Detection of Type III Secretion Toxins Encoding-genes of *Pseudomonas aeruginosa* Isolates in the West Bank-Palestine

Ghaleb Adwan[1*]

[1]*Department of Biology and Biotechnology, An-Najah National University, Nablus, Palestine.*

Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

Original Research Article

ABSTRACT

Pseudomonas aeruginosa uses Type III Secretion System (T3SS) to inject four types of secretion virulence determinants directly into the cytoplasm of host cell. This study aimed to determine the prevalence of virulence genes encoding type III secretion system toxins among *P. aeruginosa* isolates. A total of 51 isolates of *P. aeruginosa* were collected from different clinical samples in 2015-2016. Detection of gene sequences encoding type III secretion toxins ExoS, ExoT, ExoU and ExoY was performed by the multiplex PCR. Thirty-three of these *P. aeruginosa* isolates were genotyped by RAPD-PCR and Antibiogram for all isolates was also determined. Results of this research showed that the frequency of gene sequences encoding for type III secretion toxins detected by PCR in tested *P. aeruginosa* isolates was 100% and 72.5% for *exoT* and *exoY*, respectively, while *exoS* and *exoU* genes were not detected in these isolates. RAPD-PCR analyses showed that the tested isolates had 3 identical clones recovered from different hospitals and different geographical areas. The isolates of *P. aeruginosa* showed high resistance against Trimethoprim/Sulfamethoxazole (100%), Nalidixic acid (98%), Ceftriaxone (96.1%), Cefotaxim (96.1%) and Tetracycline (74.5%). To our knowledge, up to now, this is the first study documented the virulence determinants associated with *P. aeruginosa* in Palestine. Although our study is

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*Corresponding author: E-mail: adwang@najah.edu;

comprised of a relatively small number of *P. aeruginosa* isolates, it is a representative sample giving a picture of the general situation in Palestine. In conclusion, the results of this study showed high prevalence of T3SS genes among clinical isolates of *P. aeruginosa* in Palestine.

Keywords: Type III secretion system; Pseudomonas aeruginosa; RAPD-PCR; Palestine.

1. INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic, ubiquitous Gram-negative organism present in many diverse environmental settings and rarely causes disease in healthy individuals. Patients with cystic fibrosis, severe burns, neutropenia, immunocompromized, and the mechanically ventilated or have underlying diseases such as HIV or cancer are extremely susceptible to *P. aeruginosa* infections [1,2]. However, this microbial pathogen is a common cause of infection in hospitalized patients, accounting for 11-14% of all nosocomial infections [3]. Infections caused by this pathogen is associated with high case fatality rates and is a main cause of 15-20% of cases of nosocomial pneumonia [1].

Pseudomonas aeruginosa utilizes a considerable number of secreted and cell associated virulence determinants which have an important role in the pathogenesis of infection. Virulence determinants or exotoxins are either passively secreted from the cell or actively secreted via the type I secretion system (T1SS), the type II secretion system (T2SS) or the type III secretion system (T3SS) [4]. Type III secretion system is considered one of these vital virulence factors, which is present in a broad range of pathogens particularly Gram-negative bacilli, including *Shigella* spp., *Salmonella* spp., *Yersinia* spp. and *P. aeruginosa* [1,4]. *Pseudomonas aeruginosa* is able to deliver a number of exotoxins into the host cell cytoplasm via the cell contact-mediated T3SS. The Type III secretion system is composed of three separate protein complexes: the secretion apparatus, the translocation or targeting apparatus, and the secreted toxins (effector proteins) and their cognate chaperones. To date, 4 effector proteins or toxins have been identified belonged to T3SS, including ADP-ribosylating enzymes exoenzyme S (ExoS) and exoenzyme T (ExoT); an acute cytolytic factor (a phospholipase) exoenzyme U (ExoU) and an adenylate cyclase exoenzyme Y (ExoY) [1,5]. Although all clinical strains of *P. aeruginosa* have gene sequences encoding for type III secretion toxins, only few of these are capable of secreting effector proteins [6]. Several reports have shown that *exoY* and *exoT* are existing in about all clinical isolates, a significant number of clinical isolates are not capable to express either *exoS* or *exoU* [6-8]. Different researchers and authors have classified *P. aeruginosa* strains based on the expression of these toxin genes [7-9]. Comparison of the relative contributions of these effector toxins, ExoU secretion had the greatest effect on mortality, bacterial persistence in the lung, and dissemination in a mouse model of acute pneumonia, ExoS had an intermediate effect, and ExoT had a minor effect [6].

