysaccharide capsules (Murray et al., 2005), while all produce the pigment pyocyanin that gives it a greenish color when grown on minimal medium or found in infections (Govan et al., 1996; Tatterson et al., 2001).

P. aeruginosa inhabits a wide range of environmental niches due to its ability to catabolize various organic compounds found in nature. These ecological niches include water, air, soil, animals, and plants (Kominos et al., 1972; Kucera et al., 1968; Mahajan-Miklos et al., 1999). The genomic sequence of *P. aeruginosa* has been determined in PAO1 strain and was found to be about 6264 kb, encoding approximately 5,570 genes (Stover et al., 2000). The presence of many two-component regulatory systems in this relatively large genome is what provides this bacterium with this adaptability (Manson et al., 1998; Stover et al., 2000). *P. aeruginosa* is an opportunistic human pathogen causing a variety of diseases in immune-compromised hosts and patients with cystic fibrosis (Feltman et al., 2001), and is capable of causing both acute and chronic infections. Patients who suffer from acute infections include those with severe burns, while patients suffering from chronic infections include those with cystic fibrosis in which the bacterium colonizes the airways (Coburn et al., 1999). Most common clinical diseases caused by *P. aeruginosa* include primary skin infections (colonization of burn wounds), pulmonary infections (benign tracheobronchitis to severe necrotizing bronchopneumonia), urinary tract infections, ear infections, eye infections (corneal ulcers), bacteremia and endocarditis (Murray et al., 2005; Plotkowski et al., 1994;

Valente et al., 2000). In the United States it is estimated that *P. aeruginosa* causes 17% of nosocomial pneumonias, 11% of nosocomial urinary tract infections, 8% of surgical wound infections and 3% are associated with bacterimia (Feltman et al., 2000). What attributes to the ability of *P. aeruginosa* to cause nosocomial infections is its ubiquitous nature, ability to survive in moist environments, and resistance to many antibiotics (Sekiguchi et al., 2007), making it responsible for up to 11% of nosocomial infections (Berthelot et al., 2003).

2.2 Pathogenesis of *P. aeruginosa* infections

P. aeruginosa possesses many virulence factors that enable it to overcome the human innate immune system, intoxicate host cells, and alter human adaptive immune system thus creating a systemic infection or more localized, chronic infection (Kang et al., 1997).

2.3 Virulence factors

2.3.1 Extracellular toxins

The main extracellular toxins include ADP-ribosyl transferase exotoxin A (ETA), leukocidin, phospholipase C, alkaline phosphatase, elastase and alkaline protease (Hornef et al., 2000). Exotoxin A (ETA) produced by most strains of *P. aeruginosa* inhibits eukaryotic protein synthesis by inhibiting polypeptide chain elongation factor (EF2) (Khan et al., 1994). It consists of two subunits; the A subunit is catalytic while the B subunit is responsible for the binding of the toxin to the eukaryotic cell receptor (Middlebrook et al., 1977). The enzymatic activity of ETA causes severe damage to host tissues (Bitter, 2003)

2.3.2 Flagellum, pilus and alginate

The attachment and motility of the bacterium are essential mechanisms that contribute to the early steps of pathogenesis facilitating its colonization, movement in the host environment, and avoidance of the immune system. Virulence factors that are responsible for the latter functions include flagella, pili, and extrapolyssacharides (alginate) (Arora et al., 1998; Govan et al., 1996; Manson et al., 1998; Mattick, 2002).

P. aeruginosa usually has a single polar flagellum responsible for the bacterial motility (Fleiszig, 2001). During the initial colonization of the cystic fibrosis patient's airway, the flagellar cap protein FilD is involved in the adherence of *P. aeruginosa* to the human respiratory mucin (Arora et al., 1998). On the other hand, type IV pili constitute the most important adhesin, that mediate adherence and colonization of mucosal surfaces by binding to asialoganglioside GM1 on the host cell surface (Kang et al., 1997). However, the expression of the pili can be harmful to the pathogen itself since it mediates its attachment to phagocytic cells enhancing its killing. For that, bacteria have evolved regulatory mechanism that limits the expression of pili (Robinson et al., 2003). Another essential adhesin that contributes to virulence especially in patients suffering from gut derived sepsis, is the PA-I lectin/adhesin (Alverdy, 2000). PA-I lectin/adhesin enhances the adhesion of *P. aeruginosa* to intestinal epithelium. It is upregulated by local environmental factors that are unique to the intestinal tract (Wu et al., 2003). The existence of *P. aeruginosa* in a mucoid form with an over production of extracellular polysaccharide alginate, enhances its virulence and survival in various ways (Govan et al., 1996). It provides the bacterium with a barrier that helps it overcome both opsonic and nonopsonic phagocytosis (Krieg et al., 1988; Meshulam et al., 1983; Simpson et al., 1988). It also triggers the production of NO (nitric oxide) by both epithelial and endothelial cells in case of oxidative stress (Valente et al., 2000).

2.3.3 Type II and Type III secreted toxins

Another vital virulence determinant of *P. aeruginosa* is its type II and III secretion system (TTSS), which use complex secretion and translocation machinery to inject set of factors, known as effector proteins, into the cytoplasm of eukaryotic cells (Jain et al., 2004). The type II secretion machinery is responsible for the secretion of extracellular virulence factors as Exotoxin A (ExoA), elastase (LasA), and phospholipase C. The functional type II secretion machinery is encoded by the *xcp* gene cluster which in turn is controlled by quorum sensing and the virulence factors are expressed only at high bacterial density (Lee et al., 2005).

Type III secretion systems encode on the order of more than 20 proteins (Coburn et al., 1999). Psc proteins make up most of the secretion apparatus, and at least three proteins, PapB, PapD, PcrV are necessary for the translocation process (Jain et al., 2004). Many *P. aeruginosa* strains are able to induce oncosis of phagocytes via the pore forming activity of translocation proteins PopB/D and Pcr V (Berthelot et al., 2003). The genes encoding for the secretion, translocation, and regulatory machinery of this system are located on the chromosome and are designated as *psc*, *pcr*, *exs*, and *pop*. However, genes encoding for the type III effector proteins are distributed throughout the chromosome (Feltman et al., 2001). Four type III secreted toxins have been identified, ExoS, ExoT, ExoY, and ExoU (Rietsch, 2006). These cytotoxins evade the host immune response by preventing phagocytosis through the modulation of the actin cytoskeleton and inhibition of DNA synthesis leading to mammalian cell death (Wong-Beringer et al., 2008).

2.3.3.1 ExoS and ExoT

ExoS and ExoT are extracellular ADP-ribosylating enzymes (Kaufman et al., 2000; Yahr et al., 1997) responsible for altering cellular cytoskeleton components (Sato et al., 2003). ExoS and ExoT share >75% of sequence homology, and both have an ADP-ribosyltransferase (ADPRT) activity domain (ExoT has about 0.2% the ADPRT activity of ExoS) near the C-terminus and a GTPase-activating protein (GAP) domain in the N-terminal part (Hauser et al., 1999; Sato et al., 2003). ExoS and ExoT disrupt the actin cytoskeleton through two independent enzymatic activities: Rho GTPase-activating protein (GAP) activity and ADP-ribosylation (Aktories et al., 2005) (Figure 1). ExoS and ExoT have two different host targets. ExoT ADP-ribosylates Crk proteins, which leads to the inactivation of Rac1 in the integrin signalling pathway interfering with cell migration (wound healing) or phagocytosis (Aktories et al., 2005; Sun et al., 2003).

ExoS is made up of two polypeptides with molecular weight, of 53 and 49 KDa (Kulick et al., 1993). It interacts with a number of intracellular proteins such as the intermediate filament vimentin and several GTP binding proteins causing morphological alteration and cell death (Hornef et al., 2000). ExoS producing strains cause a significant decrease in DNA synthesis (Frank, 1997).

ExoS cytotoxin has bifunctional activity, it disrupts Ras and Rho mediated transduction pathways (Pederson et al., 1998). The C-terminus of ExoS disrupts eukaryotic signal transduction by preventing Ras from undergoing guanine nucleotide factor mediated nucleotide exchange (Vincent et al., 1999). The N terminus of ExoS however, possesses a GTPase activating protein (GAP) for Rho, Rac, and Cdc 42. The inactivation of the Rho GTPases will lead to the disruption of the actin cytoskeleton (Hall, 1998). The N-terminal region of ExoS possesses a perinuclear localization domain. The first nine amino acids of the ExoS are required for the export of the cytotoxin and not the membrane localization in the eukaryotic cell (Pederson et al., 2000). Two important factors that determine the type and the extent of GTPase to be modified by the cytotoxin, are the physiological state of the eukaryotic cell and the cellular processing of ExoS (Pederson et al., 2000). In the early stages of *P. aeruginosa* infection, the ADP-ribosylation of early host proteins (ERMs), ezrin, redixin, and moesin disrupts the actin cytoskeleton and phagocytic capacity of the target cell in conjunction with the expression of Rho GAP activity (Aktories et al., 2005).

2.3.3.2 ExoY and ExoU

ExoU is considered an acute cytotoxin that causes massive tissue damage through its lipase activity (Sato et al., 2003). It possesses an adenylate cyclase activity; it is delivered into eukaryotic cells through the type III secretory system. The secretion of ExoY results in an increase in the level of intracellular cAMP cell morphology changes. It also plays a role in the protection of the bacterium from local phagocytic cells. The adenylate cyclase activity of ExoY is regulated by a eukaryotic cofactor, this prevents the generation of cAMP within the bacterium before intoxication of host cells (Aktories et al., 2005; Yahr et al., 1998) (Figure 1). ExoU (PepA), is the most cytotoxic product of the type III secretory pathway (Finck-Barbancon et al., 1998). The hydrophilic ExoU protein is expressed and secreted as a 74 KDa (Finck-Barbancon et al., 1997). Strains producing ExoU are able to destroy cellular monolayers within a short course infection period, and its secretion is associated with septic shock (Allamelt et al., 2000; Kurahashi et al., 1999).

2.5 Antibiotic resistance and antibiotic susceptibility testing

P. aeruginosa is a major causative agent of nosocomial infections (Sekiguchi et al., 2007), the treatment of which could be complicated due to resistance (Goossens, 2002).

2.5.1 Targets for the main classes of antibacterial drugs

Antimicrobial agents aim at disrupting three main bacterial processes: cell wall synthesis as in the case with β -lactams that block cross linking enzymes in the peptidoglycan layer of cell walls, protein synthesis like aminoglycosides that target peptidyl transferase, and finally DNA replication/repair like fluoroquinolones that target DNA gyrase and block DNA replication (Walsh, 2000).

