An-Najah National University

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Comparison of the Distribution of Type III Exotoxin-Encoding Genes and Antibiotic Resistance Between Environmental and Clinical *Pseudomonas aeruginosa* Isolates in Northern West Bank-Palestine

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Dedication

To my Father, Mother, Sisters, Brothers and my husband Alaa Bsharat and his family, all the family members and friends.

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Above all, I would like to thank my husband, my family, specially my mother, father and my sister Bushra for their continuous love and support.

الإقرار

أنا الموقع أدناه، مقدم الرسالة التي تحمل العنوان:

Comparison of the Distribution of Type III Exotoxin-Encoding Genes and Antibiotic Resistance Between Environmental and Clinical *Pseudomonas aeruginosa* Isolates in Northern West Bank-Palestine

أقر بأن ما شملت عليه هذه الرسالة إنّما هو نتاج جهدي الخاص، باستثناء ما تمّت الإشارة إليه حيثما ورد، وأنّ هذه الرسالة ككل، أو أيّ جزء منها لم يقدّم من قبل لنيل أيّ درجة أو لقب علميّ لدى أيّ مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: **اسم الطالب:**

Signature: **التوقيع:**

Date: **التاريخ:**

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List of Abbreviations

ADPRT	ADP ribosyl transferase
°C	Celsius
CLSI	Clinical and Laboratory Standard Institute
CL	Cluster
CF	Cystic Fibrosis
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucloside triphosphate
ExoS	ADP-ribosylating enzymes exoenzyme
ExoT	Exoenzyme T
ExoY	Adenylate cyclase exoenzyme Y
ExoU	Exoenzyme U
gyrA	DNA gyrase (type II topoisomerase), subunit A
gyrB	DNA gyrase, subunit B
HCL	Hydrochloric acid
ICUs	intensive care units
I	Intermediate
L	Ladder
MDR	Multidrug-resistant
MIC	Minimum Inhibitory Concentration
MHA	Mueller Hinton agar
$MgCl_2$	Magniusim chloride
min	Minutes
MDCK	Madin-Darby Canine Kidney
NC	Negative control
NB	Nutrient broth
P. aeruginosa	*Pseudomonas aeruginosa*
PCR	Polymerase chain reaction
R	Resistant
RAPD	Random amplified polymorphic DNA
SIM	Sulfide indole motility
S	Susceptible
Sec	Second
SPSS	Statistical package for the social sciences
T1SS	Type I secretion system
T3SS	Type III secretion system
TSI	Triple sugar iron
T2SS	Type II secretion system
UPGMA	Unweighted pair group method for arithmetic average

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Abstract

Pseudomonas aeruginosa isolate is capable of producing certain virulence factors, expression of these virulence factors in a specific infection plays an essential role in the ability of this pathogen to establish and maintain infection. A total of 57 *P. aeruginosa* isolates were collected from different sources of clinical (n=47) and environmental samples (n=10) during 2018. These samples were collected from different health care centers, North West Bank-Palestine. This study aimed to compare the distribution of the type III exotoxin-encoding genes and antibiotic resistance between *P. aeruginosa* strains that were isolated from both sources, and to determine the presence of a correlation between them in both clinical and environmental isolates collected from a limited geographical area, in parallel, over a limited period of time. In addition, clone identity among the clinical and environmental strains was determined and compared using molecular typing technique. Results of this study showed that isolates recovered from clinical and environmental sources showed high resistant to Tetracycline and Kanamycin. Results of the current research using multiplex PCR technique showed that all clinical and environmental *P. aeruginosa* isolates carried type III secretion toxins-encoding genes. The

exoT gene was detected among all clinical and environmental isolates. The occurrence of *exoY* and *exoS* genes in *P. aeruginosa* isolates from both sources was 80.7% and 36.8%, respectively. Combination of the toxin genes was noted in 87% of clinical and 70% of environmental isolates. The most common combination was *exoT* and *Y*, the prevalence was 47% and 50% for clinical and environmental isolates, respectively. The *exoU* gene was not detected in isolates from both sources. Statistical analysis using Pearson Chi-Square test showed that there is no significant difference between the distribution type III secretion toxins-encoding genes and source of isolate (clinical or enivironmental). In addition, statistical analysis indicated that there was not a significant association between *P. aeruginosa* isolates type III exotoxin-encoding genes and resistant to some antibiotics such as Ciprofloxacin, Norfloxacin, Levofloxacin, Aztreonam, Tetracycline and Kanamycin. The profile of RAPD-PCR typing of 44 *P. aeruginosa* isolates (9 environmental recovered from different hospital sinks and 35 clinical isolates recovered from different hospitals were clustered into 3 clusters at 96% similarity level. Cluster-1 and cluster-2 had strains recovered from different hospitals and belonged to both environmental and clinical sources. Cluster-3 had identical RAPD-PCR profile strains, recovered from clinical sources only from different hospitals. In addition, statistical analysis showed that there was no significant difference between strains isolated from both environmental and clinical sources according to antibiotic resistance and occurrence of type III secretion toxins-encoding genes.

In conclusion, according to RAPD-PCR typing there is a high similarity between environmental and clinical *P. aeruginosa* isolates. In addition, isolates from both sources had no significant differences in antibiotic resistance and distribution of type III secretion toxins-encoding genes. This may indicate that isolates from both sources have ability to cause clinical infection.

Chapter One
Introduction

1.1. General Background

1.1.1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative, non-fermentative, oxidase-positive and motile eubacterium that ubiquitous in natures. This microorganism is considered one of the most imoprtant opportunistic human pathogen. *Pseudomonas aeruginosa* is considered one of the major causative agent of human infection, particularly in patients requiring mechanical ventilation, patient with deficient defense mechanisms, burn victims and patients with cancer and cystic fibrosis (Garau and Gomez, 2003; Valencia and Torres, 2009; Elsen *et al*., 2014; Azimi *et al*., 2016). Many studies showed that this opportunistic pathogen can cause infections in healthy persons (Hatchette *et al*., 2000; Lo *et al*., 2000; McCallum *et al*., 2002; Kang *et al*., 2005). *Pesudomonas aeruginosa* is a common microorganism in the environment, it can be a cause of community acquired infections. In addition, *P. aeruginosa* is considered a major cause of hospital-acquired infections via colonization of catheters, skin wounds, ventilator-associated pneumonia, and is also a cause of respiratory infections in inpatients with cystic fibrosis (CF) (Wagner *et al*., 2008). In intensive care units (ICUs), this opportunistic human pathogen is considered the most frequent to cause death of patients with ventilator-associated pneumonia (Barbier *et al*., 2013; Bassetti *et al*., 2012).

In contrast to other pathogenic microorganisms, genomes of clinical and environmental *P. aeruginosa* isolates are highly conserved and both clinical and environmental *P. aeruginosa* isolates are qualified for producing virulence factors and are thus potential pathogens (Hardalo and Edberg, 1997; Pirnay *et al*., 2009). The genome sequences and phenotypic characterization for four non-human *P. aeruginosa* strains collected from different sources, including: the ocean, a water-spring, a plant and from dolphin stomach, showed that these four non-human *P. aeruginosa* strains have phenotypic distinctions with regard to the production of virulence factors and motility, this phenotypic distinction is not due to genomic variation, since genomes of these strains had a very high degree of similarity and highly conserved (Grosso-Becerra *et al*., 2014). Moreover, there are other evidences had proposed no great differences between both clinical and environmental isolates in respect to the virulence factors, such as genes of clone and pilin-type distributions (Römling *et al*., 1994), pilin genes (Spangenberg *et al*., 1995), flagellin genes (Morgan *et al*., 1999), genes of haemolytic and proteolytic activities, genes of invasion of epithelial cells (Alonso *et al*., 1999), genes for multidrug efflux, type III secretion system (T3SS) and porin gene *oprD* (Pirnay *et al*., 2002).

The intrinsic and acquired resistance of *P. aeruginosa* to many different classes of antibiotics is due to adaptation of this microorganism to several mechanisms, including active efflux systems, plasmid acquisition, reduced cell wall permeability, expression of various enzymes, or by biofilm formation (Allydice-Francis and Brown, 2012). Infections caused by this

microorganism become a real health problem in hospital settings (Peterson, 2009) due to that the *P. aeruginosa* showed high level of resistance to almost all available antibiotic classes.