The prevalence of *exoS*, *exoT*, *exoU* and *exoY* in clinical and environmental *P. aeruginosa* isolates have been previously studied. In previous published reports, it was found that 100% of tested *P. aeruginosa* isolates recovered from clinical and environmental samples contained *exoT*-like sequences [6,10,11]. Lomholt et al. [12] detected the *exoT* gene in 92% of tested *P. aeruginosa* isolates. El-Solh et al. [3], showed that 41.2% *P. aeruginosa* isolates recovered from blood were expressed *exoT* gene. The prevalence of *exoS* gene among examined *P. aeruginosa* isolates was 91% [13], 96% [14], 71% [6], 66% [12], 61.9% [10], 40% [11], 52.2% [15] and 11.8% [3]. The prevalence of the *exoU* gene among *P. aeruginosa* isolates was 80% [9], 13% [16], 64% [17], 28% [6], 34% [12], 32.4% [10], 62.4% [11], 28.3 [15] and 21.2% [3]. The prevalence of the *exoY* gene among *P. aeruginosa* isolates 97% [18], 89% [6], 85.8% [10], 83.5% [11].

The frequency of these toxin genes among populations of *P. aeruginosa* isolates from different infections has not been examined previously in Palestine. The current study was conducted to determine the prevalence of genes encoding Type III secretion toxins among clinical *P. aeruginosa* isolates in the West Bank-Palestine.

2. MATERIALS AND METHODS

2.1 Bacterial Strains Collection and Identification

A total of 51 isolates of *P. aeruginosa* were collected from different clinical samples between July 2015 and March 2016 [19]. These clinical

samples included 12 urine, 18 wound swabs, 6 sputum, 1 sputum trap, 4 ear swabs, 1 nasal swab, 3 rectal swabs, 4 skin swabs, 1 blood, 1 abscess. Medical centers and number of isolates included in this study were An-Najah National University Hospital-Nablus (n=12), Rafidia Hospital-Nablus (n=8), Medicare Lab-Nablus (n=1), Patient's Friends Society-Nablus (n=2), Palestinian Medical Complex-Ramallah (n=19), AL-Amal Hospital-Jenin (n=1), Al-Shamal lab-Jenin (n=1), Ghannam Lab-Jenin (n=1), Al-Zakah Hospital Tulkarem (n=1), Thabet Hospital-Tulkarem (n=2), Al-Hussein Hospital-Beit Jala (n=2), Martyr Yasser Arafat Hospital-Salfeet (n=1). These isolates were identified by API 20 E system (Biomerieux). The isolates were subcultured in microbiology laboratory at An - Najah National University on Cetrimide agar. Gram strain and oxidase production were done for all isolates. Replicate isolates from the same patient were excluded.

2.2 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 [20]. All *P. aeruginosa* isolates were examined using disks (Oxoid) to determine resistance against Meropenem (MEM) 10 µg, Imipenem (IPM) 10 µg, Nalidixic acid (NA) 30 µg, Ceftazidime (CAZ) 30 µg, ceftriaxone (CRO) 30 µg, Cefotaxime (CTX) 30 µg, Tetracycline (TE) 30 µg, Ciprofloxacin (CIP) 5 µg, Norfloxacin (NOR) 10 µg and Trimethoprim/Sulfamethoxazole (SXT) 1.25/23.75 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℃ 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 [20].

2.3 DNA Extraction and PCR Amplification

2.3.1 DNA extraction

Pseudomonas aeruginosa genome was prepared for PCR according to the method described previously [21]. 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 spectrophotometer and the samples were stored at -20ºC for further analysis.