2.5.2 Molecular mechanisms that confer antimicrobial drug resistance

Bacteria reproduce at a very high rate at the site of infection accompanied by a high rate of mutations, where mutations could take place on average in thousand loci (Levy, 1998). If these mutations confer resistance to antibiotics, the organism will be able to grow and become the dominant variant in its population (Walsh, 2000). Resistance genes can rapidly spread through bacterial populations especially when encoded for by genes carried on plasmids (Davies, 1994).

Many gram negative and gram positive bacteria produce membrane proteins that act as an efflux pump for the antibiotic. As a result of this export, the drug will be pumped out faster than it can diffuse in, thus the concentration of the drug kept in will be too low to be effective (Levy, 1992; Paulsen et al., 1996). Others secrete enzymes that target the chemical structure of antibiotics rendering it ineffective. In penicillins and cephalosporins the β -lactam ring is deactivated by the action of the β -lactamase enzyme secreted by resistant bacteria (Philippon et al., 1985). In case of aminoglycosides, bacterial enzymes modify the structure of the antibiotic interrupting its binding to the RNA targets in ribosomes and thus preventing it from inhibiting protein synthesis (Shaw et al., 1993).

Some resistant bacteria tend to modify the target of the antimicrobial agent. In erythromycin resistant populations, bacteria tend to mono or dimethylate specific adenine residues in the peptidyl transferase loop of the 23S RNA component of the ribosome. This modification lowers the affinity of the drug to RNA (Walsh, 2000).

2.5.3 Resistance mechanisms in *P. aeruginosa* resistance

P. aeruginosa is recognized for its ability to develop high resistance against most antibiotics. This resistance is due to many mechanisms including: antibiotic modifying enzymes the genes of which are carried on plasmids and transposons and intrinsic resistance mechanisms (such as decreased outer membrane permeability and upregulation of multidrug efflux pumps) (Bryan, 1998; Poole, 2001; Woodford et al., 2007).

P. aeruginosa outer membrane possesss a major porin OprF, and possibly others such as OprB and OprD making the membrane more permeable as compared to other gram negative bacteria. This slows down the diffusion of drug through the outermembrane (Martin et al., 1993). *P. aeruginosa* is capable of pumping out detrimental chemicals, including many antibiotics, through a group of membrane-localized multidrug efflux pumps found in various Gram negative bacteria. Four efflux pumps have been identified in *P. aeruginosa*. These efflux systems have different substrate specificities along with several regulatory factors (Aries et al., 1999; Hocquet et al., 2003; Kohler et al., 1999; Kohler et al., 1997; Mao et al., 2001; Masuda et al., 2000; Okazaki et al., 1992; Sanchez et al., 2002).

It is note worthy that hypermutable strains of *P. aeruginosa* were detected in many sites of infections especially in cystic fibrosis patients; very high rate of mutations, generated clones with increased levels of resistance to various antibiotics (Oliver et al., 2000). These mutations affect many regulatory genes controlling the function of membrane permeability, efflux pumps, or other antibiotic-resistance genes (Oliver et al., 2000).

2.5.4 Treatment of multidrug resistance *P. aeruginosa*

Active surveillance of emerging resistance isolates is continuously needed for the selection of appropriate antimicrobial therapy (Obritsch et al., 2004). Nowadays, antibiotic combinations that exhibit synergistic effects are used in the treatment of hospital acquired infections to overcome treatment failures (Balke et al., 2006; Kocazeybek et al., 2002). However, for choosing the best regimens many aspects should be taken into consideration such as the source of infection, resistant phenotypes, risk factors associated with the treatment, and pharmacokinetic parameters (Rossolini et al., 2005). Antibiotic monotherapy is suggested with urinary tract infections. However, combination chemotherapy is recommended for severe infections such as endocarditis, nosocomial pneumonia, bacteraemia and cystic fibrosis (Doring et al., 2000; Hoiby, 1998; Rajten, 2001). The preferred combination is between aminoglycosides and β -lactams (Rossolini et al., 2005). The actual effectiveness of combined chemotherapy is not yet clear as the given antibiotic will not always exhibit synergistic effects (Chow, 1999).

Chapter 3

MATERIALS & METHODS

3.1. Reference strains

Reference strains used in this study were the ATCC BAA-47, PA103 and ATCC 29260, PAO1 carrying the *exoS* and *exoU* genes respectively.

3.2 Clinical isolates and storage conditions

100 isolates of *Pseudomonas aeruginosa* recovered from clinical samples provided by Dr. Araj from the American University Hospital (AUH) in Beirut were used in this study. All clinical isolates were stored in CryoBank tubes (Copan, USA) at both -20°C and -80°C. Isolates were designated as PA1 through PA100.

3.3 Identification using API 20NE strips

API 20NE strips were inoculated with bacterial suspension, with the turbidity being according to the 0.5 McFarland standard. The strips were developed after 48h of incubation at 29°C. Positive reactions were converted to a seven-digit profile number. These profiles were then compared with the index supplied by the manufacturer (API, BioMerieux).

3.4 DNA isolation and extraction

Bacterial isolates were grown on tryptone soy agar (TSA) plates 24 hours prior to DNA extraction. DNA extraction was carried on using InstaGene Matrix (BioRad) according to the manufacturer's instructions.

3.5 PCR amplifications

All PCR assays were performed on PerkinElmer GeneAmp 9700 (PerkinElmer, Wellesly, Massachusetts).

3.5.1 16S rDNA amplification

16S rDNA amplification was used as a positive PCR control to ensure the integrity of all sample DNA. The assays were performed directly on DNA. An aliquot of 1.5 µl of DNA was added to 18.5 µl of PCR mixture containing 0.4mM dNTP, 1X PCR buffer, 2.5 mM MgCl₂, 0.25 µM 27F primer (5'-AGAGTTTGATCCTGGCTCAG-3'), 0.25 µM 1492R primer (5'-GGTTACCTTGTTACGACTT-3'), 1U *AmpliTaq* Gold Taq Polymerase (Applied Biosystems). Thermal cycling included an initial denaturation of 95°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 53°C for 30 sec and 72°C for 2 min, and a final hold at 4°C. All PCR assays runs incorporated a reagent control (without template DNA). The PCR amplicons were visualized using a UV light box after electrophoresis on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide.

3.5.2 Detection of *exoS* and *exoU* genes

PCR assays, were performed for the detection of *exoS* and *exoU* genes in all samples 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. Amplification of the 2 genes was performed in separate reaction mixtures. The PCR mixture contained 2µl of template DNA, 20 µM of each pair of primers, 25 mM MgCl₂, 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 94°C), annealing (30 s at 63°C), extension (1min at 72°C), and final extension at 72°C for 10 min.

3.6 Determination of antibiotic resistance profiles and minimum inhibitory concentration by the E-test

Antimicrobial resistance and minimum inhibitory concentration (MIC) were determined for the following 5 antibiotics using the E-test (AB BIODISK, Solna, Sweden) where by each E-test strip contains a continuous gradient of antimicrobial agent applied to one side of the strip. Five antibiotics were tested: amikacin (Ak), gentamicin (Gm), tobramycin (TM), ceftriaxone (TX), ciprofloxacin (CI). MIC values that correspond to the edge of the inhibition ellipse intersecting the side of the strips were determined. Resistance or susceptibility profiles were established according to the Clinical and Laboratory Standards Institute (NCCLS).

Chapter 4

RESULTS

4.1 Identification using API 20NE

All samples identified by API 20NE showed 4 different numerical profiles all of which were *Pseudomonas aeruginosa*.

4.2 Biotypes

Morphological characteristics of each biotype are represented in Table1 and Figure 2. The 100 clinical samples divided according to the 10 different biotypes are shown in Annex 3.

4.3 Antimicrobial susceptibility testing (E test)

Four antibiotics were used to assess the antibiotic susceptibility of the 100 clinical isolates. Antibiotics tested were amikacin (AK), gentamycin (Gm), ciprofloxacin (CI), Tobramycin (TM), Ceftriaxone (TX) (Figure 3) (Annex 2).

4.3.1 Antibiotic resistance

4.3.1.1 Antibiotics and percentage of resistance isolates

The percentage of isolates resistant to each of the tested antibiotics is shown in Figure 4. Amikacin was the most effective antimicrobial agent with only 9% of the isolates being resistant while the least effective was ceftriaxone (45% of samples resistance). Intermediate resistant strains had an MIC value between that exhibited by the resistant and susceptible strains. Intermediate resistance was detected with tobramycin (3% of the isolates), with ciprofloxacin 3% of the isolates, with amikacin 6% of the isolates, with gentamicin 23% of the isolates and 45% in the case of ceftriaxone.

4.3.1.2 Multiple drug resistance

Multidrug resistance profiles (between 0 to 5) are shown in Table 3. Majority of the isolates (55%) were resistant to only one drug, while 8% showed resistance to all antibiotics used in this study. Resistance of isolates to 2, 3, and 4 drugs ranged between 5% to 18% with only 4% of the isolates being susceptible to all five antimicrobial agents used in this study (Figure 5).

4.3.1.3 Resistance versus age group, gender, and site of infection

4.3.1.3.1 Age

Age categories were classified as follows: Neonates (0-11 months); Children (1-10 years); Youth (16-24 years); Adults (25-64 years); Seniors (>65 years). The majority of the isolates in this study were recovered from infections in seniors (41%) and adults (34%), with the lowest percentage (6%) falling within isolates recovered from neonates (Figure 6). Multiple drug resistance to as high as 3 drugs was seen among seniors (>65) as compared to an average of one drug in neonates (Figure 7).

4.3.1.3.2 Gender

A significant difference based on gender could not be detected (Figure 8a). The average number of drugs to which those isolates were resistant was two drugs in both males and females (Figure 8b).

4.3.1.3.3 Site of infection

The isolates were mainly recovered from tracheal aspirates (28%), urine (21%), sputum (16%), pus (11%), and wound (7%). The remaining were distributed among bile (2%), pleural fluid (1%), catheter (1%), ear (3%), bronchial washing (3%), urethral discharge (1%), blood (3%), sinus (1%), and other tissues (2%) (Figure 9a). Resistance to four drugs was detected in samples retrieved from bile compared to an average of three drugs from tracheal aspirates. Multiple drug resistance was not seen in samples recovered from pus (Figure 9b).