Pseudomonas aeruginosa is capable of producing different types of virulence factors. These can be divided into different groups depending upon their mode of actions or mechanism of delivery to the host cell. The major virulence factors of *P. aeruginosa* include those involved in adherence (flagella, type IV pili, lipopolysaccharides), antiphagocytosis (alginate) and iron uptake (siderophores pyocynin and pyoverdine). Secreted exotoxins are another type of virulence factors that either passively secreted from the *P. aeruginosa* cell or actively by different types of secretion systems including the type I secretion system (T1SS), the type II secretion system (T2SS) or the type III secretion system (T3SS) (Bradbury *et al*., 2010; Kiseleva and Novik, 2015). The expression of these virulence factors in a specific *Pseudomonas* infection is of fundamental importance in the ability of *P. aeruginosa* strain to set up and maintain infection (Bradbury *et al*., 2010).

1.1.2. Type III secretion system (T3SS)

1.1.2.1. Effector toxins of T3SS

Generally, most clinical *P. aeruginosa* isolates have ability to produce many vital virulence factors and involved a specific specialized system associated with the cell wall of bacteria, which is utilized to inject the virulence toxins into cytoplasm of the infected host cells. This is known as

the T3SS. In general, T3SS is existing in most pathogenic Gram-negative bacillus, including *Escherichia coli*, *Salmonella* spp., *Yersinia* spp., and *Shigella* spp. (Ajayi *et al*., 2003; Trautmann *et al*., 2005; Bradbury *et al*., 2010; El-Solh *et al*., 2012; Sawa *et al*., 2014).

The T3SS is composed of a complex syringe-like system, exists on the surface of bacteria and involved five distinguished groups of proteins. These proteins are: the needle complex (needle-like nanomachine), the translocation apparatus or tool, regulator proteins, chaperones and effector toxins. The needle complex protein has ability to transport the effector toxins from the bacterial cytoplasm into the outside of bacterial. The translocation apparatus is a membrane pore that takes the effector proteins secreted by the needle complex and conveys them across the target host cell plasma membrane. In *P. aeruginosa*, the T3SS has three proteins for translocation, these including PopB, PopD and PcrV (Galle *et al*., 2012). These three translocation proteins are secreted by the T3SS and are surely required for the pore formation and the translocation of effectors proteins toward the host cell membrane (Galle *et al*., 2012). *Pseudomonas aeruginosa* strains have four recognised secretion effector toxins including ADP-ribosylating enzymes exoenzyme (ExoS), exoenzyme T (ExoT), adenylate cyclase exoenzyme Y (ExoY) and acute cytolytic factor (a phospholipase) exoenzyme U (ExoU). Although the T3SS is not required for the infection, the *P. aeruginosa* T3SS promotes disease severity in a several of animal models, including keratitis, peritonitis, burn infections, bacteremia, acute pneumonia and gut-derived sepsis in the setting of

neutropenia (Hauser, 2009). It was reported that these effector proteins alter a variety of cellular functions for pathogen's advantage such as destroy signal transduction pathways and avoid host cell innate immunity depending on the T3SS effectors encoded (Engel and Balachandran, 2009).

1.1.2.1a. ADP-ribosylating enzymes exoenzyme S (ExoS)

It has been shown that ExoS is considered one of the major cytotoxin involved in many functions such as colonization, invasion and spreading during infection (Lee *et al*., 2005). It was shown that increased concentrations of ExoS protein get together with increased pulmonary spoilage in cystic fibrosis patients, in animal models and *in vitro* cytotoxicity (Galle *et al*., 2012). In addition, ExoS ADP-ribosylates small Ras-like proteins, have ability to inhibit internalization, DNA synthesis and induction apoptotic-like cell death (Vance *et al*., 2005; Engel ,2003). It has also been reported that expression of the ADP ribosyl transferase (ADPRT) domain of ExoS protein is toxic to cultured cells (Barbieri and Sun; 2004).

1.1.2.1b. Exoenzyme T (ExoT)

Product of *exoT* gene or expression of *exoT* gene intervenes with phagocytic process of host cell (Vance *et al*., 2005; Barbieri and Sun; 2004), induction the death in *Galleria mellonella* expressed *exoT* gene (Miyata *et al*., 2003), protects culture cells from T3SS-dependent lysis *in vitro* (Lee *et al*., 2005), implicated in wound healing lateness and cytokinesis inhibition (Galle *et al*., 2012), and apoptosis stimulation in HeLa cells (Shafikhani *et al*., 2008). In addition, it has been shown in mice

that secretion of *ExoT* gene is associated with disseminating the infection from the mice lungs to the liver (Shaver and Haucer, 2004).

1.1.2.1c. Acute cytolytic factor (a phospholipase) exoenzyme U (ExoU)

The ExoU effector protein has activity of phospholipase A2 enzyme and it has been considered as a protein belong to the family of phospholipases (Sitkiewicz *et al*., 2007). This protein is capable of causing necrosis by eukaryotic cell lysis membrane induction (Gendrin *et al*., 2012). The ExoU effector protein is considered the most potent cytotoxin, it occurs in approximately 30% of *P. aeruginosa* strains that are associated with highly severe infections, including septicemia (Elsen *et al*., 2014). Also, this ExoU effector protein has significant remarkably rapid and fulminant cytotoxic effects (Vance *et al*., 2005; Engel, 2003; Finnan *et al*., 2004). In particular, ExoU in murine models has a correlationship with lung damage and severe cytotoxicity in both macrophages and epithelial cells (Finck-Barbancon *et al*., 1997), and has association with acute and severe *P. aeruginosa* infections in humans where products of *exoU* gene can kill neutrophils specifically. It has been shown that *exoU* gene recombinant *P. aeruginosa* strains and can express products of *exoU* gene, these strains showed a high pathogenicity in a murine model of acute pneumonia and systemic spread (Allewelt *et al*., 2000). In *P. aeruginosa* strains causing bloodstream infection, it has been proposed an association between the product of *exoU* gene and invasive infection (Wareham and Curtis, 2007). In contrast, in BALB/c mice no correlation was noticed with colonization and invasion (Lin *et al*., 2006). It has been shown that deletion or mutation in *exoU* gene

leads to severe reduction in the *P. aeruginosa* strains toxicity in the lung infections, product of *exoU* gene has been involved as a factor associated with septic shock and disease severity and mortality in pneumonia (Engel, 2003; Vance *et al*., 2005; Wong-Beringer *et al*., 2008).

1.1.2.d. Adenylate cyclase exoenzyme Y (ExoY)

ExoY is an adenylate cyclase that requires an unidentified host cell cofactor for it activity. Its role in virulence remains in doubt, the product of *exoY* gene still had a little important effect in murine pneumonia (Lee *et al*., 2005), but it had a major effect on cytotoxicity in MDCK cells (Lin et al., 2006), the product of ExoY gene when expressed in yeast cells had been shown to be toxic (Arnoldo *et al*., 2008) and shown to induce cell rounding upon cocultivation with eukaryotic immune cells (Cowell *et al*., 2005).