2.3.2 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 described previously [1]. Primer sequences and size of amplicons are presented in Table 1. The PCR reaction mix with a final volume of 25 µl, was performed with 12.5 µl of PCR premix (ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$, Sigma), 0.4 µM of each primer and 3 µl (100-200 ng) of DNA template. A negative control without DNA templates was also included. The cycling conditions were: initial denaturation at 94ºC 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 72ºC for 5 min.

Table 1. 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	118	[1]
	Exo S R: TTC GGC GTC ACT GTG GAT GC		
exoT	Exo T F: AAT CGC CGT CCA ACT GCA TGC G	152	[1]
	Exo T R: TGT TCG CCG AGG TAC TGC TC		
exoU	Exo U F: CCG TTG TGG TGC CGT TGA AG	134	[1]
	Exo U R: CCA GAT GTT CAC CGA CTC GC		
exoY	Exo Y F: CGG ATT CTA TGG CAG GGA GG	289	[1]
	Exo Y R: GCC CTT GAT GCA CTC GAC CA		

The PCR products were then detected by electrophoresis through 2.5% 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.

2.3.3 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) composed of 10 mM PCR buffer pH 8.3; 3 mM $MgCl_2$; 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 s, 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 through 1.0% 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). The number of different bands in each fingerprint was considered for comparison of bacterial strains as previously described [22], based on the following criteria: identical clones (no different band), "closely related clones" (have 1 different band), "possibility different clones" (have two different bands), "different clones" (have three or more different bands).

2.4 Statistical Analysis

Generated data were analyzed by percentages. A two-tailed Z-test to find association between antibiotic resistance and TTSS[+] isolates (A *P* value of < 0.05 was considered to be dependent). Data were analyzed by use of SPSS software (version 19).

3. RESULTS AND DISCUSSION

Type III secretion system is considered one of the most important virulence factor in *P. aeruginosa* isolates. This complex bacterial structure facilitates the secretion of specific virulence determinants into target host cell cytoplasm by the cell contact-mediated T3SS. In patients with ventilator-associated pneumonia, clinical isolates of *P. aeruginosa* recovered from these patients, have ability to produce type III secretion exotoxins and are linked with worse clinical outcomes. The existence of ExoS, ExoT, or ExoU toxin associates with a higher relative risk of mortality [23].

3.1 Identification of Bacteria

All of *P. aeruginosa* isolates were identified by API 20 E system in laboratories of hospitals from where the isolates were collected. In microbiology laboratory at An-Najah National University, all isolates inoculated on Cetrimide and Mueller Hinton agar had pyocynin pigment (Blue-Green) and pyoverdine (Yellow-Green fluorescent). Gram stain showed that *P. aeruginosa* was Gram negative bacteria and had rod shape. All isolates were oxidase positive.

3.2 Antibacterial Susceptibility

The isolates of *P. aeruginosa* in this research showed high resistance against Trimethoprim/ Sulfamethoxazole (100%), Nalidixic acid (98%), Ceftriaxone (96.1%), Cefotaxim (96.1%) and Tetracycline (74.5%), while showed low resistance against Norfloxacin (19.6%), Ciprofloxacin (21.6%) and Ceftazidime (25.5%). Antibiotic resistance profile against the tested clinical *P. aeruginosa* isolates in this study is presented in Fig. 1. The major cause of high prevalence of antibiotic resistance among clinical isolates of *P. aeruginosa* in Palestine may be due to selective pressure of antibiotic imposed by the high rate and misuse of antimicrobial agents.