4.4 PCR assays

4.4.1 16S rDNA amplification

Amplification of the 16S rDNA was the positive PCR control employed to ensure the integrity of all DNA samples used for the detection of *exoS* and *exoU* toxin genes. The size of all the PCR bands obtained after gel electrophoresis had the expected 1500 bp size (Figure 10).

4.4.2 Detection of *exoS* and *exoU* toxin genes

A total of 100 isolates were screened for the presence of two exotoxins, *exoS* and *exoU*. The expected amplicon sizes of 250 bp for *exoU* and 370 bp for *exoS* were obtained using PA103 and PAO1 reference strains, respectively (Figures 11a and 11b). The detailed distribution of the exotoxins is shown in Annex 3. Results obtained in this study revealed that 8% of the samples had none of the exotoxin genes, 94% had either *exoS* or *exoU*, while only 4% harbored both genes (Figure 12). Moreover, the prevalence of *exoS* and *exoU* was almost equal among the 100 tested clinical isolates. The *exoS* positive samples were mainly recovered from urine (67%) and tracheal aspirates (61%). However, the majority of samples isolated from other infection sites including sputum (50%), pus (64%), wound (71%), blood (67%), ear (100%), and other tissues (50%) harbored the *exoU* gene (Figure 13). Resistance to different antibiotics was mainly detected in isolates harboring the *exoS* gene with 78% of the positive (*exoS*) isolates being resistance to Ak, 75% resistance to TM, 63% resistant to GM, 72% to CI, and 48% to TX. On the other hand, 25% of *exoU* positive isolates were resistant to TM and 44% resistant to TX (Table 2) (Figure14). Multiple drug resistance was another important aspect with resistance to 3 drugs being detected among *exoS* positive samples and resistance to an average of 2 drugs among *exoU* positive samples (Figure15).

Figure 2. *Pseudomonas aeruginosa* biotypes 1 to 10 classified based on differences in the morphological characteristics of the colonies.

Figure 3. Antimicrobial Susceptibility test (E-test) for *P. aeruginosa* susceptible to amikacin (Ak), tobramycin (TM), ceftriaxone (TX), ciprofloxacin (CI), and gentamicin (Gm).

Table1.The characteristic features of colonies representing the different assigned biotypes.

Biotype	Shape	Margin	Elevation	Pigmentation	Texture	Appearance	Optical Property	Other
B1	Irregular	Rhizoid	Convex	Green	Rough	Shiny	Opaque	
B2	Irregular	Rhizoid	Convex	Green	Smooth	Shiny	Opaque	
B3	Irregular	Rhizoid	Convex	Green	Smooth	Shiny	Opaque	Mucoid
B4	Irregular	Rhizoid	Flat	Creamy	Rough	Dull	Opaque	
B5	Circular	Rhizoid	Convex	Dark cream	Rough	Shiny	Opaque	Flourescent Green
B6	Irregular	Rhizoid	Convex	Green	Rough	Shiny	Opaque	
B7	Circular	Entire	Convex	Creamy	Rough	Shiny	Opaque	
B8	Circular	Entire	Convex	Creamy	Rough	Dull	Opaque	
B9	Circular	Entire	Convex	Creamy	Rough	Shiny	Opaque	Flourescent Green
B10	Irregular	Rhizoid	Convex	Green	Rough	Dull	Opaque	

Table 2. Multiple drug resistance detected in *P. aeruginosa* clinical isolates.

No. of Drugs	% of Resistance Isolates
0	4
1	55
2	18
3	9
4	5
5	8

Figure 4. Resistance and/or intermediate resistance detected within *P. aeruginosa* clinical isolates using five different antimicrobial agents (AK: amikacin; TM: tobramycin; CI: ciprofloxacin; GM: gentamicin; TX: ceftriaxone).

Figure 5. Distribution of multidrug resistance among *P. aeruginosa* clinical isolates.

Figure 6. *P. aeruginosa* infections versus age group. Age categories were classified as follows: Neonates (0-11 months); Children (1-10 years); Youth (16-24 years); Adults (25-64 years); Seniors (>65 years).

Figure 7. Multiple drug resistance versus age.

Figure 8a. *P. aeruginosa* infection in females versus males.

Figure 8b. Average multiple drug resistance in males versus females.

Figure 9a. The distribution of *P. aeruginosa* isolates undertaken in this study within different sites of infection.

Figure 9b. Average multiple drug resistance versus site of infection.

Table 3. Resistance versus toxigenicity within the studied isolates

Antibiotic	% of resistance (n=100)	% of resistant samples harboring <i>exoS</i>	% of resistant samples harboring <i>exoU</i>
AK	9	78	22
TM	12	75	25
GM	16	63	37
CI	18	72	28
TX	45	48	44

Figure10. 16S rRNA gene amplification

1.5% agarose gel electrophoresis showing PCR products after 16S rDNA amplification. Lane 1: DNA ladder; Lanes 2 through 15 show 16S rDNA products 1500bp for samples PA9, PA14, PA21, PA28, PA36, PA43, PA45, PA53, PA59, PA62, PA63, PA65, PA70, PA72

Figure 11a. PCR assay for the detection of *exoU* gene using PAO1 reference strain as a positive control.

1.5 % agarose gel electrophoresis showing the product of the PCR assay. Lane 1: DNA ladder; Lane2 to 21 contains the following samples PA102, PA103, PA104, PA105, PA106, PA107, PA108, PA109, PA110, PA111, PA112, PA113, PA116, PA118, PA119, PA120, PA121, PA122, PA123, PA124, PA125 showing band of 250bp, Lane 22: *exoU* positive control showing band of 250 bp each, Lane 23: negative control.

Figure 11b. PCR assay for the detection of *exoS* gene using PA103 reference strain as a positive control.

1.5 % agarose gel electrophoresis showing the product of the PCR assay. Lane 1: DNA ladder; Lane2 to 21 contains the following samples PA102, PA103, PA104, PA105, PA106, PA107, PA108, PA109, PA110, PA111, PA112, PA113, PA116, PA118, PA119, PA120, PA121, PA122, PA123, PA124, PA125 showing band of 250bp, Lane 22: *exoS* positive control showing band of 370 bp each, Lane 23: negative control.

Figure 12. Prevalence of *exoS* and *exoU* toxin genes among the tested sample.

Figure 13. *exoS* and *exoU* distribution within *P. aeruginosa* clinical isolates versus sites of infection.

Figure 14. *exoS* and *exoU* genes versus antimicrobial resistance.

Figure 15. Average multiple drug resistance among isolates harboring either *exoS* or *exoU*

Chapter 5

DISCUSSION

The incidence of infections caused by *P. aeruginosa* has increased in recent decades (Bertrand et al., 2001). It can cause significant morbidity and mortality especially in immunocompromised hosts particularly in patients suffering from cystic fibrosis and neutropenia. It colonizes the gastrointestinal tract and is associated with urinary tract infections, biliary tract infections and severe sepsis (Sajjad Raja et al., 2007). *P. aeruginosa* possesses an outstanding capacity to develop resistance to any antimicrobial agent (Gutiérrez et al., 2007). These high rates of resistance to antibiotics cause therapeutic complications associated with treatment failure and death (Di Martino et al., 2002). Aminoglycosides (amikacin, gentamicin, and tobramycin) are a class of important antibiotics widely used for the treatment of bacterial infections including those covered by *P. aeruginosa* (Hoffman et al., 2005). However, the rate of antibiotic resistance, especially to β -lactams, aminoglycosides and fluoroquinolones is increasing in many parts of the world (Sekiguchi et al., 2007). Local and regional surveillance of antibiotic resistance provides better understanding of the global trends in antibiotic resistance. This will help physicians to make the right choice of antibiotic for the treatment of specific organism infection (Al-Tawfik., 2007).

In this study, a total of 100 *P. aeruginosa* clinical isolates collected from a major tertiary care center in Lebanon (AUB-MC) during the year 2007 were analyzed to evaluate their antibiotic susceptibility patterns to five different antimicrobial agents; amikacin, gentamicin, and tobramycin (aminoglycosides), ceftriaxone (cephalosporin), and ciprofloxacin (fluoroquinolone).

Highest resistance was reported against ceftriaxone with 45% of the isolates showing total resistance. Similarly, resistance to ceftriaxone up to 49% was reported in Egypt (Gad et al., 2007). In Turkey however, Yetkin et al. (2006) found that 88% of the isolates were resistant to ceftriaxone, as opposed to 98.1 % reported by Bayram et al. (2006) and 83% in an intensive care unit in the USA between the years 1990-1993.

Fluoroquinolones (FQ) are becoming the most commonly prescribed antibiotics especially within adults in the USA for the treatment of *P. aeruginosa* infections (Wong-Beringer, 2008). The extensive use of the FQ class is leading to the rapid emergence of resistance among *P. aeruginosa* strains, where resistance to ciprofloxacin tripled from 11% during 1990-1993 to 32% in the year 2000 (Hsu et al., 2005). This increase in resistance to ciprofloxacin was also seen by Neuhauser et al. (2003) from 11% in the 1990 to 32% in 2000.

In our study, 18 % of the samples were resistant to ciprofloxacin with additional 3% demonstrating intermediate resistance. A higher resistance (57.7%) was detected among isolates collected from Tripoli, Lebanon by Hamze et al. (2004) in a study that was conducted between 1998 and 2001. Similar results were found in the USA by the 1997 ASCP group (1999) where resistance was up to 20%, in Turkey (34%), and Iran (39%) (Gad et al., 2007; Yetkin et al., 2006). Sánchez-Romero et al. (2007) also detected an increase in ciprofloxacin resistance (23%-28%) from 1998 to 2003. In contrast, a study conducted in Saudi Arabia between 2001-2005 showed a reduction in the number of resistant strains (Habib Babay, 2007). It is noteworthy that in our study 14% of the isolates demonstrating multidrug resistance (resistance to 3, 4, or 5 drugs), exhibited resistance to ciprofloxacin. Hence, ciprofloxacin resistant strains might be displaying cross resistance to other antipseudomonal agents as was previously reported by Hsu et al. (2005) and Neuhauser et al. (2003). This emerging resistance to fluoroquinolones is most probably mediated through antibiotic efflux mechanisms (Gad et al., 2007). Among the aminoglycosides tested, resistance to gentamicin was highest in this study,

16% of total resistance and 23% of intermediate resistance. Similar results were obtained by Al-Tawfiq (2007) with samples collected from a hospital between 1998-2003 in Saudi Arabia; while interestingly enough Hamza et al. (2004) detected 42.5% of average rate of resistance in strains tested between 1996-2001. In Turkey on the other hand, resistance to gentamicin was 49% (Yetkin et al., 2006), >30% in Spain (Gutiérrez et al., 2007; Sánchez-Romero et al., 2007), 12.8% in a tertiary care hospital in Malaysia, and almost 95% in Canada (Sligl et al., 2006).