1.2. Literature Review

The occurrence rate of *exoS*, *exoT*, *exoU* and *exoY* in clinical and environmental *P. aeruginosa* isolates has been previously reported. In several previous published studies, it was found that all tested clinical and environmental *P. aeruginosa* isolates had *exoT*-like sequences (Feltman *et al*., 2001; Strateva *et al*., 2010; Gawish *et al*., 2013; Adwan, 2017). In other studies, the occurrence rate of *exoT* gene sequences in *P. aeruginosa* isolates recovered from both environmental and clinical sources had a range from 5%-92% (Lomholt *et al*., 2001; El-Solh *et al*., 2012; Azimi *et al*., 2016). The occurrence rate of the *exoY* gene among *P. aeruginosa* isolates had a range from 55%-97% (Dacheux *et al*., 2000; Feltman *et al*.,

2001; Strateva *et al*., 2010; Gawish *et al*., 2013; Azimi *et al*., 2016; Adwan, 2017). Several studies showed that the occurrence rate of the *exoS* gene sequences among *P. aeruginosa* isolates had a range 0%-96% (Woods *et al*., 1986; Rumbaugh *et al*., 1999; Feltman *et al*., 2001; Lomholt *et al*., 2001; Berthelot *et al*., 2003; Strateva *et al*., 2011; El-Solh *et al*., 2012; Gawish *et al*., 2013; Azimi *et al*., 2016; Adwan, 2017). Finally, the occurrence rate of *exoU* gene sequences reported in previously published studies among *P. aeruginosa* isolates had a range from 0%-80% (Fleiszig *et al*., 1997; Allewelt *et al*., 2000; Hirakata *et al*. 2000; Feltman *et al*., 2001; Lomholt *et al*., 2001; Berthelot *et al*., 2003; Strateva *et al*., 2011; El-Solh *et al*., 2012; Gawish *et al*., 2013; Azimi *et al*., 2016; Adwan, 2017).

Pseudomonas aeruginosa isolated from different vegetables showed that 64% of the isolates harboured an exoenzyme gene. It has been shown that *exoS, exoT, exoU,* and *exoY* gene sequences were detected in 6%, 40%, 33%, and 30% of *P. aeruginosa* isolates, respectively. Also, the study showed that the *exoS* gene was not found in combination with other exoenzyme genes. The *exoT, exoU,* and *exoY* gene sequences were found in a combination in 14% of total tested *P. aeruginosa* isolates (Allydice-Francis and Brown, 2012). *Pseudomonas aeruginosa* isolates recovered from hospital environments showed that all of these isolates carried exoenzyme gene sequences. The *exoY* and *exoT* gene sequences were detected in all *P. aeruginosa*, while 80% and 25.7% for *exoS* and *exoU* gene sequences, respectively (Btadbury *et al*., 2010). In addition, it has been noted that *P. aeruginosa* isolates recovered from the environmental

sources were found to be significantly more likely to carry *exoU* gene sequences than other isolates (Btadbury *et al*., 2010). It appears that *exoU* gene transmitted horizontally on plasmid and then integrated into the *P. aeruginosa* chromosome (Kulasekara *et al*., 2006). In other study, it has been shown that there was no significant difference in the occurrence rate of type III exoenzyme gene sequences between both nosocomial and environmental *P. aeruginosa* isolates or between isolates recovered from different clinical infection sites (Gawish *et al*., 2013).

A correlation between the antimicrobial resistance of the *P. aeruginosa* and an *exoU*$^{+ve}$ genotype has been reported in different clinical studies. Analysis the antimicrobial resistance profiles and genotypes of the studied *P. aeruginosa* isolates that recovered from patients suffered from cystic fibrosis and non-cystic fibrosiss (Mitov *et al*., 2010). The authors found that the occurrence rate of *exoU* gene sequences was more significant among multidrug-resistant (MDR) *P. aeruginosa* strains; even the occurrence rate of *exoS* gene sequences among tested *P. aeruginosa* isolates was higher than the occurrence rate of *exoU* gene sequences. In other study, it has been shown that *P. aeruginosa exoU*$^{+ve}$ isolates recovered from blood samples were significantly more likely to be MDR, these strains were resistant to different antibiotic classes including: cephems, carbapenems, fluoroquinolones, and gentamicin (Garey *et al*., 2008). A high frequency of antibiotic resistance in the T3SS^{+ve} *P. aeruginosa* isolates was observed to ciprofloxacin (59%), cefipime (35%), and gentamycin (38%). In addition, it has been observed a high resistant

frequency for gentamicin among *P. aeruginosa* isolates producing ExoU toxin but not ExoS toxin (El-Solh *et al*., 2012). Some studies have linked between the clinical MDR *P. aeruginosa* and T3SS phenotypes **(**Zhu *et al*., 2006; Wong-Beringer *et al*., 2008). The association between fluoroquinolone-resistant and clinical isolates of T3SS^{+ve} *P. aeruginosa* was published previously (Wong-Beringer *et al*., 2008; Agnello and Wong-Beringer, 2012). It was found a higher proportion of *exoU*$^{+ve}$ *P. aeruginosa* isolates were more likely to be fluoroquinolone-resistant in comparison to exoS^{+ve} *P. aeruginosa* strains and about all *exoU*$^{+}$ *P. aeruginosa* strains expressed ExoU toxin (Agnello and Wong-Beringer, 2012; Cho *et al*., 2014).

1.3. Aims of the Study

This study aimed to compare the distribution of the type III exotoxin-encoding and antibiotic resistance between *P. aeruginosa* strains that were isolated from clinical and environmental sources, and to explore the presence of a correlation between them, isolates collected from a limited geographical area, in parallel, over a limited period of time. In addition, clone identity among the clinical and environmental strains was determined and compared using molecular technique.

Chapter Two
Materials and Methods

2.1. Bacterial Isolates Collection and Identification

A total of 57 isolates of *P. aeruginosa* were collected from different sources of clinical (n=47) and environmental samples (n=10) during 2018 (Table 2.1). Both clinical and environmental samples were collected from different health care centers, North West Bank-Palestine. These centers were An-Najah National University Hospital-Nablus, Alwatany Hospital-Nablus, Tubas Turk Hospital –Tubas, Thabet Thabet Hospital-Tulkarm and Jenin Hospital -Jenin. These isolates were identified using biochemical tests such as culture of these isolates on MacConkey agar. Gram stain was carried out for all isolates, detection of growth at 42°C in addition to other biochemical tests such as indole production, citrate utilization, oxidase test, urease test, glucose fermentation, H2S production and motility test.

Table 2.1. A sample source of 57 of *P. aeruginosa* isolates collected from different hospitals.

Hospital	Sample source (n)												Total
	wound	urine	tissue	Blood	Bed sore	nasal	fluid	skin	sputum	swabs	umbilicus	Environment (sink)	
N	5	4	1	3	1	0	0	0	0	0	0	0	14
W	0	0	0	0	1	0	0	0	0	0	0	0	1
T	1	1	0	0	0	3	0	0	0	0	0	3	8
J	1	7	0	1	0	0	1	2	4	3	0	7	26
TH	2	1	0	0	0	0	0	0	1	3	1	0	8
Total	9	13	1	4	2	3	1	2	5	6	1	10	57

N: An-Najah National University Hospital; W: Alwatany Hospital; T: Tubas Turk Hospital; J: Jenin Hospital; TH: Thabet Hospital.

2.2. Media Preparation

2.2.1. MacConkey agar

The preparation of MacConkey agar (BioMaxima) was performed according to the manufacturer's instructions. A two-liter flask containing a total of 1 L of deionized H_2O and 51.55 gram of the medium was heated and stirred until completely dissolved. The solution was allowed to boil for one minute, autoclaved at 121°C for 15 minutes. Then the melted medium was allowed to cool (nearly to 50-55°C). After that, it was poured in Petri dishes to have approximately 25-30 ml in each, then they were covered and allowed to solidify and sorted at 4°C in refrigerator.

2.2.2. Mueller Hinton agar (MHA)

The preparation of MHA (HIMEDIA, INDIA) was performed according to the manufacturer's instructions. A two liter flask containing a total of 1 L of deionized H_2O and 38 gram MHA, was heated and stirred until completely dissolved. The melted medium was allowed to boil for one minute, autoclaved at 121°C for 15 minutes. Then the medium was allowed to cool (nearly to 50-55°C). After that, the medium was poured in Petri dishes to have approximately 20-30 ml in each. Then, they were covered and allowed to solidify and sorted at 4°C in refrigerator.

2.2.3. Nutrient broth (NB)

Nutrient broth (ACUMEDIA, USA) was prepared according to manufacturer's instructions labeled on the bottle. In a 0.5 L bottle, 2 g of NB and 248 ml deionized water were mixed and dissolved well. The broth was then distributed into tubes to have 7 ml each and plugged with cotton. The tubes were autoclaved at 121°C for 15min, allowed to cool and then kept at 4-6°C in refrigerator.