3.3 Type III Secretion Toxins-encoding Genes

In this research, the prevalence *exoT* gene among clinical isolates of *P. aeruginosa* was 100%. Result of this research is in agreement with other previous studies [6,10,11], where the prevalence *exoT* gene recovered from clinical and environmental *P. aeruginosa* samples in these studies was 100%. This result is in contrast to other studies previously published, which showed that 41.2% of *P. aeruginosa* isolates recovered from blood were expressed *exoT* gene [3] and 5% of *P. aeruginosa* isolates recovered from different clinical sources [24]. The prevalence *exoT* gene recovered from clinical

and environmental *P. aeruginosa* ranged from 5%-100% [3,6,10-12,24]. Product of *exoT* gene interferes with phagocytic activity of host cell [25], is also involved in delayed wound healing [26], protects cultured cells from TTSS-dependent lysis in vitro [27], death induction in *Galleria mellonella* expressed *exoT* gene [28], inhibits cytokinesis [26] and induction of apoptosis in HeLa cells [29]. In mice, ExoT secretion is associated with dissemination of infection from the mice to the liver [30]. Results of gene sequences encoding for type III secretion toxins detected by PCR are presented in Fig. 2.

The prevalence *exoY* gene recovered from *P. aeruginosa* isolates ranged from 55%-97%. In current study, the prevalence *exoY* gene among clinical isolates of *P. aeruginosa* was 72.5%. Result of this study is close to previously published results by others, in these studies it was shown that the prevalence was 85.8% [10] and 83.5% [11]. In contrast, the prevalence of the *exoY* gene among *P. aeruginosa* isolates was lower than that found in other studies, 97% [18] and 89% [6] and higher than that found in other reports such as [24], which was 55%. Product of *exoY* gene had a little significant effect on pathology in murine pneumonia [27], had a considerable effect on cytotoxicity in MDCK cells [31], was shown to be toxic in yeast cells [32], and shown to induce cell rounding in eukaryotic immune cells [33]. Results of gene sequences encoding for type III secretion toxins detected by PCR are presented in Fig. 2.

In this study, the *exoU* sequences were not detected in these isolates. The prevalence *exoU* gene among *P. aeruginosa* isolates ranged from 13%-80% [3,6,9-12,15-17]. The ExoU has significant cytotoxic capabilities with remarkably rapid and fulminant cytotoxic effects [25,34,35]. In particular, ExoU correlates with lung damage in murine models and acute cytotoxicity in epithelial cells and macrophages [7], and is associated with severe *P. aeruginosa* infectious diseases in human where ExoU secretion specifically kills neutrophils. Recombinant *P. aeruginosa* strains expressed *exoU* gene, showed significantly increased virulence in a murine model of acute pneumonia and systemic spread [17]. It has been proposed an association between ExoU secretion and invasive infection in *P. aeruginosa* strains causing bloodstream infection [36], in contrast, no correlation was noticed with colonization and invasion in BALB/c mice [31]. It has been shown that deletion of *exoU* leads to decrease in

the toxicity of *P. aeruginosa* strains in the lung [34].
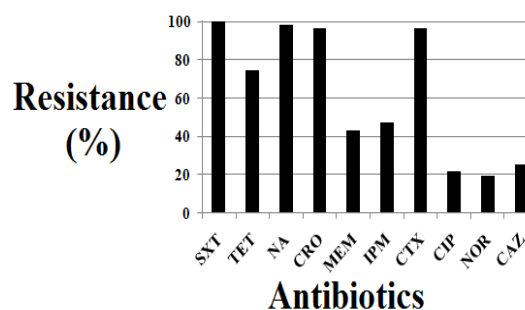
**Fig. 1. Antibiotic resistance of 51 *P. aeruginosa* isolates collected from different clinical samples**
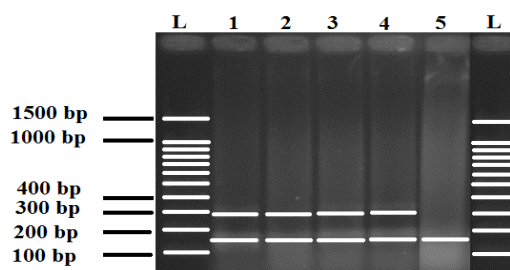
Fig. 2. Multiplex PCR profiles specific for T3SS. Lanes L represent the ladder; lanes 1,2,3,4 represent *exoT* (152 bp) and *exoY* (289 bp) genes and lane 5 represents *exoT* (152 bp) gene

Unlike *exoT*, *exoS*-like sequences were not found in all clinical isolates of *P. aeruginosa* in this study. The prevalence *ExoS* gene among tested *P. aeruginosa* isolates in previous published studies ranged from 11.8%-96% [3,6,10-15]. 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 [27]. Increased concentrations of ExoS get together with increased pulmonary damage in cystic fibrosis patients and in animal models and *in vitro* cytotoxicity [26]. In addition, ExoS ADP-ribosylates small Ras-like proteins, inhibiting internalization and DNA synthesis and inducing apoptotic-like cell death [25,34].

