



Full Length Research Paper

Characterization of *P. aeruginosa* isolates recovered from different clinical specimens

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Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen and a leading cause of hospital-acquired infections. Characterization of the isolates from different infection sites might help to control infections caused by the pathogen. The aim of the present work is to characterize *P. aeruginosa* isolates recovered from different clinical specimens at two hospitals in Cairo with regard to their antibiogram, genotypes and virulence factors. The highest antimicrobial resistance pattern was exhibited by isolates from sputum. Resistance rate recorded for sputum samples to different in-use antibiotics was 80, 80-100, 36, 54 and 54% for Penicillins, Cephems, Carbapenems, Aminoglycosides and Fluoroquinolones, respectively. Phenotypic detection of virulence factors in *P. aeruginosa* isolates included detection of protease, lecithinase, DNase, hemolysin and pyocyanin revealed that, each isolate had at least one virulence factor. Protease and lecithinase were the most commonly detected, where 68 and 66% of the isolates showed positive protease and lecithinase activities respectively. Random amplified polymorphic DNA (RAPD) genotyping using 2 random primers revealed 22 and 14 different genetic profiles. Phylogenetic trees based on genetic distances showed 3 clusters with obvious similarity between some isolates, indicating common sources of infection. No association could be found between clustering pattern of the isolates, their antibiogram and virulence.

Key words: *Pseudomonas aeruginosa*, antibiogram, genotypes, random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR).

INTRODUCTION

Pseudomonas aeruginosa is a Gram negative highly opportunistic pathogenic bacteria commonly isolated from

hospitalized patients. It is a leading cause of nosocomial infections particularly in immunocompromised patients

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Table 1. Number of *P. aeruginosa* isolates recovered from different clinical sources.

Source	Number of isolates (percentage)	Hospital/number of isolates
Urine	12 (26.7)	Al- AzharUniversity (5); Kasr Al Ainy (7)
Wound	8 (17.8)	Al- AzharUniversity (8); Kasr Al Ainy (0)
Sputum	11(24.4)	Al- AzharUniversity (5); Kasr Al Ainy (6)
Abscess	11(24.4)	Al-AzharUniversity (9); Kasr Al Ainy (2)
Stool	3 (6.7)	Al-AzharUniversity (2); Kasr Al Ainy (1)

(Zenone and Souillet, 1996). *P. aeruginosa* accounts for 10 to 22.5% of hospital acquired infections in Asia-Pacific, Europe, Latin America, United States, Canada and Italy (Gales et al., 2001; Simonetti et al., 2013). It causes infections such as bacteraemia, urinary tract infections, lung infections, cystic fibrosis, wound infections especially of thermal burns, surgical wound infections, and otitis media (Pollak, 1998; Schaechter et al., 2009).

It is widely known that *P. aeruginosa* infections cause significant morbidity and mortality due to the organism ability to adapt easily to environmental changes, to develop resistance to antibiotics and to produce a wide variety of virulence factors (Van Delden and Iglewski, 1998), such as the formation of pyocyanin, hemolysin, gelatinase and biofilm which lead to tissue damage and protect *P. aeruginosa* against the recognition of the immune system and action of antibiotics (Cevahir et al., 2008; Ciofu et al., 2008; Todar, 2009); in addition *P. aeruginosa* is capable of producing other virulence factors as lipase, lecithinase, DNase and protease.

P. aeruginosa has different mechanisms of resistance, which generates multi-drug resistant isolates or pan-resistant isolates (Jácome et al., 2012). Several mechanisms of resistance which the pathogen develops include multidrug efflux pump, production of β -lactamases and aminoglycoside modifying enzymes (Mahmoud et al., 2013) which reflect the increasing rate of multidrug resistant strains worldwide causing a serious problem in hospital settings (Lim et al., 2009).

The molecular studies aimed to determine diversity among the pathogen isolates, polymorphism of certain of its genes and also genetic comparison of *P. aeruginosa* isolates from different hosts and environments (Kiewitz and Tummler, 2000; Martin et al., 1999; Rumi et al., 2001; Onasanya et al., 2010).