2.2.4. Sulfide indole motility (SIM) medium

SIM medium (Acumedia, USA) was prepared according to the manufacturer's instructions. A 250 ml flask containing 97 ml deionized water and 3 g of SIM agar were heated and stirred until dissolved. Medium was dispensed into tubes to give a depth of about 7-10 cm. The tubes were plugged tightly with cotton then covered with a piece of aluminum foil, then autoclaved at 121°C for 15 min, allowed to cool by leaving the tubes on a rack to form deep agar, and then stored in refrigerator at 4°C.

2.2.5. Triple sugar iron (TSI) agar

TSI agar (Acumedia, USA) was prepared according to the manufacturer's instructions. A 250 ml flask containing 93.5 ml deionized water and 6.5 g of TSI agar were mixed thoroughly and heated to dissolve. Ten ml of Triple sugar iron medium was dispensed into tubes, plugged with a piece of cotton which then covered with aluminum foil. The tubes were autoclaved

at 121°C for 15 min, and then the tubes were left to cool and solidify in a slant position. Finally, the tubes were stored in refrigerator at 4°C.

2.2.6. Simmons citrate agar

Simmons citrate agar (Acumedia) was prepared according to the manufacturer's instructions. A 0.5 L bottle containing 250 ml deionized water and 11.25 g of Simmons citrate agar was heated and stirred until dissolved. Ten ml of Simmons citrate agar was dispensed into tubes, autoclaved at 121°C for 15 min. The medium was prepared as slant agar tubes and then stored in refrigerator at 4°C.

2.2.7. Urea agar medium

Urea agar (Oxoid) was prepared according to the manufacturer's instructions. A 0.5 L bottle containing 4.8g of urea agar base medium and 190 ml of distilled water. The mixture boiled to dissolve completely, then sterilized by autoclaving at 121°C for 15 min. The medium cooled, 10 ml of sterile 40% urea solution SR0020 was added aseptically to the solution and mixed well. Then, the urea agar was dispensed into sterile tubes each contains 10ml and allowed to set in the slant position. After that, the tubes stored in refrigerator at 4°C.

2.3. Sample Identification

2.3.1. Gram staining

Gram staining was performed to distinguish Gram-positive bacteria from Gram-negative bacteria. A thin smear of bacteria was made on a clean glass slide by picking the isolates from marked colonies after 24 hours incubation on Meuller-Hinton agar plate and mixed with a drop of distilled water. The smear was heat fixed by passing through a flame 2 or 3 times. Care was taken to avoid air bubbles formation and overheating to prevent distortions of the glass slide. After cooling, the slide was flooded with primary stain (crystal violet) and left for one minute then washed with tap water. Gram's iodine solution was then added for one minute and again washed off with tap water, decolorized with acetone alcohol from 10 to 20 seconds and then again washed with tap water. Finally, the slide was flooded with a counter stain (safranin) for 1 minute, washed with tap water and blotted to dry with tissue paper. The smear was observed under 100x objective lens of the light microscope (Cappuccino and Sherman, 1996).

2.3.2. Indole test

The SIM tube used to detect the presence of tryptophanase enzyme that catabolizes tryptophan producing indole. This was carried out by adding 10 drops of Kovac's reagent after 24 h of inoculation. Incase of indole positive bacteria, Kovac's reagent will combine with indole producing a red color at the top of the agar tube. The absence of red color change after the addition

of Kovac's reagent, indicates an indole negative bacteria (Cappuccino and Sherman, 1996).

2.3.3. Motility test

In the SIM agar deep tube inoculated by mean of stab method, a motile organism, caused a turbidity or diffused growth and the stab line is unclear. In case the organism was nonmotile, the growth was confined to the stab line and the medium showed very clear (Johnson and case, 1998).

2.3.4. H_2S production

The formulation of SIM Medium is also designed to allow the detection of sulfide production. The medium contains ferrous ammonium sulfate and sodium thiosulfate, which together serve as indicators for the production of hydrogen sulfide. Hydrogen sulfide production is detected when ferrous sulfide, a black precipitate, is produced as a result of ferrous ammonium sulfate reacting with H_2S gas.

2.3.5. Citrate utilization test

Citrate utilization test was carried out by stabbing the butt and streaking the slant of Simmons citrate agar. The tube was then incubated at 37°C for 24 h. Citrate positive result was indicated by either change in the color of the medium from green to blue or growth of bacteria in the medium or both. Lack of growth on citrate medium and lack of color change indicates a citrate negative result (Cappuccino and Sherman, 1996).

2.3.6. Oxidase test

Oxidase disks were used in this test, take one colony from the bacteria then touch oxidase disk. Formation of an intense blue color within 10 second is indicative of positive result (Cappuccino and Sherman, 1996).

2.3.7. Urease test

Incubation a tube of urea agar with the bacteria at 37°C for 24 hours if the bacteria produce urease the pH become alkaline and the color changed from orange-pink to dark pink this indicates positive results (Cappuccino and Sherman, 1996).

2.3.8. Triple sugar iron test

This test was carried out by stabbing the butt and streaking the slant of TSI agar tube by a zig-zag pattern. The tube was then incubated at 37°C for 24 h. bacterial cells sub-cultured on TSI agar showed the whole media had a yellow color due to fermentation of glucose and lactose (acid/acid), splitting

of the agar (gas production), and no blackening of the agar (No H2S production) (Cappuccino and Sherman, 1996).

2.3.9. Growth at 42°C

Each *Pseudomonas aeruginosa* isolate was subcultured on MacConkey agar plate and incubated at 42°C for 24 h.

2.4. Antibacterial Susceptibility Test

Antimicrobial sensitivity testing was conducted according to instructions determined by the Clinical and Laboratory Standard Institute (CLSI) using the disk diffusion method (CLSI, 2016). All *P. aeruginosa* isolates were examined using disks (Oxoid) to determine resistance against, Ciprofloxacin (5 µg), Kanamycin (30 µg), Tetracycline (30 µg), Norfloxacin (10 µg), Aztreonam (30 µg) and levofloxacin (5 µg). Mueller Hinton agar plates were seeded with a 6-8 h old culture of the *P. aeruginosa* strains, antibiotic disks were placed on the Muller-Hinton agar plates containing the inoculum. Then, the plates were incubated at 37°C for 24 h. The inhibition zones were measured and the isolates were classified as resistant or susceptible according to the criteria recommended by CLSI guidelines (CLSI, 2016). Isolates resistance to three or more drugs were considered multi-drug resistant (MDR).

2.5. DNA Extraction and PCR Amplification

2.5.1. DNA extraction

P. aeruginosa genome was prepared for PCR according to the method described previously (Adwan *et al*., 2013). Briefly, cells were scraped off an overnight MHA plate, washed with 800 µl of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]), then the pellet was resuspended in 400 µl of sterile double distilled H_2O, and boiled for 10-15 min. Then, the cells were incubated on ice for 10 min. The debris was pelleted by centrifugation at 11,500 X *g* for 5 min. Concentration of DNA was

determined using a nanodrop spectrophotometer (Genova Nano, Jenway) and the DNA samples were stored at -20ºC for further analysis.

2.5.2. PCR amplification of type III secretion toxins-encoding genes

Detection of gene sequences encoding for type III secretion toxins (ExoS, ExoT, ExoU and ExoY) was performed by the multiplex PCR using specific oligonucleotide primer sets as described previously (Ajayi *et al*., 2003). Primer sequences and size of amplicons are presented in Table 2.2 The PCR reaction mix with a final volume of 25 µl, was performed with 12.5 µl of PCR premix (ReadyMix^{TM} Taq PCR Reaction Mix with $MgCl_{2,}$ Sigma), 0.3 µM of each primer, 0.3 mM dNTP, 2.25 mM $MgCl_{2,}$ 1.25 U Taq DNA polymarase and 3 µl (100-200 ng) of DNA template. A negative control without DNA template was also included. The cycling conditions were: initial denaturation at 94 ºC for 3 min; followed by 36 cycles of denaturation at 94ºC for 40 s, annealing at 56ºC for 40 s and extension at 72ºC for 1 min; were followed by a single final extension step at 72 ºC for 5min. The PCR products were then detected by electrophoresis through 1.5% agarose gels to determine the size of amplified fragment after staining with a final concentration 0.5 µg/ml of ethidium bromide dye. Positive control strain encoding type III secretion toxins (department collection) was used.