Analysis of fluoroquinolones and carbapenems resistance profile and gene sequences encoding for T3SS genes is presented in Fig. 4. Antibiotic resistance profiles for fluoroquinolones and carbapenems were grouped into four clusters

(C1-C4), some of these clusters had sub clusters as C3A and C3b for C3 and C4A, C4B, C4C and C4D for C4. In this study, it was found that 85.7% of the isolates carried *exoT* gene alone were sensitive to Ciprofloxacin and/or Norfloxacin. These results indicate the presence of statistically significant difference at (*P*= 0.00016) between the isolates sensitive and resistant to Ciprofloxacin and/or Norfloxacin and carried *exoT* gene alone in favor of sensitive isolates. The higher frequency of sensitive to both Ciprofloxacin and Norfloxacin (78.4%) in T3SS+ isolates was detected in this study. These results indicate the presence of statistically significant difference at (*P*= 0) between the isolates sensitive to both Ciprofloxacin and Norfloxacin and resistant to Ciprofloxacin and/or Norfloxacin TTSS+ isolates in favor of sensitive isolates. In contrast, a higher frequency of resistance to ciprofloxacin (59%) in the TTSS+ isolates was observed in previously published study [3]. In previous study, *exoU* gene carriage
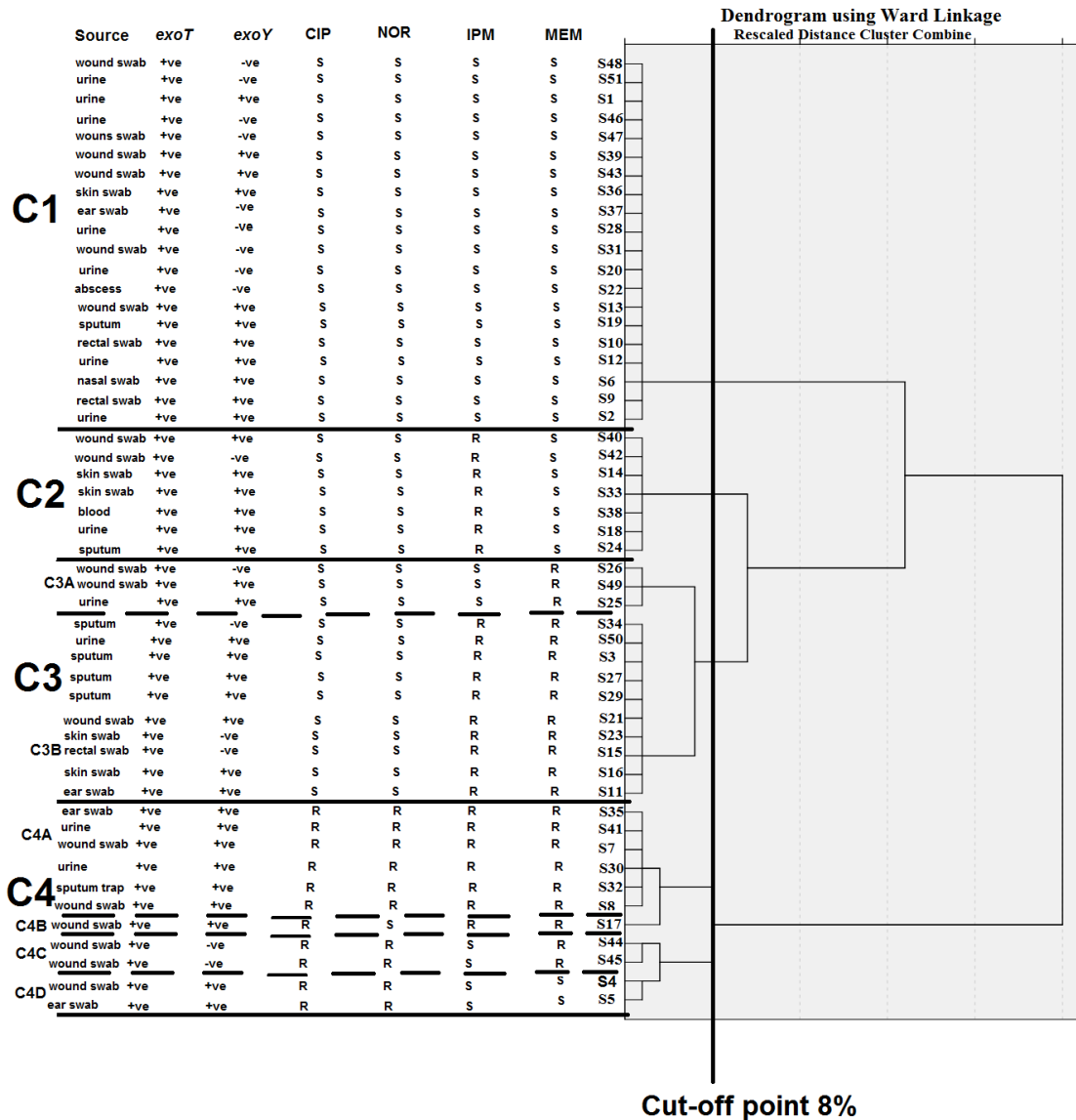
Fig. 3. Dendrogram of 51 clinical isolates of *P. aeruginosa* based on the UPGMA method derived from analysis of fluoroquinolones and carbapenems resistance profile and gene sequences encoding for T3SS at 80% similarity level
CIP: Ciprofloxacin; NOR: Norfloxacin; IPM: Imipenem; MEM: Meropenem; C: Cluster
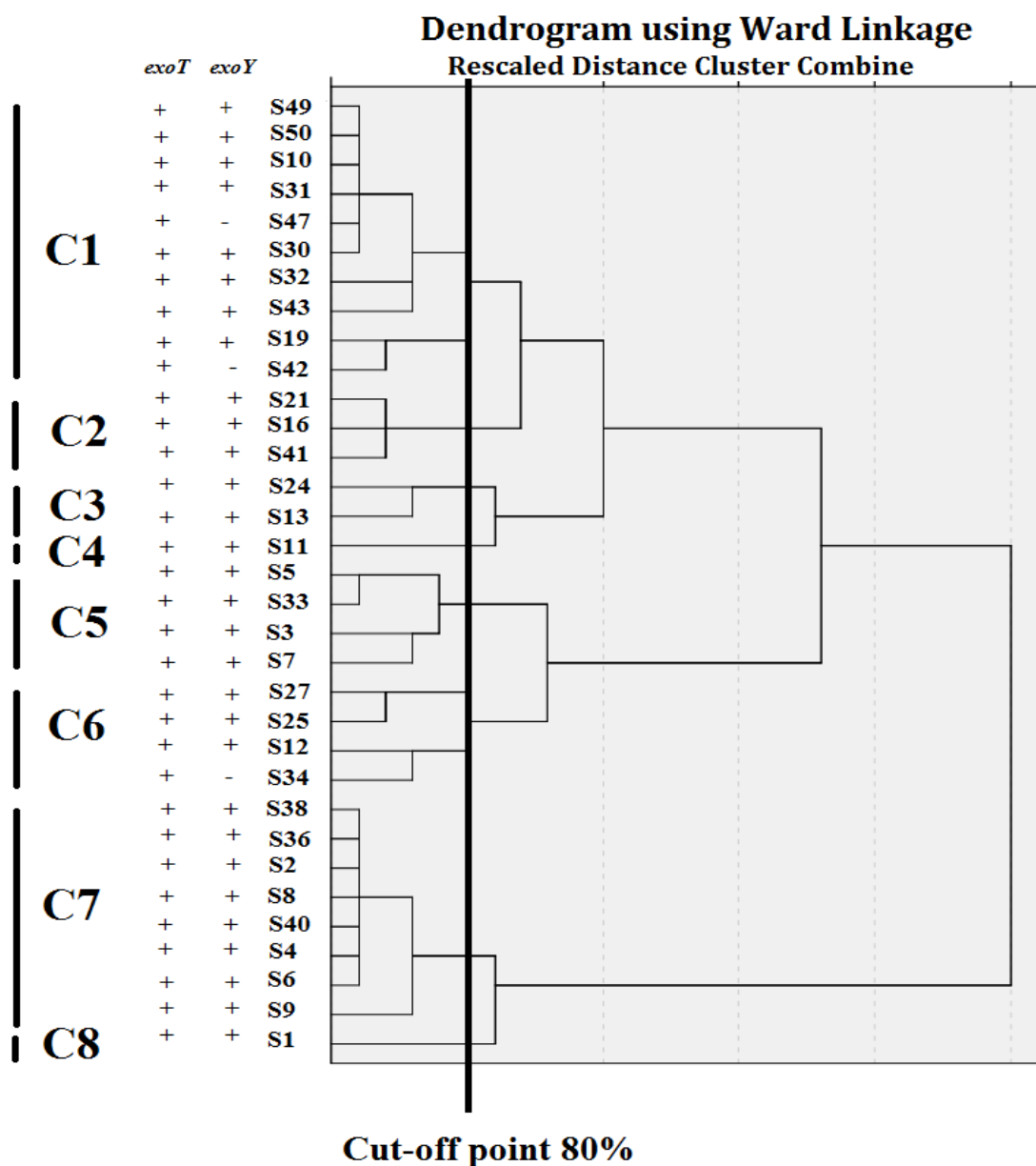
Fig. 4. Dendrogram of 33 clinical isolates of *P. aeruginosa* harbored gene sequences encoding for T3SS based on the UPGMA method derived from analysis of the RAPD-PCR profiles at 80% similarity level
C: Cluster; (+): Gene is present; (-): Gene is absent