Lower resistance was detected against the other two aminoglycosides in this study being 9% for amikacin and 12% for tobramycin. Higher resistance rates were reported by Hamze et al. (2004), with 37.8% resistance to tobramycin and 31% resistance to amikacin. Low levels of resistance to amikacin (6.7%) were seen in Malaysia (Sajjad Raja, 2007), <10% in Spain in the year 2003 (Sánchez-Romero et al., 2007), and 11% to amikacin and 31% to tobramycin in Turkey (Yetkin et al., 2006). Similar results were also seen in Saudi Arabia between 2001-2005 (Habib Babay, 2007), while Friedlend et al. (2002) revealed that amikacin was the most effective antibiotic used against infections caused by *P. aeruginosa*.

This clearly indicates the variation in resistance patterns among *P. aeruginosa* clinical isolates, where of special interest is the difference seen between our results and between those reported by Hamze et al. (2004), with a lower percentage of resistance being detected in our case for both amikacin (9% vs 31%) and tobramycin (12% vs 37.8%). This difference could be mainly correlated to geographical attributes and to the methodology used for testing the antimicrobial susceptibility; Hamze et al used the agar disk diffusion compared to the E-test used in this study. However, amikacin still appears to be the most effective antimicrobial agent compared to ceftriaxone against which high worldwide resistance was observed (Habib Babay, 2007; Hamze et al., 2004; Gad et al., 2007; Friedlend et al., 2002; Sánchez-Romero et al., 2007; Yetkin et al., 2006).

P. aeruginosa was mainly recovered from tracheal aspirate (28%) and urinary tract infections (21%). Similar results were reported by Neuhauser et al. (2003) with 31.6% of the isolates being recovered from the respiratory tract and 24.9% from wounds as compared to only 7% being isolated from wound in this study and as was also obtained by Wonger-Beringer et al. (2008).

Morphological variation is a common phenomenon among gram negative bacteria being mainly associated with environmental conditions. *P. aeruginosa* resistant strains had smaller colonies compared to non resistant strains even on antibiotic free medium (Drenkard et al., 2002). In chronic infection, such as cystic fibrosis patients, *P. aeruginosa* exists as multiple variants. Different colony types will not always reflect differences in the genetic makeup, thus all recovered variants should be tested (Foweraker et al., 2005). In this study, 10 different biotypes were recovered based on morphological differences; however, we could not detect any correlation between, morphology, virulence, and antibiotic resistance.

The *exoS* and *exoU* genes encoding for the cytotoxins in *Pseudomonas aeruginosa* exist as variable traits depending on the disease site and/or background. In a mouse model of acute pneumonia, secretion of ExoU had the greatest impact on mortality, bacterial persistence and dissemination in the lung, followed by ExoS and ExoT (Wong-Beringer et al., 2008). Nearly all strains contain the genes for at least a part of the secretion apparatus, as well as for one or more effector proteins (Shaver et al., 2004). In this study two PCR assays were used to screen for *exoS* and *exoU* toxin genes and results obtained revealed that 47% of the isolates harbored *exoU* compared to 48% having the *exoS*. This was in contrast to the results reported by Winstanley et al. (2005), where the distribution of these two genes among the 63 isolates screened was 59% for *exoU* and 38% for *exoS*. Nearly all isolates possess either *exoS* or *exoU*, which was also the case in a study conducted by Schulert et al. (2003). Reasons behind this dichotomy are not clear (Schulert et al., 2003).

Results from this study also revealed that 61% of samples isolated from tracheal aspirates harbored *exoS* gene while 32% harbored the *exoU* genes. Similar results were obtained with Feltman et al. (2001), where 25% of samples isolated from trachea had the *exoU* compared to 75% having the *exoS*. However in their study, *exoS* was prevalent in isolates recovered from urine samples (67% *exoS* vs. 38% *exoU*). Similar results were also reported by Feltman et al. (2001) (70% for *exoS* vs 35 % for *exoU*).

Isolates recovered from sputum in this study had a higher percentage of *exoU* gene compared to *exoS* (50% vs 38% gene). These results are in contrast to the findings of Wong-Beringer et al. (2008) and Feltman et al. (2001). The prevalence of genes encoding the two effector proteins however, is usually independent of the site of infection in the case of wound, blood, sputum, and pus, but not for isolates from urine and tracheal aspirate (Feltman et al., 2001; Wong-Beringer et al., 2008). This could be possibly due to the persistence of infection in these site of infection associated with chronic diseases as in the case of cystic fibrosis and neutropenia.

Some studies suggest that antibiotic resistance in *P. aeruginosa* could be associated with increased virulence. In *P. aeruginosa* infections resistance to Flouroquinolones was accompanied observed in higher percentages with samples harboring the *exoU* gene (Hauser et al., 2003; Hsu et al., 2005). In this study ciprofloxacin resistant strains harbored mainly *exoS*, which did not agree with results obtained by Wong-Beringer et al. (2008). This could be explained by the fact that isolates harboring the *exoU* gene are more virulent thus are under selective pressure of developing antimicrobial resistance due to extensive treatment strategies.

Prevalence of multidrug resistance strains with multidrug resistant features will remain to constitute a major risk for hospitalized patients, thus a continuous surveillance on resistant patterns is necessary for the identification of new emerging trends that will help in rapid control of potential outbreaks.

As future work, detection of the presence of the genes encoding cytotoxins and the secretion of the active effector proteins is necessary for determining the actual virulence and pathogenesis of clinical isolates. Subtyping by pulse field gel electrophoresis is needed for epidemiological purposes.

Chapter 6

CONCLUSIONS

- The least effective drug against *P. aeruginosa* was ceftriaxone due to high percentage of resistance detected (45%), while the most effective was amikacin with 9% of the isolates being resistant.
- Only 4% of isolates were susceptible to the five tested antibiotics, only 8% showed multidrug resistance, 5% of which were isolated from tracheal aspirate.
- Multidrug resistant strains, had ciprofloxacin resistance in common.
- Infections caused by *P. aeruginosa* were mostly seen in seniors (>65 years) 41% and adults (34%).
- Infections were almost equally detected in males (49%) and females (51%) with both showing resistant to an average of two drugs.
- Majority of the sample (90%) had one of the studied exotoxins, with 48% harboring *exoS* and 46% harboring *exoU*.
- *exoS* was mainly detected in isolates recovered from tracheal aspirate and urine, while isolates from other sites of infection harbored *exoU* gene.
- Resistance was detected in *exoS* positive with a general tendency of exhibiting resistance to two drugs while resistance to only one drug was detected with *exoU* positive ones.
- No direct correlation could be detected between gender and toxigenicity

- Continuous surveillance on resistance patterns and characterization of recent *P. aeruginosa* clinical isolates in Lebanon is crucial for effective and rapid control of potential outbreaks.

Chapter 7

BIBIOGRAPHY

- 1997 ASCP Susceptibility Testing Group. (1999). United States Geographic Bacteria **Susceptibility Patterns**. *Diagnostic Microbiology and Infectious Disease*, 35, 143–151.
- Aires, J. R., Kohler, T., Nikaido, H., & Plesiat, P. (1999). Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrobial Agents and Chemotherapy*, 43, 2624-8.
- Aktories, K. & Barbieri, J. T. (April 8, 2005). Bacterial cytotoxins: Targeting eukaryotic switches. *Nature Reviews Microbiology*, Retrieved May 18, 2008, from Medline database.
- Allewelt, M., Coleman, F.T., Grout, M., Priebe, G.P., & Pier, G.B. (2000). Acquisition of expression of the *Pseudomonas aeruginosa* ExoU cytotoxin leads to increased bacterial virulence in a murine model of acute pneumonia and systemic spread. *Infection and Immunity*, 68(7), 3998-4004.
- Al-Tawfiq, J.A. (2005). Occurrence and antimicrobial resistance pattern of inpatient and outpatient isolates of *Pseudomonas aeruginosa* in a Saudi Arabian hospital: 1998-2003. *International Journal of Infectious Diseases*, 11, 109-114.
- Alverdy, J., Holbrook, C., Rocha, F., Seiden, L., Licheng Wu, R., Musch, M., et al. (2000). Gut-derived sepsis occurs when the right pathogen with the right virulence genes meets the right host. *Annals of Surgery*, 232, 480-489.

- Arora, S. K., Ritchings, B. W., Almira, E. C., Lory, S., & Ramphal, R. (1998). The *Pseudomonas aeruginosa* flagellar cap protein, FliD, is responsible for mucin adhesion. *Infection and Immunity*, 66, 1000-1007.
- Baker, C.N., Stocker, S.A., Culver, D.H., & Thornsberry, C. (1991). Comparison of the *E* test to Agar dilution, broth microdilution, and agar diffusion susceptibility testing techniques by using a special challenge set of bacteria. *Journal of Clinical Microbiology*, 29(3), 533-538.
- Balke, B., Hogardt, M., Schmoldt, S., Hoy, L., Weissbrodt, H., & Haussler, S. (2006). Evaluation of the *E* test for the assessment of synergy of antibiotic combinations against multiresistant *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *European Journal of Clinical Microbiology and Infectious Diseases*, 25, 25-30.
- Bayram, A. & Balci, I. (2006). Patterns of antimicrobial resistance in a surgical intensive care unit of a university hospital in Turkey. *Journal of Infectious Diseases*, 6, 155.
- Berthelot, P., Attree, I., Plesiat, P., Chabert, J., De Bentzmann, S., Pozzetto, B., et al. (2003). Genotypic and phenotypic analysis of type III secretion system in a Cohort of *Pseudomonas aeruginosa* bacteremia isolates: Evidence for a possible association between O serotypes and *exo* genes. *Journal of Infectious Diseases*, 188, 512-518.
- Bertrand, X., Thouverez, M., Talon, D., Boillot, A., Capellier, G., Floriot, C., et al. (2001). Endemicity, molecular diversity and colonization routes of *Pseudomonas aeruginosa* in intensive care units. *Intensive Care Medicine*, 27, 1263-1268.