Table 2.2. Target genes for PCR amplification, amplicon size and primer sequences that were used in this study.

Target gene	Primer sequence (5′→3′)	Amplicon size (bp)	Reference
exoS	Exo S F: GCG AGG TCA GCA GAG TAT CG Exo S R: TTC GGC GTC ACT GTG GAT GC	118	Ajayi *et al*., 2003
exoT	Exo T F: AAT CGC CGT CCA ACT GCA TGC G Exo T R: TGT TCG CCG AGG TAC TGC TC	152	Ajayi *et al*., 2003
exoU	Exo U F: CCG TTG TGG TGC CGT TGA AG Exo U R: CCA GAT GTT CAC CGA CTC GC	134	Ajayi *et al*., 2003
exoY	Exo Y F: CGG ATT CTA TGG CAG GGA GG Exo Y R: GCC CTT GAT GCA CTC GAC CA	289	Ajayi *et al*., 2003

2.5.3. Random amplified polymorphic DNA (RAPD) PCR

Random amplified polymorphic DNA PCR was performed using Primer RAPD 208: 5`-ACG GCC GAC C-3`. Each PCR reaction mix (25 µl) was composed of 10 mM PCR buffer pH 8.3; 3 mM MgCl2; 0.4 mM of each dNTP; 0.8 µM primer; 1.5U of Taq DNA polymerase and 3 µl (100-200 ng) of DNA template. DNA amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation for 3 min at 94ºC; followed by 35 cycles of denaturation at 94ºC for 50 seconds, annealing at 45ºC for 1 min and extension at 72ºC for 1 min, were followed by a final extension step at 72ºC for 5 min. The PCR products were analyzed by electrophoresis using 1.7% agarose gel. The gel image was scored using binary scoring system that recorded the absence and presence of bands as 0 and 1, respectively. A binary matrix was analyzed by the unweighted pair group method for arithmetic averages (UPGMA), using SPSS statistical software version 20 (IBM).

2.6. Statistical Analysis

The association between the antibiotic resistance and occurrence of type III secretion toxins-encoding genes in *P. aeruginosa* isolates, the correlation between type III secretion toxins-encoding genes and source of isolate (clinical or environmental) was evaluated by the Fisher exact test using SPSS version 20. *P* values larger than 0.05 were regarded as not significant.

Chapter Three
Results

3.1. Identification of Isolates

Clinical *P. aeruginosa* isolates were identified by API 20 E system in laboratories of hospitals from where the isolates were collected. Environmental isolates were identified by biochemical tests at An-Najah National University. All isolates of *P. aeruginosa* pathogen (clinical and environmental) inoculated on MacConkey agar had brown colonies while on Mueller Hinton agar had pyocynin pigment (Blue-Green) and pyoverdine (Yellow-Green fluorescent). Gram stain showed that *P. aeruginosa* was Gram negative bacteria (red or pink color) and had rod shape. Isolates of this pathogen had ability to grow at 42ºC. Biochemically, all isolates were oxidase positive, citrate positive, indole negative, urease negative, H_2S production negative and glucose fermentation negative.

3.2. Antibiotic Susceptibility

A total of 57 isolates (47 clinical isolates and 10 environmental isolates) of *P. aeruginosa* were tested for antibiotic susceptibility. Isolates recovered from clinical sources showed high resistant to Tetracycline and Kanamycin with a range from 62%-68%. The same thing, isolates recovered from environmental sources demonstrated high resistant to Tetracycline and Kanamycin with a range from 60%-80%. Clinical isolates showed low resistant rate (17%) against Aztreonam, while environmental isolates showed low resistant rate (20%) against Norfloxacin. Statistical analysis

showed that there was no significant difference between strains isolated from both environmental and clinical sources according to antibiotic resistance. Fisher exact test showed that the *p* values were 0.2597, 1, 0.727, 0.084, 1 and 1 for Ciprofloxacin, Norfloxacin, Levofloxacin, Aztreonam, Tetracycline and Kanamycin resistance, respectively. The antibiotic resistance profile of 57 *P. aeruginosa* isolates recovered from clinical and environmental sources is presented in Table 3.1. Results in this study demonstrated that 42% of *P. aeruginosa* isolates were MDR.

Table 3.1. Antibiotic resistance profile of 57 *P. aeruginosa* isolates collected from different clinical and environmental sources.

Antibiotic	Antibiotic resistance n (%)								
	Clinical source n=47			Environmental source n=10			Total n=57		
	S	I	R	S	I	R	S	I	R
Ciprofloxacin	27 (57.4 %)	3 (6.3 %)	17 (36.3 %)	3 (30 %)	2 (20 %)	5 (50 %)	30 (52.6 %)	5 (8.7 %)	22 (38.7 %)
Norfloxacin	27 (56 %)	6 (12 %)	14 (32 %)	6 (60 %)	2 (20 %)	2 (20 %)	33 (57 %)	8 (14 %)	16 (29 %)
Levofloxacin	27 (57 %)	1 (2 %)	19 (41 %)	6 (60 %)	1 (10 %)	3 (30 %)	33 (58 %)	2 (3.5 %)	22 (38.5 %)
Aztreonam	25 (53.1 %)	14 (29.7 %)	8 (17.2 %)	3 (30 %)	2 (20 %)	5 (50 %)	28 (49.1 %)	16 (28%)	13 (22.9 %)
Tetracycline	9(19.1 %)	6 (12.7%)	32 (68.2%)	2 (20 %)	2 (20 %)	6 (60 %)	11 (19.2 %)	8 (14 %)	38 (66.8 %)
Kanamycin	7(14 %)	12 (24 %)	28 (62 %)	1 (10 %)	1 (10 %)	8 (80 %)	8(14.1 %)	13 (22.8 %)	36 (63.1 %)

n: number of isolates; S: Susceptible; I: Intermediate; R: Resistant

3.3. Detection type III secretion toxins-encoding genes

Results of this research using PCR technique showed that all clinical and environmental *P. aeruginosa* isolates carried type III secretion toxins-encoding genes. The *exoT* gene was detected among all clinical and environmental isolates. The occurrence of *exoY* and *exoS* genes in *P. aeruginosa* isolates from both sources was 80.7% and 36.8%, respectively. Combination of the toxin genes was noted in 87% of clinical and 70% of environmental isolates. The most common combination was *exoT and Y*, the prevalence was 47% and 50% for clinical and environmental isolates, respectively. The *exoU* gene was not found in these isolates. Results of type III secretion toxins-encoding genes detected by multiplex-PCR are presented Table 3.2 and Figure 3.1. Statistical analysis using Fisher exact test showed there was no significant difference between the occurrence of type III secretion toxins-encoding genes and the source of isolates (clinical and environmental isolates). Fisher exact test showed that *P*-values were 1.00, 0.295 and 0.387 for the occurrence of *exo T*, *exo Y* and *exo S*, respectively. In addition, statistical analysis indicated that there was not a significant association between the presence of *exoT*, *exoY*, *exoS P. aeruginosa* isolates recovered from clinical and environmental sources and resistance to some antibiotics such as Ciprofloxacin, Norfloxacin, Levofloxacin, Aztreonam, Tetracycline and Kanamycin. Association between *exoT*, *exoY*, *exoS* toxin genes and resistant to some antibiotics are presented in Tables 3.3, 3.4 and 3.5. Results in Table 3.5 indicate the presence of statistically significant difference at P=0.046 between the

absence and presence of *exoS* gene in isolates resistant to antibiotic Levofloxacin in favor of absence of *exoS* gene.

Table 3.2: Prevalence of type III secretion toxins-encoding genes in clinical and environmental isolates .