One of the molecular techniques widely used in these studies include RAPD-PCR, a method based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence (Williams et al., 1990). The main advantage of RAPD-PCR over other molecular genotyping techniques is its simplicity, low cost and its ability to generate a large number of genetic markers without the requirement for cloning, sequencing or any other form of molecular characterization of the

genome of the species (Kumar and Gurusubramanian, 2011).

The investigation of genotypes of the *P. aeruginosa* isolates, characterization of their antibiotic resistance pattern and virulence factors might be useful to identify the clonal relationship between isolates from different clinical specimens and to control infections caused by the pathogen.

Therefore, the aim of this study is to evaluate the antimicrobial resistance pattern, genetic diversity and virulence factors of strains of *P. aeruginosa* isolated from different sources of infections at two Egyptian hospitals.

MATERIALS AND METHODS

Specimen collection

A total of 45 *P. aeruginosa* isolates were obtained from the bacteriology laboratory of Al- Azhar University and Kasr Al Ainy Egyptian hospitals. Isolates were recovered from urine, wound, stool, abscess and sputum specimens as shown in Table 1. Isolates were identified according to the standard microbiological tests using gram staining, cetrimide agar (Oxoid, UK), *Pseudomonas* P (Difco, USA), *Pseudomonas* F (Difco, USA), Oxidase test and API 20NE (API BioMérieux, France).

Antibiotic susceptibility testing

Susceptibility test was performed according to Kirby- Bauer disc diffusion test (CLSI 2011) using the following antibiotic discs (Oxoid, UK): Amoxicillin/clavulanic Acid (AMC 30 μ g), Amikacin (AK 30 μ g), Ciprofloxacin (CIP, 5 μ g), Ceftriaxone (CRO 30 μ g), Trimethoprim sulfamethoxazole (SXT), Cefotaxime (CTX 30 μ g), Imipenem (IPM 10 μ g), Meropenem (MEM 10 μ g), Chloramphenicol (C 30 μ g), and Tetracycline (TE 30 μ g). Inhibition zone was recorded in mm. The susceptibility pattern was determined using the CLSI interpretation chart as susceptible (S), intermediate (I) and resistant (R).

Phenotypic detection of virulence factors

Determination of enzymatic activities

Enzymatic activities were evaluated by spot inoculation of an overnight culture of the organisms in various media: Skim milk agar for the protease activity, trypticase soy agar (TSA) supplemented with egg yolk for the lecithinase activity, DNase agar for the DNase activity (Matar et al., 2005). Plates were incubated for 24 or 48 h at

37°C. A positive result around the inoculum spot is indicated by a clear halo for protease activity, white precipitate for lecithinase activity and clear zone after adding 1 N HCl for DNase activity.

Test for pyocyanin

Test was performed according to the method of Huerta et al. (2008). Cultures were grown in Luria broth at 37°C at 120 rpm for 16 to 18 h. Cultures were centrifuged at 10,000 g at 4°C for 15 min. Cell free supernatants and cell pellets were separated. 3 ml of culture supernatant was mixed with 1.2 ml of chloroform. Mixture was incubated for 30 min at room temperature. Absorbance of chloroform layer was measured at 690 nm.

Production of hemolysin

Hemolysin production was assayed by growing the isolate overnight for 16 h in nutrient broth and spot inoculation of 10 µl onto sheep blood agar. Incubation done was at 37°C overnight. Hemolysin production was verified by the presence of clear haemolytic halo around the spot (Santo et al., 2006).

RAPD genotyping

Total DNA from *P. aeruginosa* isolates was obtained by suspending 1 to 5 colonies in 100 µl of nuclease free water. Suspension was heated at 99°C for 10 min, and then cooled on ice. After centrifugation at 13000 rpm for 5 min, 5 µl of supernatant was used as template in 25 µl PCR reaction (Nunes et al., 1999). Amplification was performed using primer 208 (5' ACGGCCGACC 3') (Campbell et al., 2000), or primer 1281 (5' AACGCGCAAC 3') (Akopyanz et al., 1992). RAPD PCR mix (25 µl) consisted of 10 x reaction buffer, 3 mM MgCl₂, 200 µM dNTP, 20 pm primer and 2 U Taq DNA polymerase, 5 µl DNA template.