- Bitter, W. (2003). Secretins of *Pseudomonas aeruginosa*: Large holes in the outer membrane. *Archives of Microbiology*, 179, 307-314.
- Bryan, L. E. (1988). General mechanisms of resistance to antibiotics. *Journal of Antimicrobial and Chemotherapy*, 22 (Suppl A), 1-15.
- Bussiere, D.E., Muchmore S.W., Dealwis, C.G., Schluckebier, G., Nienaber, V.L., Edalji, R.P., et al. (1998). Crystal structure of ErmC', an rRNA methyltransferase which mediates antibiotic resistance in bacteria. *Biochemistry*, 37, 7103-7112.
- Chow, J.W. & Yu, V.L. (1999). Combination antibiotic therapy versus monotherapy for gram-negative bacteraemia: A commentary. *International Journal of Antimicrobial Agents*, 11, 7-12.
- Coburn J. & Frank, D.W. (1999). Macrophages and epithelial cells respond differently to the *Pseudomonas aeruginosa* type III secretion system. *Infection and Immunity*, 67(6), 3151-3154.
- Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial Biofilms: A common cause of persistent infections. *Science*, 284, 1318-1322.
- Davies, J. (1994). Inactivation of antibiotics and the dissemination of resistance genes. *Science*, 264, 375-382.
- Di Martino, P., Gagnière, H., Berry, H., & Bret, L. (2002). 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 and Infection*, 4, 613-620.
- Doring, G., Conway, S.P., Heijerman, H.G., Hodson, M.E., Hoiby, N., Smyth, A., et al. (2000). Antibiotic therapy against *Pseudomonas aeruginosa* in cystic fibrosis: A European consensus. *European Respiratory Journal*, 16, 749-767.

- Drenkard, E. (2003). Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. *Microbes and Infection*, 5, 1213-1219.
- Drenkard, E. & Ausubel, F.M. (2002). *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature*, 416, 740-743.
- Feltman, H., Schulert, G., Khan, S., Jain, M., Peterson, L., & A.R. Hauser. (2001). Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. *Microbiology*, 147, 2659-2669
- Finck-Barbancon, V., Goranson, J., Zhu, L., Sawa, T., Wiener-Kronish, J. P., Fleiszig, et al. (1997). ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. *Molecular Microbiology*, 25, 547-57.
- Finck-Barbancon, V., Yahr, T.L. & Frank, D.W. (1998). Identification and characterization of SpcU, a chaperone required for efficient secretion of the ExoU cytotoxin. *Journal of Bacteriology*, 180(23), 6224-6231.
- Fleiszig, S. M., Arora, S. K., Van, R., & Ramphal, R. (2001). FlhA, a component of the flagellum assembly apparatus of *Pseudomonas aeruginosa*, plays a role in internalization by corneal epithelial cells. *Infection and Immunity*, 69, 4931-7.
- Foweraker, J.E., Laughton, C.R., Brown, D.F.J., & Bilton, D. (2005). Phenotypic variability of *Pseudomonas aeruginosa* in sputa from patients with acute infective exacerbation of cystic fibrosis and its impact on the validity of antimicrobial susceptibility testing. *Journal of Antimicrobial Chemotherapy*, 55, 921-927.
- Frank, D. W. 1997. The exoenzyme S regulon of *Pseudomonas aeruginosa*. *Molecular Microbiology*, 26, 621-9.
- Friedland, I., Stinson, L., Ikaidi, M., Harm, S., & Woods, G.L. (2003). Phenotypic antimicrobial resistance patterns in *Pseudomonas aeruginosa* and *Acinetobacter*: Results of multicenter intensive care unit surveillance study, 1995-2000. *Diagnostic Microbiology and Infectious Disease*, 45, 245-250.

- Gad, G.F., El-Domany, R.A., Zaki, S. & Ashour, H.M. (2007). Characterization of *Pseudomonas aeruginosa* isolated from clinical and environmental samples in Minia, Egypt: Prevalence, antibiogram and resistance mechanisms. *Journal of Antimicrobial Chemotherapy*, 60, 1010-1017.
- Goossens, H. (2003). Susceptibility of multi-drug-resistant *Pseudomonas aeruginosa* in intensive care units: Results from the European MYSTIC study group. *Clinical Microbiology and Infection*, 9, 980-983.
- Govan, J. R. & Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: Mucooid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiological Reviews*, 60, 539-74.
- Gutiérrez, O., Juan, C., Cercenado, E., Navarero, F., Bouza, E., Coll, P., et al. (2007). Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Spanish hospitals. *Antimicrobial Agents and Chemotherapy*, 51(12), 4329-4335.
- Habib Babay, H.A. (2007). Antimicrobial Resistance among clinical isolates of *Pseudomonas aeruginosa* from Patients in a Teaching Hospital, Riyadh, Saudi Arabia, 2001-2005. *Japanese Journal of Infectious Diseases*, 60, 123-125.
- Hadadi, A., Rasoulinejad, M., Maleki, Z., Yonesian, M., Shirani, A., & Kourorian, Z. (2008). Antimicrobial resistance pattern of Gram-negative bacilli of nosocomial origin at 2 university hospitals in Iran. *Diagnostic Microbiology and Infectious Disease*, 60, 301-305.
- Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science*, 279, 509-514.
- Hamze, M., Dabboussi, F., & Izard, D. (2004). A 4-year study of *Pseudomonas aeruginosa* susceptibility to antibiotics (1998-2001) in northern Lebanon. *Médecine et Maladies Infectieuses*, 34, 321-324.

- Hauser, A.R. & Engel, J. N. (1999). *Pseudomonas aeruginosa* Induces Type-III-Secretion-Mediated Apoptosis of Macrophages and Epithelial Cells. *Infection and Immunity*, 67 (10), 5530–5537.
- He, J., Baldini, R.L., Deziel, E., Saucier, M., Zhang, Q., Liberati, N.T., Lee, D., Urbach, J., Goodman, H.M., & Rahme, L.G. (2004). The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proceedings of the National Academy of Sciences*, 101, 2530-2535.
- Hocquet, D., Vogne, C., El Garch, F., Vejux, A., Gotoh, N., Lee, A., et al. (2003). MexXY-OprM efflux pump is necessary for adaptive resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrobial Agents and Chemotherapy*, 47, 1371-5.
- Hoffman, L.R., D'Argenio, D.A., MacCoss, M.J., Zhang, Z., Jones, R.A., & Miller, S.I. (2005). Aminoglycosides antibiotics induce bacterial biofilm formation. *Nature*, 436, 1171-1175.
- Hoiby, N. (2002). New antimicrobials in the management of cystic fibrosis. *Journal of Antimicrobial Chemotherapy*, 49, 235-238.
- Hornef, M.W., Roggenkamp, A., Geiger, A.M., Hogardt, M., Jacobi, C.A. & Heesemann, J. (2000). Triggering the ExoS regulon of *Pseudomonas aeruginosa*: A GFP-reporter analysis of exoenzyme (Exo) S, ExoT and ExoU synthesis. *Microbial Pathogenesis*, 29, 329-343.
- Hsu, D.I., Okamoto, M.P., Murthy, R., & Wong-Beringer, A. (2005). Fluoroquinolone-resistant *Pseudomonas aeruginosa*: risk factors for acquisition and impact on outcomes. *Journal of Antimicrobial Chemotherapy*, 55, 535-541.

- Jain, M., Ramirez, D., Seshadri, R., Cullina, J.F., Powers, C.A., Schulert, G.S., et al. (2004). Type III secretion phenotypes of *Pseudomonas aeruginosa* strains change during infection of individuals with cystic fibrosis. *Journal of Clinical Microbiology*, 42(11), 5229-5237.
- Kang, P.J., Hauser, A.R., Apodaca, G., Fleiszig, S.M.J., Wiener-Kronish, J., Mostov, K., et al. (1997). Identification of *Pseudomonas aeruginosa* genes required for epithelial cell injury. *Molecular Microbiology*, 24(6), 1249-1262
- Kaufman, M.R., Jia, J., Zeng, L., Ha, U., Chow, M., & Jin, S. (2000). *Pseudomonas aeruginosa* mediated apoptosis requires the ADP-ribosylating activity of ExoS. *Microbiology*, 146, 2531-2541.
- Khan, A.A. & Cerniglia, C.E. (1994). Detection of *Pseudomonas aeruginosa* from clinical and environmental samples by amplification of the Exotoxin A gene using PCR. *Applied and Environmental Microbiology*, 60(10), 3739-3745.
- Kohler, T., Epp, S. F., Curty, L. K., & Pechere, J. C. (1999). Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. *Journal of Bacteriology*, 181, 6300-5.
- Kohler, T., Michea-Hamzehpour, M., Henze, U., Gotoh, N., Curty, L. K., & Pechere, J. C. (1997). Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. *Molecular Microbiology*, 23, 345-54.
- Kocazeybek, B.S., Arabaci, U., Erenturk, S., & Akdur, H. (2002). Investigation of various antibiotic combinations using the E-test Method in Multiresistant *Pseudomonas aeruginosa* strains. *Chemotherapy*, 48, 31-35.
- Kominos, S. D., Copeland, C. E., Grosiak, B., & Postic, B. (1972). Introduction of *Pseudomonas aeruginosa* into a hospital via vegetables. *Applied Microbiology*, 24, 567-70.

- Krieg, D. P., Helmke, R. J., German, V. F., & Mangos, J. A. (1988). Resistance of mucoid *Pseudomonas aeruginosa* to nonopsonic phagocytosis by alveolar macrophages *in vitro*. *Infection and Immunity*, 56, 3173-9.
- Kucera, M. & Lysenko, O. (1968). The mechanism of pathogenicity of *Pseudomonas aeruginosa*. V. Isolation and properties of the proteinases toxic for larvae of the greater wax moth *Galleria mellonella* L. *Folia Microbiology*, 13, 288-94.
- Kulich, S.M., Frank, D.W. & Barbieri, J.T. (1993). Purification and characterization of Exoenzyme S from *Pseudomonas aeruginosa* 388. *Infection and Immunity*, 61(1), 307-313.
- Kurahashi, K., Kajikawa, O., Sawa, T., Ohara, M., Gropper, M.A., Frank, D.W., et al. (1999). Pathogenesis of septic shock in *Pseudomonas aeruginosa* pneumonia. *The Journal of Clinical Investigation*, 104(6), 743-750.
- Lee, V.T., Smith, R.S., Tummler, B. & Lory, S. (2005). Activities of *Pseudomonas aeruginosa* effectors secreted by the type III secretion system *in vitro* and during infection. *Infection and Immunity*, 73(3), 1695-1705.
- Levy, S.B. (1992). Active efflux mechanisms for antimicrobial resistance. *Antimicrobial Agents and Chemotherapy*, 36, 695-703.
- Levy, S.B. (1998). The challenge of antibiotic resistance. *Scientific American*, 275, 46-53.
- Mah, T. F., Pitts, B., Pellock, B., Walker, G. C., Stewart, P. S., & O'Toole, G. A. (2003). A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature*, 426, 306-10.
- Mahajan-Miklos, S., Tan, M. W., Rahme, L. G., & Ausubel, F. M. (1999). Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa*-*Caenorhabditis elegans* pathogenesis model. *Cell*, 96, 47-56.