Source of *P. aeruginosa* isolate	**type III secretion toxins-encoding genes in combination** n (%)			
	exoT	*exoT and Y*	*exoS and T*	*exoS, Y and T*
Clinical n (47)	6 (13%)	22 (47%)	2 (4%)	17 (36%)
Environmental n (10)	3 (30%)	5 (50%)	0 (0%)	2 (20%)

Source of *P. aeruginosa* isolate	**type III secretion toxins-encoding genes** n (%)			
	exoT	*exoS*	*exoY*	*exoU*
Clinical n (47)	47 (100%)	19 (40.4%)	39 (83%)	0 (0%)
Environmental n (10)	10 (100%)	2 (20%)	7 (70%)	0 (0%)
Total	57 (100%)	21 (36.8%)	46 (80.7%)	0 (0%)

n: number of isolates

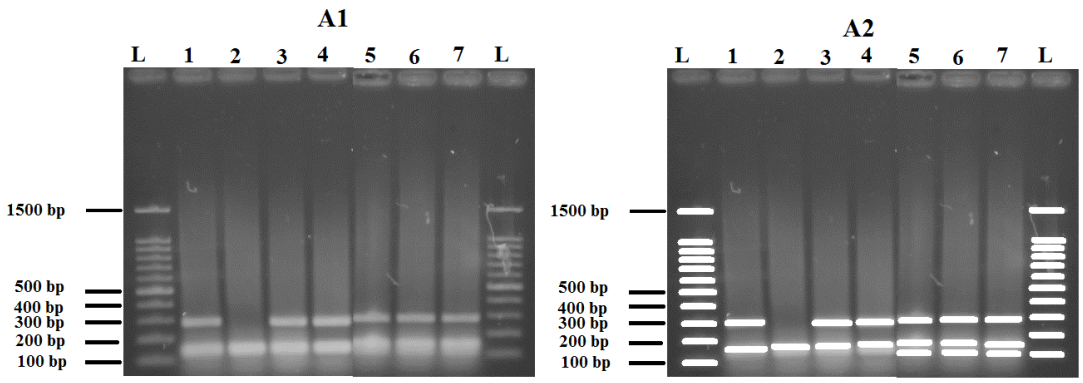

Figure 3.1: A1. Multiplex PCR profiles specific for T3SS. Lanes L represent 100 bp ladder; lanes 1,3 and 4 represent *exoT* (152 bp) and *exoY* (289 bp) genes; lane 2 represents *exoT* (152 bp) gene; lanes 5, 6 and 7 represent *exoS* (118 bp), *exoT* (152 bp) and *exoY* (289 bp) genes. Figure A2: It is the same as A1 but bands are demarcated to be obvious.

Table 3.3: Antibiotic susceptibility profile according to the presence or absence of *exoT* gene.

Antibiotics	Sensitive isolates		Intermediate isolates		Resistant isolates		*P* Pearson Chi-Square (fisher's exact test)
	***exoT*$^{+ve}$**	***exoT*$^{-ve}$**	***exoT*$^{+ve}$**	***exoT*$^{-ve}$**	***exoT*$^{+ve}$**	***exoT*$^{-ve}$**	
Ciprofloxacin	30	0	5	0	21	1	*p*-value=0.423
Norfloxacin	33	0	8	0	15	1	*p*-value=0.326
Levofloxacin	33	0	2	0	21	1	*p*-value=0.400
Aztreonam	28	0	16	0	12	1	*p*-value=0.317
Tetracycline	11	0	8	0	37	1	*p*-value=1.00
Kanamycin	8	0	13	0	35	1	*p*-value=1.00

Table 3.4: Antibiotic susceptibility according to the presence or absence of *exoY* gene.

Antibiotics	Sensitive isolates		Intermediate isolates		Resistant isolates		*P* Pearson Chi-Square (fisher's exact test)
	***exoY*$^{+ve}$**	***exoY*$^{-ve}$**	***exoY*$^{+ve}$**	***exoY*$^{-ve}$**	***exoY*$^{+ve}$**	***exoY*$^{-ve}$**	
Ciprofloxacin	20	10	5	0	18	4	*p*-value=0.343
Norfloxacin	24	9	7	1	13	3	*p*-value=0.726
Levofloxacin	23	10	1	1	19	3	*p*-value=0.203
Aztreonam	21	7	12	4	10	3	*p*-value=1.00
Tetracycline	7	4	4	4	29	9	*p*-value=0.450
Kanamycin	5	3	10	3	28	8	*p*-value=0.391

Table 3.5: Antibiotic susceptibility according to the presence or absence of *exoS* gene.

Antibiotics	Sensitive isolates		Intermediate isolates		Resistant isolates		*P* Pearson Chi-Square (fisher's exact test)
	***exoS*$^{+ve}$**	***exoS*$^{-ve}$**	***exoS*$^{+ve}$**	***exoS*$^{-ve}$**	***exoS*$^{+ve}$**	***exoS*$^{-ve}$**	
Ciprofloxacin	11	19	3	2	6	16	*p*-value=0.558
Norfloxacin	15	18	1	7	3	13	*p*-value=0.113
Levofloxacin	15	18	0	2	4	18	*p*-value=0.046
Aztreonam	9	19	5	11	5	8	*p*-value=0.733
Tetracycline	3	8	5	3	11	27	*p*-value=1.00
Kanamycin	3	5	4	9	12	24	*p*-value=1.00

3.4. RAPD-PCR Typing

The profile of RAPD-PCR typing of 44 *P. aeruginosa* isolates (9 environmental recovered from different hospital sinks and 35 clinical isolates recovered from different hospitals) were clustered into 3 groups (clusters) at 96% similarity level. Strains located in cluster-1 (CL1), which had identical RAPD-PCR profile were recovered from different hospitals and belonged to both environmental (8 isolates) and clinical (19 isolates) sources. Strains located in cluster-2 (CL2), which had identical RAPD-PCR profile were recovered from different hospitals and belonged to both environmental (1 isolate) and clinical (4 isolates) sources. The strains of the last cluster (CL3) also had identical RAPD-PCR profile, recovered from clinical (12 isolates) sources only. Identical clones in the same cluster have been detected among strains from different hospitals. Results of RAPD-PCR analyses are presented in Figures 3.2 and 3.3.