Thermal cycler program was as follows: Initial denaturation of 5 min at 94°C, followed by (I) 4 cycles with each cycle consisting of 5 min at 94°C, 5 min at 36°C and 5 min at 72°C. (II) 30 cycles with each cycle consisting of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C and followed by a final extension step at 72°C for 10 min. RAPD products were then separated by electrophoresis in 1% agarose gel at 80 v for 1 h. The RAPD fingerprints were analysed both by naked eye and by computer with Gel Compar II software with 1Kb DNA ladder. The bands for each strain, were scored as absent (0) or present (1) in order to construct a genotype dendrogram. The similarities between fingerprints were determined by construction of a similarity matrix using the Jaccard coefficient and a dendrogram generated using unweighted pair group method with an average linkage (UPGMA) algorithm. Primer efficiency was calculated for each primer by using the following equation as described by Graham and McNicol (1995):

$$\text{Primer efficiency} = \frac{\text{Total No. of bands produced by all primers}}{\text{No of total bands of primer}} \times 100.$$

RESULTS

Among the forty five *P. aeruginosa* isolates, 26.7% were recovered from urine, 24.4% from both sputum and abscess, 17.8% from wound and 6.7% from stool (Table 1). Isolates were characterized with regard to antimicrobial

susceptibility profile, virulence factors and RAPD typing.

Antimicrobial profile

In this study, antimicrobial susceptibility profiles to 10 antibiotics, representing eight different classes, showed high rate of antibiotic resistance. It was observed that the percentage resistance to Ceftriaxone, Trimethoprim Sulfamethoxazole, amoxicillin/clavulanic acid, cefotaxime, meropenem, imipenem, amikacin, chloramphenicol, tetracycline, and ciprofloxacin was 95, 95, 84, 82, 22, 15%, 17, 48, 35 and 20%, respectively (Figure 1).

Virulence factors

Phenotypic detection of virulence factors in *P. aeruginosa* isolates included protease, lecithinase, DNase, hemolysin and pyocyanin. Protease and lecithinase activities were the most commonly detected virulence factors, where 68 and 66% of the isolates showed positive results for protease and lecithinase activities respectively. While only, 37, 22 and 13% of the isolates were positive for DNase, hemolysin and pyocyanin, respectively (Figure 3).

Recorded virulence factors exhibited variation according to the isolation source. The highest protease production was observed in isolates from sputum, while isolates from abscess and urine showed the highest lecithinase and DNase production respectively (Figure 4).

RAPD profile

Genetic analysis of 45 *P. aeruginosa* isolates revealed 22, 14 different profiles using primers 208 and 1281, respectively. Fingerprints consisted of 1 to 7 amplification bands ranging in size from 100 to 3 Kb. Some isolates showed the same profile bands. Figure 5 shows a representative banding profile of *P. aeruginosa* isolates following RAPD-PCR.

The dendrograms created by UPGMA using primer 208 and primer 1281 are shown in Figures 6 and 7, respectively.

Three major clusters A, B and C are identified in both dendrograms. Each cluster contained one or two groups which are further subdivided into subgroups. Sixty one-percent of isolates were placed in cluster C for dendrogram created using primer 208 and 70% of the isolates were grouped in cluster B for dendrogram created using primer 1281, while cluster A and cluster C contained only one isolate and three isolates in dendrograms created using primer 208 and 1281, respectively. Some isolates were placed in the same subgroups with genetic