- Mao, W., Warren, M. S., Lee, A., Mistry, A., & Lomovskaya, O. (2001). MexXY-OprM efflux pump is required for antagonism of aminoglycosides by divalent cations in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, 45, 2001-7.
- Manson, M. D., Armitage, J. P., Hoch, J. A., & Macnab, R. M. (1998). Bacterial locomotion and signal transduction. *Journal of Bacteriology*, 180, 1009-22.
- Martin, N. L., Rawling, E. G., Wong, R. S., Rosok, M., & Hancock, R. E. (1993). Conservation of surface epitopes in *Pseudomonas aeruginosa* outer membrane porin protein OprF. *FEMS Microbiology Letters*, 113, 261-6.
- Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H., & Nishino, T. (2000). Contribution of the MexX-MexY-oprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, 44, 2242-6.
- Mattick, J. S. (2002). Type IV pili and twitching motility. *Annual Reviews of Microbiology*, 56, 289-314.
- Meshulam, T., Obedeau, N., Merzbach, D., & Sobel, J. D. (1984). Phagocytosis of mucoid and nonmucoid strains of *Pseudomonas aeruginosa*. *Clinical Immunology Immunopathology*, 32, 151-65.
- Middlebrook, J.L. & Dorland, R.B. (1977). Response of cultured mammalian cells to the exotoxins of *Pseudomonas aeruginosa* and *Corynebacterium diphtheria*: Differential cytotoxicity. *Canadian Journal of Microbiology*, 23, 183-189.
- Murray, P.R., Rosenthal, K.S., & Pfaller, M.A. (2005). *Medical microbiology* (5th ed., pp. 357-360). United States of America: Elsevier.
- Neuhauser, M.M., Weinstein, R.A., Rydman, R., Danziger, L.H., Karam, G., & Quinn, J.P. (2003). Antibiotic resistance among gram-negative bacilli in US intensive care units: implications for fluoroquinolone use. *Journal of the American Medical Association*, 289, 885-888.

- Obritsch, M. D., Fish, D. N., MacLaren, R., & Jung, R. (2004). National Surveillance of Antimicrobial Resistance in *Pseudomonas aeruginosa* Isolates Obtained from Intensive Care Unit Patients from 1993 to 2002. *Antimicrobial Agents and Chemotherapy*, 48(12), 4606-4610.
- Okamoto, K., Gotoh, N., & Nishino, T. (2001). *Pseudomonas aeruginosa* reveals high intrinsic resistance to penem antibiotics: Penem resistance mechanisms and their interplay. *Antimicrobial Agents and chemotherapy*, 45(7), 1964-1971.
- Okazaki, T., and K. Hirai. 1992. Cloning and nucleotide sequence of the *Pseudomonas aeruginosa nfxB* gene, conferring resistance to new quinolones. *FEMS Microbiology Letters*, 76, 197-202.
- Oliver, A., Canton, R., Campo, P., Baquero, F., & Blazquez, J. 2000. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science*. 288, 1251-4.
- Paulsen, I.T., Brown, M.H., & Skurray, R.A. (1996). Proton-dependent multidrug efflux systems. *Microbiological Reviews*, 20, 575-608.
- Pearson, J.P., Gray, K.M., Passador, L., Tucker, K.D., Eberhard, A., Iglewski, B.H., et al. (1994). Structure of the autoinducer required for the expression of *Pseudomonas aeruginosa* virulence genes. *Biochemistry*, 91, 197-201.
- Pearson, J.P., Van Delden, C., & Iglewski, B.H. (1999). Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* Cell-to-Cell Signals. *Journal of Bacteriology*, 181(4), 1203-1210.
- Pederson, K.J., Pal, S., Vallis, A.J., Frank, D.W. & Barbieri, J.T. (2000). Intracellular localization and processing of *Pseudomonas aeruginosa* ExoS in eukaryotic cells. *Molecular Microbiology*, 37(2), 287-299.

- Pederson, K.J. & Barbieri, J.T. (1998). Intracellular expression of the ADP-ribosyltransferase domain of *Pseudomonas* exoenzyme S is cytotoxic to eukaryotic cells. *Molecular Microbiology*, 30(4), 751-759.
- Philippon, A., Labia, R., & Jacoby, G. (1985). Extended spectrum β -lactamases. *Antimicrobial Agents and Chemotherapy*, 28, 302-307.
- Plotkowski, M., Saliba, A., Pereira, S.H.M., Cervante, M.P., & Bajolet-Laudinat, O. (1994). *Pseudomonas aeruginosa* selective adherence to and entry into human epithelial cells. *Infection and Immunity*, 62(12), 5456-5463.
- Poole, K. (2001). Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. *Journal of Molecular Microbiology and Biotechnology*, 3, 255-264.
- Rambaugh, K.P., Griswold, J.A., & Hamood, A.N. (2000). The role of quorum sensing in the in vivo virulence of *Pseudomonas aeruginosa*. *Microbes and Infection*, 2, 1721-1731.
- Ratjen, F. (2001). Changes in strategies for optimal antibacterial therapy in cystic fibrosis. *International Journal of Antimicrobial Agents*, 17, 93-96.
- Reinhardt, A., Köhler, T., Wood, P., Rohner, P., Dumas, J., Ricou, B., et al. (2007). Development and persistence of antimicrobial resistance in *Pseudomonas aeruginosa*: A longitudinal observation in mechanically ventilated patients. *Antimicrobial Agents and Chemotherapy*, 51(4), 1341-1350.
- Rietsch, A. & Mekalanos, J.J. (2006). Metabolic regulation of type III secretion gene expression in *Pseudomonas aeruginosa*. *Molecular Microbiology*, 59(3), 807-820.

- Robinson, P., Carzino, R., Armstrong, D. & Olinsky, A. (2003). *Pseudomonas* cross-infection from cystic fibrosis patients to non-cystic fibrosis patients: Implications for inpatient care of respiratory patients. *Journal of Clinical Microbiology*, 41(12), 5741.
- Rossolini, G.M. & Mantengoli, E. (2005). Treatment and control of severe infections caused by multiresistant *Pseudomonas aeruginosa*. *Clinical Microbiology and Infection*, 11 (Suppl.4), 17-32.
- Sajjad Raja, N. & Nihar Singh, N. (2007). Antimicrobial susceptibility pattern of clinical isolates of *Pseudomonas aeruginosa* in a tertiary care hospital. *The Journal of Microbiology, Immunology and Infection*, 40, 45-49.
- Saliba, A.M., De Assis, M.C., Nishi, R., Raymond, B., De A. Marques, E., Lopes, U.G., et al. (2005). Implications of oxidative stress in the cytotoxicity of *Pseudomonas aeruginosa* ExoU. *Microbes and Infection*, 8, 450-459.
- Sanchez, P., Rojo, F., & Martinez, J. L. (2002). Transcriptional regulation of mexR, the repressor of *Pseudomonas aeruginosa* mexAB-oprM multidrug efflux pump. *FEMS Microbiology Letters*, 207, 63-8.
- Sanchez-Romero, I., Cercendo, E., Cuevas, O., Garcia-Escribano, N., Garcia-Martinez, J., Bouza, E., et al. (2007). Evolution of the antimicrobial resistance of *Pseudomonas aeruginosa* in Spain: Second National Study (2003). *Sociedad Española de Quimioterapia Noticias*, 20(2), 222-229.
- Sato, H., Frank, D. W., Hillard, C. J., Feix, J. B., Pankhaniya, R. R., Moriyama, K., et al (2003). The mechanism of action of the *Pseudomonas aeruginosa*-encoded type III cytotoxin, ExoU. *The EMBO Journal*, 22, 2959-69

- Schulert, G.S., Feltman, H., Rabin, S.D.P., Martin, C.G., Battle, S.E., Rello, J., et al. (2003). Secretion of the toxin ExoU is a marker of highly virulent *Pseudomonas aeruginosa* isolates obtained from patients with hospital-acquired pneumonia. *The Journal of Infectious Diseases*, 188, 1695-1706.
- Sekiguchi, J., Asagi, T., Miyoshi-Akiyama, T., Kasai, A., Mizugushi, Y., Araake, M., et al. (2007). Outbreaks of multidrug-resistant *Pseudomonas aeruginosa* in community hospitals in Japan. *Journal of Clinical Microbiology*, 45(3), 979-989.
- Shaver, C.M. & Hauser, A.R. (2004). Relative contributions of *Pseudomonas aeruginosa* ExoU, ExoS, and ExoT to virulence in the lung. *Infection and Immunity*, 72(12), 6969-6977.
- Shaw, K. J., Rather, P. N., Hare, R. S., & Miller, G. H. (1993). Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiology Reviews*, 57, 138-63.
- Simpson, J. A., Smith, S. E., & Dean, R. T. (1988). Alginate inhibition of the uptake of *Pseudomonas aeruginosa* by macrophages. *Journal of General Microbiology*, 134, 29-36.
- Sligl, W., Taylor, G. & Brindley, P.G. (2006). Five years of nosocomial gram-negative bacteremia in a general intensive care unit: Epidemiology, antimicrobial susceptibility patterns, and outcomes. *International Journal of infectious diseases*, 10, 320-325.
- Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrenner, P., Hickey, M. J., et al. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature*, 406, 959-64.
- Sun, J. & Barbieri, J.T. (2003). *Pseudomonas aeruginosa* ExoT ADP-ribosylates CT10 regulator of kinase (Crk) proteins. *Journal of Biological Chemistry*, 278, 32794-32800.