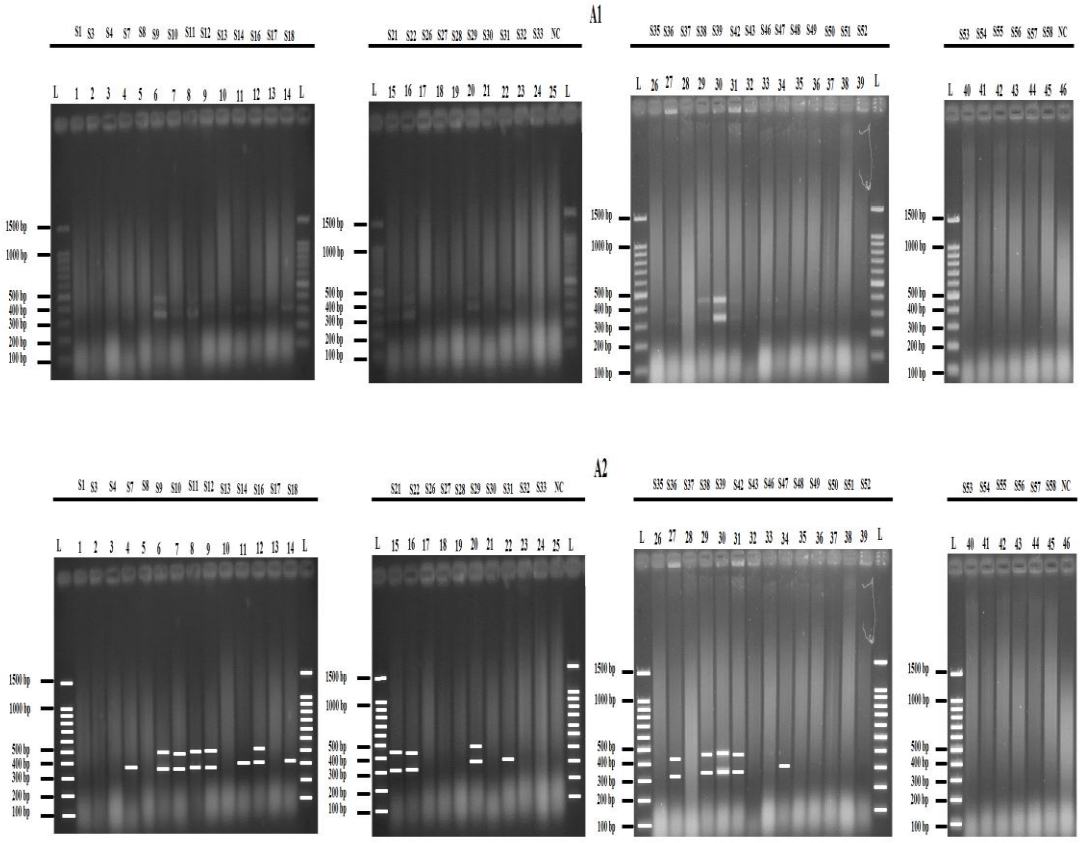

Figure 3.2: A1: RAPD-PCR profile of 44 *P. aeruginosa* isolates (35 clinical isolates and 9 environmental isolates) recovered on 1.7% agarose. Lanes represent DNA 100-bp ladder, lanes 25 and 46 represent negative control (NC), lanes 10, 11, 21, 40, 41, 42, 43, 44 and 45 represent RAPD-PCR profiles for environmental samples S13, S14, S30, S53, S54, S55, S56, S57 and S58, respectively, while other lanes represent RAPD-PCR profiles for clinical samples. **Figure 3.1**: A2: It is the same as A1 but bands are demarcated to be obvious.

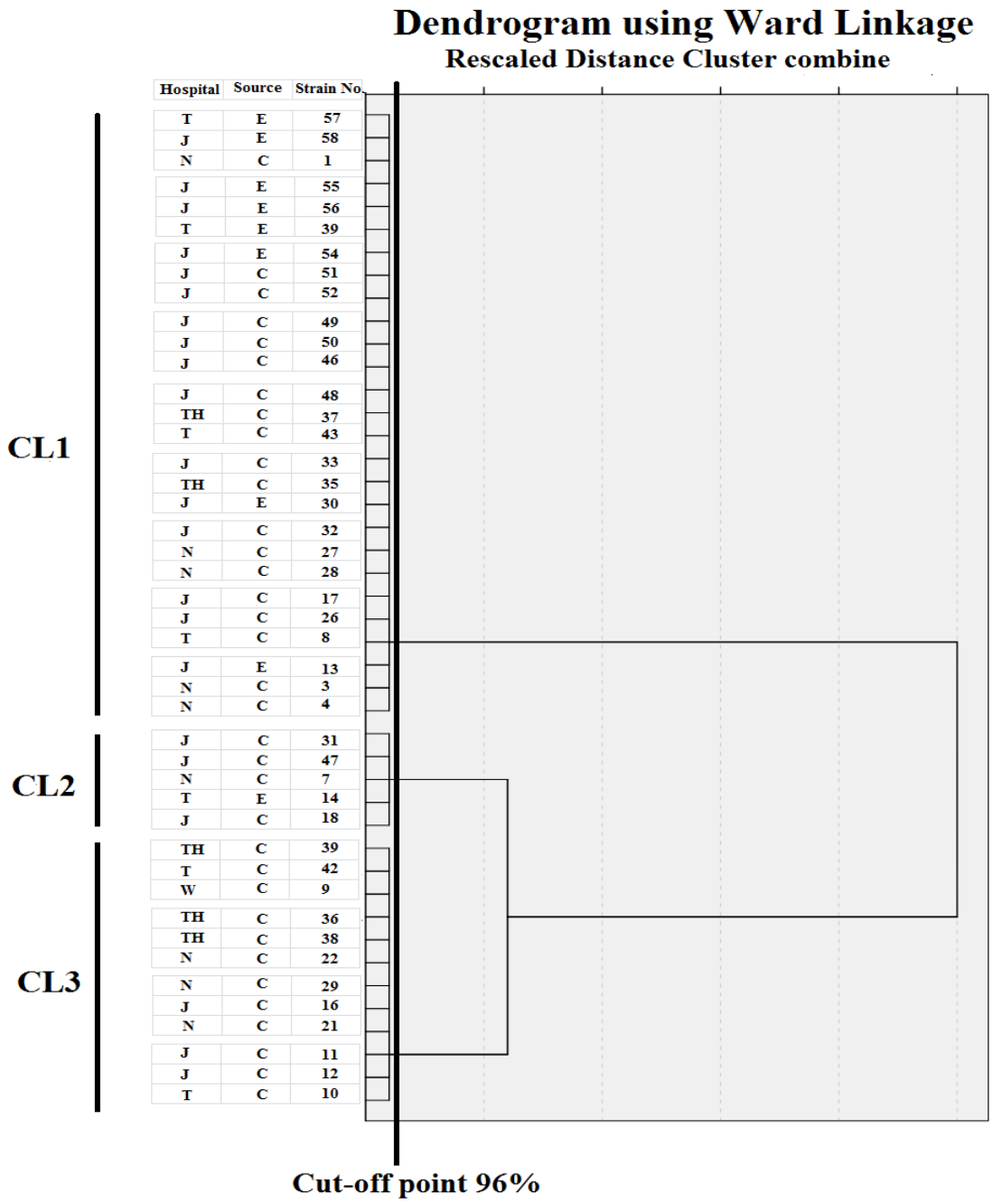

Figure 3.3: Dendrogram of 44 *P. aeruginosa* isolates recovered from clinical (35 isolates) and environmental (9 isolates) sources based on the UPGMA method derived from analysis of the RAPD-PCR profiles at a 96% similarity level.

CL: Cluster; E: Environmental sample; C: clinical sample; N: An-Najah National University Hospital; W: Alwatany Hospital; T: Tubas Turk Hospital; J: Jenin Hospital; TH: Thabet Hospital.

Chapter Four
Discussion

P. aeruginosa is one of the major causes of both nosocomial and community-acquired infections in humans, this due to its potency to cause a different types of diseases and its high-level resistance to many classes of antibiotics. The resistance of *P. aeruginosa* to several classes of antibiotics is due to adaptation to several different mechanisms (Allydice-Francis and Brown, 2012).

Results of this study showed that all isolates recovered from both environmental and clinical sources had a high resistant to Tetracycline and Kanamycin. Results of previous study in Palestine showed that clinical isolates had a high resistant to different tested antibiotics including Tetracycline (Adwan *et al*., 2016; Adwan, 2017). Results reported previously showed that *P. aeruginosa* isolates from different sources had resistance to many different classes of antimicrobial agents (Allydice-Francis and Brown, 2012; Azimi *et al*., 2016). The major cause of high prevalence of antibiotic resistance among clinical isolates of *P. aeruginosa* in Palestine may be due to selective pressure resulting from uncontrolled, extensive incorrect and misuse of antibiotics inside and outside hospitals. This is promoted by the lack of national antibiotic policy and over-the-counter antibiotic availability in Palestine (Adwan *et al*., 2016).

Results of the current study showed that all isolates recovered from both environmental and clinical sources were $exoT^{+ve}$, this result is an agreement

with other studies published elsewhere (Feltman *et al*., 2001; Strateva *et al*., 2010; Gawish *et al*., 2013; Adwan, 2017). The occurrence rate of *exoT* gene in this study was 100% which is in contrast to other studies previously published, which showed that the occurrence rate of this gene in *P. aeruginosa* isolates recovered from both environmental and clinical sources had a range from 5%-92% (Lomholt *et al*., 2001; El-Solh *et al*., 2012; Azimi *et al*., 2016).

In the present study, the occurrence rate of *exoY* gene among *P. aeruginosa* isolates recovered from clinical and environmental sources was 80.7%. Result of this study is close to previously published results in Palestine (Adwan, 2017), which showed that 72.5% of *P. aeruginosa* recovered from clinical isolates were $exoY^{+ve}$. Also this result is an agreement with other studies published elsewhere (Strateva *et al*., 2011; Gawish *et al*., 2013), which showed that the occurrence rate of this gene had a range from 83.5%-85.8%. The occurrence rate of *exoY* gene among *P. aeruginosa* isolates in the current study was 80,7% which is inconsistent with other previously published reports elsewhere, which showed that the occurrence rate of this gene was lower than found in other studies, 97% (Dacheux et al., 2000) and 89% (Finck-Barbancon et al., 1997) and higher than that found in other reports such as Azimi et al., 2016, which was 55%.