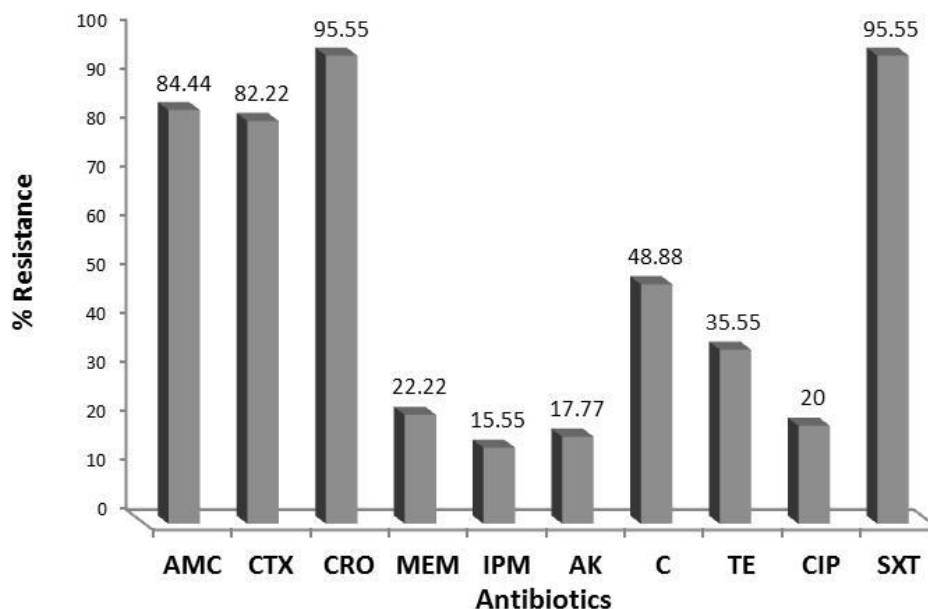


Figure 1. Antibiotic susceptibility profile for *P. aeruginosa* isolates.

distance of 0, indicating genetic similarity between them. Genetic distance ranged from 0 to 0.5 and from 0 to 0.25 for dendrograms created using primer 208 and 1281, respectively.

DISCUSSION

In this study, we characterized and typed *P. aeruginosa* strains isolated from different clinical sources from Egyptian hospitals according to their antibiogram, genetic fingerprint and virulence factors. The antibiotics were selected to represent different classes. High rate of antibiotic resistance was observed compared to other studies of Ali and Balkhy in Gulf Cooperation Council countries (2012), Yayan et al. (2015) in Germany, and Bonfiglio et al. (1998) in Italy. These results might be justified by the extensive usage of antibiotics in Egyptian hospitals. Different resistance patterns were shown, where the most common pattern exhibited by 53% of the isolates was Amoxicillin/ clavulanic acid (R), Cefotaxime (R), Ceftriaxone (R), Trimethoprim sulfamethoxazole (R), Amikacin (S), Imipenem (S), Meropenem (S), and Ciprofloxacin (S). While the least common pattern shown by 13% was Amoxicillin/clavulanic acid (R), Cefotaxime (R), Ceftriaxone (R), Amikacin (R), Imipenem (R), Meropenem (R), Trimethoprim sulfamethoxazole (R), Ciprofloxacin (R), Tetracycline (R) and Chloramphenicol (S). Resistance rate recorded for sputum samples to different in-use antibiotics was 80, 80-100, 36, 54 and 54% for Penicillins, Cephalosporins, Carbapenems, Aminoglycosides and Fluoroquinolones respectively, indicating different mechanisms of resistance used by

these isolates. Resistance rate of sputum samples was higher than those reported by Fatima et al. (2012) in Pakistan, who studied the susceptibility profile of *P. aeruginosa* isolates from patients with lower respiratory tract infection.

It is well known that, *P. aeruginosa* depends upon extracellular enzymes and toxins that break down physical barriers and damage host cells to invade tissues and overcome host immune defence (Cevahir et al., 2008). Among the enzymes and proteins involved in invasion are proteases, lecithinase, hemolysins and pyocyanins pigments. Phenotypic detection of extracellular virulence factors revealed the presence of the tested virulence factors, including lecithinase, protease, pyocyanin, hemolysin and DNase in variable percentages, where the most prevalent were lecithinase and protease. In this study, the results were in agreement with a study of Mashhadani (2004) in Iraq who reported that *P. aeruginosa* isolates from different clinical sources were positive for protease production (100%). In this study, the recorded DNase activity was higher in urine isolates. The results disagreed with Holban et al. (2013) who reported the absence of DNase in all tested UTI isolates and who justified that by the absence of viscous secretions and neutrophils extracellular traps in UTI infections due to its physiology which are the important stresses for DNase production. In our study, urine and sputum isolates were the most virulent strains expressing all tested virulence markers. This finding was also reported by Holban et al. (2013), that *P. aeruginosa* isolates from blood and respiratory tract isolates were the most virulent and was exhibiting the entire spectrum of