- Tatterson, L. E., J. F. Poschet, A. Firoved, J. Skidmore, & V. Deretic. 2001. CFTR and *Pseudomonas* infections in cystic fibrosis. *Frontiers in Bioscience*, 6, 890-7.
- Valente, E., Cristina Assis, M., Alvim, I.M.P., Pereira, G.M.B., & Plotkowski, M.C. (2000). *Pseudomonas aeruginosa* induces apoptosis in human endothelial cells. *Microbial Pathogenesis*, 29, 345-356.
- Vincent, T.S., Fraylick, J.E., McGuffie, E.M., & Olson, J.C. (1999). ADP-ribosylation of oncogenic Ras proteins by *Pseudomonas aeruginosa* exoenzyme S in vivo. *Molecular Microbiology*, 32(5), 1054-1064.
- Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance. *Nature*, 406, 775-781
- Winstanely, C., Kaye, S.B., Neal, T.J., Chilton, H.J., Miksch, S., Anthony Hart, C., et al. (2005). Genotypic and phenotypic characteristics of *Pseudomonas aeruginosa* isolates associated with ulcerative keratitis. *Journal of Medical Microbiology*, 54, 519-526.
- Wong-Beringer, A., Wiener-Kronish, J., Lynch, S., & Flanagan, J. (2008). Comparison of type III secretion system virulence among fluoroquinolone-susceptible and resistant clinical isolates of *Pseudomonas aeruginosa*. *Clinical Microbiology and Infection*, 14, 330-336.
- Woodford, N. & Ellington, M.J. (2007). The emergence of antibiotic resistance by mutation. *Clinical Microbiology and Infection*, 13, 5-18.
- Wozniak, D. J., Wyckoff, T. J., Starkey, M., Keyser, R., Azadi, P., O'Toole, G. A., et al. (2003). Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proceedings of the National Academy of Sciences*, 100, 7907-12.

- Wu, L., Holbrook, C., Zaborina, O., Ploplys, E., Rocha, F., Pelham, D., et al. (2003). *Pseudomonas aeruginosa* expresses a lethal virulence determinant, the PA-I Lectin/Adhesin, in the intestinal tract of a stressed host. *Annals of Surgery*, 238, 754-764.
- Yahr, T.L., Mende-Mueller, L.M., Friese, M.B., & Frank, D.W. (1997). Identification of type III secreted products of the *Pseudomonas aeruginosa* Exoenzyme S regulon. *Journal of Bacteriology*, 179(22), 7165-7168.
- Yahr, T.L., Vallis, A.J., Hancock, M.K., Barbieri, J.T. & Frank, D.W. (1998). ExoY, an adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III system. *Proceedings of the National Academy of Sciences*, 95, 13899-13904.
- Yetkin, G., Otlu, B., Cicek, A., Kuzucu, C., & Durmaz, R. (2006). Clinical, microbiological, and epidemiological characteristics of *Pseudomonas aeruginosa* infections in a University Hospital, Malatya, Turkey. *American Journal of Infection Control*, 34 (4), 188-192
- Yoon, S. S., Hennigan, R. F., Hilliard, G. M., Ochsner, U. A., Parvatiyar, K., Kamani, M. C., et al. (2002). *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Developmental Cell*, 3, 593-603.
- Zaborina, O., Kohler, J.E., Wang, Y., Bethel, C., Shevchenko, O., Wu, L., et al. (2006). Identification of multi-drug resistant *Pseudomonas aeruginosa* clinical isolates that are highly disruptive to the intestinal epithelial barrier. *Annals of Clinical Microbiology*, 5(14).

Annex 1. Demographics of the 100 *Pseudomonas aeruginosa* clinical isolates collected from Beirut Medical Center (AUB-MC).

Strain	Gender	Age	Site of infection	Date
3	F	85	fluid pleural tap	28/03/2007
4	M	60	bile	02/04/2007
5	M	60	urine	07/04/2007
9	M	77	catheter	27/03/2007
14	M	78	Tracheal aspirate	29/04/2007
15	F	82	Tracheal aspirate	24/04/2007
16	F	82	Tracheal aspirate	20/04/2007
17	F	22M	swab, ear	20/04/2007
19	F	66	Tracheal aspirate	06/11/2007
20	M	50	Pus	20/04/2007
21	F	5	Tracheal aspirate	30/04/2007
25	M	47	Urine	03/05/2007
26	F	85	Tracheal aspirate	30/04/2007
28	F	62	Tracheal aspirate	09/05/2007
29	M	4	Wound	02/05/2007
32	M	23	Pus	05/05/2007
34	F	31	Ear	03/05/2007
36	M	58	Other	18/06/2007
37	F	21M	Sputum	10/05/2007
43	F	5	Tracheal aspirate	31/05/2007
44	M	37	Tracheal aspirate	30/05/2007
45	M	22	aspirate-tracheal	03/06/2007
47	F	19	Pus	22/05/2007
51	M	37	Tracheal aspirate	31/05/2007
53	F	82	Tracheal aspirate	28/05/2007
59	M	58	Sputum	26-6-2007
60	F	69	Tracheal aspirate	25/06/2007
61	M	72	bronchial washing	23/06/2007
62	M	71	Tracheal aspirate	27/06/2007
63	F	33	Pus	27-6-2007
64	M	71	Tracheal aspirate	22-06-2007
65	M	82	Tracheal aspirate	13-06-2007
67	F	68	Tracheal aspirate	16-06-2007
68	M	22	swab-pus	13-06-2007
69	M	15M	Tracheal aspirate	14-06-2007
70	M	58	Sputum	14-06-2007
72	M	51	Urethral discharge	19-06-2007
93	F	77	Urine	26-8-2007
94	M	72	Sputum	09/09/2007
95	M	61	Sputum	09/10/2007
96	M	57	Sputum	09/10/2007

Annex 1. Demographics of the 100 *Pseudomonas aeruginosa* clinical isolates collected from Beirut Medical Center (AUB-MC).

Strain	Gender	Age	Site of infection	Date
97	F	72	URINE	09/08/2007
98	M	68	URINE	09/05/2007
99	F	31	Pus	09/01/2007
100	M	74Y	Sputum	09/11/2007
102	M	53	Urine	09/09/2007
103	F	7W	Urine	17-9-2007
104	M	72	bronchial washing	16-9-2007
105	F	57	URINE	17-9-2007
106	M	57	bronchial washing	16-09-2007
107	F	2M	Tracheal aspirate	15-09-2007
108	F	16	Pus	17-9-2007
109	F	3M	Urine	19-9-2007
110	F	84	Urine	18-9-2007
111	F	37	Urine	21-9-2007
112	F	3M	Blood	24/9/2007
113	F	1Y	Urine	25-9-2007
116	F	21	Pus	10/01/2007
118	F	2Y	Tracheal aspirate	10/03/2007
119	M	7W	Urine	10/04/2007
120	F	3	Tracheal aspirate	10/02/2007
121	F	10	Urine	OCT.07
122	M	51	Sputum	10/09/2007
123	F	86	Urine	OCT.07
124	F	30	Urine	13-10-2007
125	F	72	Wound	13-10-2007
126	F	3	Tracheal aspirate	10/10/2007
132	M	71	Blood	10/09/2007
133	M	7W	Urine	OCT.07
135	F	77	Urine	OCT.07
137	F	63	Blood	10/09/2007
138	F	60	Bile	10/08/2007
139	F	63	Urine	OCT.07
140	M	38	Wound	17-10-2007
141	M	85	Sputum	10/11/2007
142	F	75	Urine	OCT.07
144	F	70Y	Sputum	13-11-2007
147	F	76	tissue, other	11/09/2007
148	F	46	Sinus	11/12/2007
152	M	68	Urine	11/08/2007

Annex 1. Demographics of the 100 *Pseudomonas aeruginosa* clinical isolates collected from Beirut Medical Center (AUB-MC).

Strain	Gender	Age	Site of infection	Date
154	M	73	Pus	24-10-2007
155	M	47	Tracheal aspirate	11/03/2007
156	F	68	Wound	02/04/2008
157	F	77	Tracheal aspirate	30-10-2007
158	F	90	Pus	30-10-2--7
159	M	80Y	Tracheal aspirate	31-10-2007
160	M	21	Wound	11/02/2007
161	M	79	Sputum	27-10-2007
162	F	38	Pus	13-11-2007
163	M	47	Wound	26-10-2007
166	F	65	Sputum	11/07/2007
167	M	70	Sputum	11/06/2007
168	M	47	Tracheal aspirate	11/06/2007
169	M	79Y	Sputum	11/03/2007
170	F	44	Ear	11/04/2007
172	M	22	Sputum	27-11-2007
174	F	63	Sputum	29-11-2007
175	M	47	Tracheal aspirate	29-11-2007
176	M	72	Tracheal aspirate	27-11-2007
177	M	76	Wound	27-11-2--7

Annex 2. E-test results for 100 *P. aeruginosa* clinical isolates.

Strain	AK	TM	GM	CI	TX
3	S	R	R	R	I
MIC µl/ml	4	16	24	>32	12
4	S	R	I	I	S
MIC µl/ml	1	>256	7	1.5	6
5	R	R	R	R	R
MIC µl/ml	48	>256	>256	>32	>256
9	R	S	I	R	R
MIC µl/ml	>256	1	6	>32	>256
14	S	S	S	S	R
MIC µl/ml	3	0.5	3	0.094	>256
15	R	R	R	R	R
MIC µl/ml	>256	>1024	>1024	>32	>256
16	R	R	R	R	R
MIC µl/ml	>256	>1024	>1024	>32	>256
17	S	S	S	S	I
MIC µl/ml	3	0.5	3	0.064	15
19	S	S	S	S	R
MIC µl/ml	3	0.5	1.5	0.064	54
20	S	S	I	S	R
MIC µl/ml	12	3	6	0.25	>256
21	S	S	S	S	R
MIC µl/ml	4	1	4	0.064	>256
25	S	S	S	S	I
MIC µl/ml	3	0.38	1.5	0.064	12
26	S	S	S	S	R
MIC µl/ml	4	1	4	0.125	96
28	R	R	R	R	R
MIC µl/ml	>256	>1024	>1024	>32	>256
29	S	S	S	S	I
MIC µl/ml	3	0.75	3	0.16	12
32	s	S	S	S	R
MIC µl/ml	3	0.5	2	0.38	>256

Annex 2. E-test results for 100 *P. aeruginosa* clinical isolates.