Results of this study also demonstrated that the occurrence rate of *exoS* gene among *P. aeruginosa* isolates recovered from clinical and environmental sources was 36.8%. Results of the current study according to the presence of *exoS* gene sequences among *P. aeruginosa* isolates

recovered from clinical were unlike the previous study (Adwan, 2017). In this study, *exoS* gene was not found in all clinical isolates of *P. aeruginosa*. The occurrence rate of *ExoS*-like sequences among tested *P. aeruginosa* isolates in previous published studies had a range from 0%-96% (Woods *et al*., 1986; Rumbaugh *et al*., 1999; Feltman *et al*., 2001; Lomholt *et al*., 2001; Berthelot *et al*., 2003; Strateva *et al*., 2011; El-Solh *et al*., 2012; Gawish *et al*., 2013; Adwan, 2017). Product of this gene is considered a main cytotoxin involved in colonization, invasion, and spreading the infection (Lee *et al*., 2005). Increasing the product concentration of this gene will result to raise pulmonary damage in cystic fibrosis patients and in animal models and *in vitro* cytotoxicity and to induce apoptotic-like cell death (Lee *et al*., 2005; Engel, 2003).

In this study, all *P. aeruginosa* isolates recovered from clinical and environmental sources *were* $exoU^{-ve}$. This result is consistent with a previous report published previously (Adwan, 2017), which showed that all clinical *P. aeruginosa* isolates were $exoU^{-ve}$. The occurrence rate of *exoU* gene sequences among *P. aeruginosa* isolates had a range from 0%-80% (Fleiszig *et al*., 1997; Allewelt *et al*., 2000; Hirakata *et al*. 2000; Feltman *et al*., 2001; Lomholt *et al*., 2001; Berthelot *et al*., 2003; Strateva *et al*., 2011; El-Solh *et al*., 2012; Gawish *et al*., 2013; Adwan, 2017). Secretion of *exoU* gene has specificity to human neutrophils, thus product of this gene is associated with severe *P. aeruginosa* infectious diseases in humans (Finck-Barbancon *et al*., 1997). In addition, it has been shown that strains of *P. aeruginosa* with deleted or mutated *exoU* gene results in a decrease in the

toxicity of these strains in the lung infection (Engel, 2003). In addition, it was proposed that there is an association between the product of *ExoU* gene and invasive infection in *P. aeruginosa* strains causing bloodstream infections in humans (Wareham and Curtis, 2007).

In this study, these was no association between resistance of studied antibiotics and *P. aeruginosa* isolates recovered from both clinical and environmental sources carried type III secretion toxins-encoding genes. Only statistically significant difference at *P*=0.047 was demonstrated between the absence and presence of *exoS* gene in *P. aeruginosa* isolates resistant to antibiotic Norfloxacin in favor of the absence of *exoS* gene. In recent study (Adwan, 2017), results showed that no association between Ciprofloxacin, Norfloxacin, Meropenem and Imipenem resistance and clinical *P. aeruginosa* isolates carried type III secretion toxins-encoding genes. Results of the current study is in contrast to study published previously (Wong-Beringer et al., 2008), which showed an association between *P. aeruginosa* isolates carried *exoU* gene and fluoroquinolone resistance. A significant association between clinical *P. aeruginosa isolates* carried *exoU* gene sequences and fluoroquinolone resistance has been proposed (Cho *et al*., 2014). In other report, it was demonstrated no significant association between ciprofloxacin resistance and *P. aeruginosa* isolates had *exoU* gene (Bradbury *et al*., 2010).

RAPD-PCR typing of *P. aeruginosa* genomes of both environmental and clinical isolates showed that the genome of these strains of this pathogen is highly conserved (96% similarity) and all *P. aeruginosa* isolates whether

these strains isolated from clinical or environmental sources are able to produce virulence factors and are thus potential pathogens. Cluster 1 and 2 have identical strains isolated from both environmental and clinical sources. This indicates that strains of environmental origin may cause human infection. These results are in accordance to other results published previously (Hardalo and Edberg, 1997; Pirnay *et al*., 2009). Moreover, other evidences have proposed that there are no great differences between environmental and clinical isolates regarding the virulence aspects (Römling *et al*., 1994; Spangenberg *et al*., 1995; Morgan *et al*., 1999; Alonso *et al*., 1999; Pirnay *et al*., 2002). Identical clones have been detected among strains from different hospitals. This may be due to medical referrals and transportation of patients among different hospitals (Adwan , 2017).

In conclusion, according to RAPD-PCR typing there is a high similarity between *P. aeruginosa* strains isolated from both environmental and clinical and both clustered together. In addition, strains from both sources had no significant differences in antibiotic resistance and occurence of type III secretion toxins-encoding genes. This may indicate that strains from both sources have ability to cause clinical infection. At the end of this research, I recommend to compare the protein profile between clinical and environmental *pseudomonas aeruginosa* isolates samples and expand the study to include more hospitals and more samples.

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جامعة النجاح الوطنية

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مقارنة بين توزيع الجينات المشفرة لسموم النوع الثالث ومقاومة المضادات الحيوية في سلالات الزائفة الزنجارية (*P. aeruginosa*) من مصادر سريرية و بيئية في شمال الضفة الغربية – فلسطين

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الملخص

بكتيريا الزائفة الزنجارية تنتج مجموعة من مسببات الامراض او السموم ومنها سموم النوع الثالث التي تساعدها على احداث المرض وزيادة شدة المرض في بعض الأحيان. تم جمع وعزل 57 عينة (47 من مصادر سريرية و 10 عينات من مصادر بيئية) من مستشفيات مختلفة في شمال الضفة الغربية. هدف البحث الى مقارنة توزيع الجينات المشفرة لسموم النوع الثالث في العزلات السريرية والبيئية للزائفة الزنجارية في نفس الفترة الزمنية ومقارنة مدى مقاومتها للمضادات الحيوية باستخدام التقنيات الجزيئية. أظهرت نتائج هذا البحث أن جميع العزلات سواء كانت من مصادر بيئية او سريرية أن لديها مقاومة عالية ضد Tetracycline and Kanamycin وأن جميعها تحمل الجينات المشفرة لسموم النوع الثالث وجميعها تحمل جين *exo T*. نسبة وجود *exo y* و*exo S* في جميع العزلات كانت 80,7% و 36,8 %على التوالي. وهناك عزلات تحمل أكثر من جين في نفس الوقت تصل نسبتها في العزلات السريرية الى 87% وفي العزلات البيئية الى 70% وأكثر تواجد كان لجين *exo T* مع جين *exo Y* حيث وصلت نسبة العزلات السريرية والبيئية التي تحمل كلا الجينين الى 47% و 50% على التوالي. التحليل الاحصائي لنتائج هذا البحث اثبت انه لا يوجد اختلاف في توزيع الجينات المشفره لسموم النوع الثالث في العزلات السريرية والبيئية ولا في مقاومتها للمضادات الحيوية. استنتجنا من نتائج RAPD-PCR typing ل 44 عينة (35 سريرية و9 بيئية) بأن هناك تشابه كبير بين هذه العزلات بنسبة 96% وأن جميع العزلات توزعت في ثلاث مجموعات كل مجموعة تحتوي على عزلات سريرية وبيئية من مستشفيات مختلفة

ما عدا المجموعة الثالثة تحتوي فقط على عزلات سريرية وهناك سلالة مشتركة بين جميع المستشفيات سواء كانت العينات من مصادر بيئية او سريرية لا تختلف في توزيع الجينات المشفرة لسموم النوع الثالث ولا في مدى مقاومتها للمضادات الحيوية وهذا يدل على أنها قادرة أن تسبب عدوى سواء كانت من مصادر سريرية او بيئية.