and fluoroquinolone resistance in some isolates of *P. aeruginosa* has been suggested [37], while in other study, it was found no significant association between ciprofloxacin resistance and the presence of *exoU* gene [4]. In current study, it was found no association between Meropenem and/or Imipenem resistance and isolates with T3SS⁺ genotypes at ($P= 0.16452$). In addition, no association between Ciprofloxacin and/or Norfloxacin resistance and isolates carried both *exoT* and *exoY* gene or these carried only *exoT* gene ($P= 0.4354$). Also no association between Meropenem and/or Imipenem resistance and isolates carried either both *exoT* and *exoY* genes or carried only *exoT* gene ($P= 0.54186$).

3.4 RAPD-PCR Typing

RAPD-PCR typing of 33 of these *P. aeruginosa* isolates which harbored gene sequences

encoding for type III secretion toxins were genetically diverse and consisted of a heterogeneous population with a total of 8 RAPD-PCR clusters (C1-C8) at an 80% similarity level. Some of these clusters have also divided into subclusters. Identical clones have been detected between strains from different hospitals and regions such as in C1 (S10, S30, S31, S47, S49 and S50), C5 (S5 and S33) and C7 (S2, S4, S6, S8, S36, S38 and S48). All strains of identical clones in C5 and C7 carried both *exoT* and *exoY* genes, while in C1 one strain (S47) carried only *exoT* gene. Results of RAPD-PCR profiles are presented in Fig. 4. This may be due to medical referrals and transportation of patients among different hospitals.

4. CONCLUSION

To our knowledge, up to now, this is the first study documented the virulence determinants associated with *P. aeruginosa* in Palestine. Although our study is comprised of a relatively small number of *P. aeruginosa* isolates, it is a representative sample giving a picture of the general situation in Palestine. In conclusion, the results of this study showed high prevalence of T3SS genes among clinical isolates of *P. aeruginosa* in Palestine.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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