Strain	AK	TM	GM	CI	TX
34	S	S	I	R	R
MIC µl/ml	16	1.5	6	>32	>256
36	S	S	I	S	I
MIC µl/ml	4	1.5	6	0.125	16
37	S	S	S	S	R
MIC µl/ml	4	0.75	3	0.125	>256
43	S	S	S	S	R
MIC µl/ml	6	2	4	0.125	>256
44	S	S	S	S	R
MIC µl/ml	2	0.38	1	0.38	>256
45	R	R	R	R	R
MIC µl/ml	>256	>1024	>1024	>32	>256
47	S	S	S	S	R
MIC µl/ml	4	0.75	3	0.25	16
51	S	S	S	S	R
MIC µl/ml	4	1.5	2	0.38	>256
53	R	R	R	R	R
MIC µl/ml	>256	>1024	>1024	>32	>256
59	R	R	R	R	R
MIC µl/ml	>256	>1024	>1024	>32	>256
60	S	S	S	S	I
MIC µl/ml	3	0.75	3	0.16	16
61	I	S	I	S	I
MIC µl/ml	24	2	8	0.38	12
62	S	S	S	S	I
MIC µl/ml	1	0.25	0.38	0.064	12
63	S	S	S	S	I
MIC µl/ml	4	0.75	4	0.125	32
64	S	S	S	S	I
MIC µl/ml	1	0.16	0.25	0.064	16
65	S	S	S	S	I
MIC µl/ml	4	1	4	0.047	16
67	S	S	S	S	I
MIC µl/ml	2	0.25	0.75	1.5	12
68	S	S	R	R	R
MIC µl/ml	6	1.5	>1024	>32	>256

Annex 2. E-test results for 100 *P. aeruginosa* clinical isolates.

Strain	AK	TM	GM	CI	TX
69	I	S	I	S	R
MIC µl/ml	24	4	8	0.75	>256
70	S	S	I	S	R
MIC µl/ml	12	2	6	0.5	>256
72	S	S	I	S	I
MIC µl/ml	8	1.5	6	0.38	12
93	S	S	S	S	S
MIC µl/ml	3	0.5	1.5	0.5	8
94	S	S	S	S	R
MIC µl/ml	3	0.5	1.5	0.125	>256
95	S	S	I	S	I
MIC µl/ml	12	1.5	6	0.16	12
96	S	S	S	S	S
MIC µl/ml	4	0.5	1.5	0.125	6
97	S	S	I	S	I
MIC µl/ml	8	0.75	6	0.125	16
98	S	S	S	S	I
MIC µl/ml	8	1	4	0.064	16
99	S	S	S	S	I
MIC µl/ml	3	0.5	1.5	0.125	32
100	I	S	R	S	R
MIC µl/ml	24	4	24	0.25	>256
102	S	S	I	S	S
MIC µl/ml	12	1.5	8	0.25	8
103	S	S	I	S	I
MIC µl/ml	16	3	8	0.094	12
104	S	S	I	S	R
MIC µl/ml	4	1.5	6	0.094	>256
105	S	S	S	S	I
MIC µl/ml	4	0.38	2	0.19	12
106	S	S	S	S	I
MIC µl/ml	6	0.38	4	0.25	16
107	S	S	S	S	I
MIC µl/ml	3	0.5	2	0.125	12
108	S	S	S	S	I
MIC µl/ml	6	0.5	1.5	0.16	12

Annex 2. E-test results for 100 *P. aeruginosa* clinical isolates.

Strain	AK	TM	GM	CI	TX
109	S	S	S	S	R
MIC µl/ml	8	1	4	0.94	64
110	S	S	I	S	R
MIC µl/ml	12	4	6	1	35
111	S	S	S	S	R
MIC µl/ml	2	0.25	2	0.125	>256
112	S	S	S	S	S
MIC µl/ml	3	0.75	1.5	0.125	8
113	S	S	S	S	I
MIC µl/ml	3	4	4	0.125	32
116	S	S	S	S	I
MIC µl/ml	12	2	4	0.19	25
118	S	S	S	S	S
MIC µl/ml	4	0.38	1.5	0.19	8
119	S	S	S	S	I
MIC µl/ml	3	0.125	0.75	0.125	16
120	S	S	S	S	I
MIC µl/ml	6	1	1.5	0.125	24
121	S	S	R	S	R
MIC µl/ml	12	4	12	0.25	>256
122	S	S	S	S	R
MIC µl/ml	0.75	3	0.5	1.5	>256
123	S	S	S	S	I
MIC µl/ml	8	1	2	0.16	24
124	S	S	S	S	I
MIC µl/ml	6	3	3	0.125	16
125	S	I	R	S	R
MIC µl/ml	8	8	128	0.25	>256
126	S	S	S	S	I
MIC µl/ml	6	1	0.75	0.47	12
132	S	S	I	S	I
MIC µl/ml	12	1.5	6	0.38	12
133	S	S	S	S	R
MIC µl/ml	8	1.5	3	0.094	>256
135	S	R	R	R	R
MIC µl/ml	8	12	16	>32	>256
137	S	S	S	S	I
MIC µl/ml	6	1	1.5	0.094	24

Annex 2. E-test results for 100 *P. aeruginosa* clinical isolates.

Strain	AK	TM	GM	CI	TX
138	I	R	R	R	R
MIC µl/ml	32	>1024	>1024	>32	>256
139	S	S	S	S	I
MIC µl/ml	1.5	0.75	1.5	1	16
140	S	S	S	S	I
MIC µl/ml	4	1	1.5	0.19	24
141	S	S	S	S	R
MIC µl/ml	6	1	4	0.5	64
142	S	S	S	S	R
MIC µl/ml	1.5	1	3	0.38	34
144	S	S	S	S	I
MIC µl/ml	4	1	4	0.094	16
147	S	S	S	S	I
MIC µl/ml	8	1	4	0.5	16
148	S	S	S	S	I
MIC µl/ml	6	2	4	0.125	16
152	S	R	R	R	R
MIC µl/ml	16	96	>1024	>32	>256
154	S	S	S	S	I
MIC µl/ml	8	0.75	3	0.25	16
155	S	S	I	S	R
MIC µl/ml	12	2	6	0.19	96
156	I	S	R	S	I
MIC µl/ml	24	4	12	0.032	24
157	I	S	I	R	I
MIC µl/ml	24	3	6	>32	16
158	S	S	S	I	I
MIC µl/ml	3	0.38	1.5	2	32
159	S	S	S	S	R
MIC µl/ml	12	1.5	4	0.5	>256
160	S	S	S	S	R
MIC µl/ml	4	2	4	0.125	>256

Annex 2. E-test results for 100 *P. aeruginosa* clinical isolates.

Strain	AK	TM	GM	CI	TX
161	S	S	I	S	R
MIC µl/ml	16	4	8	0.64	>256
162	S	S	S	S	R
MIC µl/ml	6	0.75	2	0.25	>256
163	S	S	S	R	S
MIC µl/ml	0.5	0.125	0.38	4	4
166	S	S	S	S	I
MIC µl/ml	12	2	4	0.5	12
167	S	S	S	S	S
MIC µl/ml	4	0.094	1	0.094	8
168	R	S	S	R	S
MIC µl/ml	>256	4	1	4	0.016
169	S	I	S	R	S
MIC µl/ml	4	8	3	>32	0.016
170	S	S	S	S	I
MIC µl/ml	4	0.38	4	0.125	12
172	S	S	S	S	I
MIC µl/ml	6	0.38	2	0.25	16
174	S	S	S	S	I
MIC µl/ml	4	0.38	1.5	0.19	12
175	S	S	I	I	I
MIC µl/ml	12	1.5	6	2	25
176	S	S	I	S	R
MIC µl/ml	12	3	6	0.16	>256
177	S	S	S	S	R
MIC µl/ml	12	1.5	3	0.5	34

Annex 3. *Distribution of exoS and exoU genes among the 100 P. aeruginosa clinical isolates.*

Strain	ExoS	ExoU
PA3	+	-
PA4	-	+
PA5	+	-
PA9	+	-
PA14	+	-
PA15	+	-
PA16	+	-
PA17	-	+
PA19	-	+
PA20	+	+
PA21	+	+
PA25	+	-
PA26	+	-
PA28	-	-
PA29	-	-
PA32	-	+
PA34	-	+
PA36	-	+
PA37	-	-
PA43	-	-
PA44	-	+
PA45	+	+
PA47	-	+
PA51	-	+
PA53	+	-
PA59	-	+
PA60	+	-
PA61	-	+
PA62	+	-
PA63	+	-
PA64	-	-
PA65	-	-
PA67	+	-
PA68	-	+
PA69	-	+
PA70	-	+
PA72	+	-
PA93	-	+
PA94	-	-
PA95	+	-
PA96	+	-

Annex 3. Distribution of *exoS* and *exoU* genes among the 100 *P. aeruginosa* clinical isolates.

Strain	ExoS	ExoU
PA97	+	-
PA98	+	-
PA99	-	+
PA100	-	+
PA102	-	+
PA103	+	-
PA104	-	+
PA105	-	+
PA106	+	-
PA107	+	-
PA108	+	-
PA109	+	-
PA110	-	+
PA111	-	+
PA112	+	-
PA113	-	+
PA116	-	+
PA118	-	+
PA119	+	-
PA120	-	+
PA121	+	-
PA122	+	-
PA123	+	-
PA124	-	+
PA125	-	+
PA126	-	+
PA132	-	+
PA133	+	-
PA135	+	-
PA137	-	+
PA138	+	-
PA139	-	+
PA140	-	+
PA141	-	+
PA142	+	-
PA144	-	+
PA147	-	+
PA148	-	+
PA152	+	-
PA154	+	-
PA155	+	-

Annex 3. Distribution of *exoS* and *exoU* genes among the 100 *P. aeruginosa* clinical isolates.

Strain	ExoS	ExoU
PA156	-	+
PA157	+	-
PA158	-	+
PA159	+	-
PA160	-	+
PA161	-	-
PA162	+	-
PA163	+	-
PA166	+	-
PA167	-	+
PA168	+	-
PA169	-	+
PA170	-	+
PA172	+	-
PA174	-	+
PA175	+	-
PA176	+	-
PA177	-	+

Annex 4. Distribution of the isolates in the different biotypes based on their morphological characteristics.

Biotypes	Samples
B1	PA4,PA5,PA14, PA19, PA25, PA34, PA47, PA51, PA62, PA63, PA64, PA72, PA97, PA98, PA99, PA104, PA106, PA108, PA110, PA 113, PA 119, PA120, PA123, PA124, PA126, PA133, PA135, PA139,PA140, PA141, PA142, PA156, PA157, PA158, PA160, PA169, PA170, PA172, PA174, PA96, PA148, PA176
B2	PA100, PA121, PA162
B3	PA61, PA161, PA67
B4	PA15, PA16, PA17, PA65, PA68, PA70, PA93, PA94, PA95, PA103, PA107, PA112, PA144, PA147, PA155, PA163, PA167, PA168, PA21, PA26, PA29, PA32, PA69, PA102, PA125, PA137, PA159, PA166, PA175
B5	PA3, PA9, PA109, PA154, PA177
B6	PA44
B7	PA28, PA43, PA45, PA53, PA59, PA60, PA122
B8	PA105
B9	PA118
B10	PA20, PA36, PA111, PA116, PA132, PA138, PA152