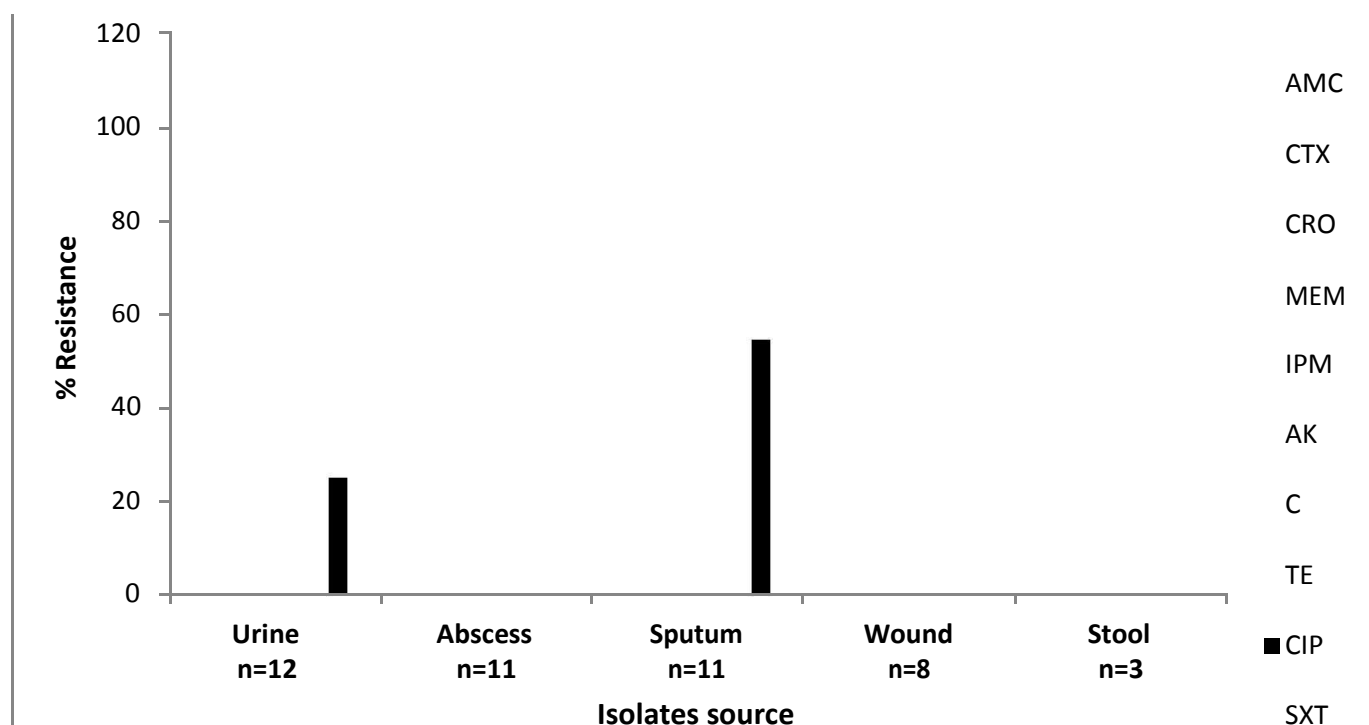


Figure 2. Antibiotics susceptibility profile of *P. aeruginosa* isolates in relation to clinical sources.

tested virulence factors. Variation of expressed virulence factors by *P. aeruginosa* isolates could be justified by the difference in signalling molecules present in different anatomical sites within the host and that could affect bacterial modulation of virulence factors for better persistence (Hughes and Sperandio, 2008).

Analysing the results of RAPD genotyping revealed that, the higher number of RAPD pattern was shown with primer 208 (22 patterns) compared with primer 1281 (14 patterns). Also, primer 208 showed 13 bands, compared to 9 bands for primer 1281. No monomorphic bands were shown for each primer. Primer 208 was more efficient (59%), whereas efficiency of primer 1281 was 40%. The efficiency of the primer reflects the availability of sequences complementary to the primer in the genome that allows base pairing between the primer and genomic DNA (Karp and Edward, 1997). These results were in agreement with the suggestion of Fristch et al. (1993) who reported that high efficiency of the primer is always correlating with the GC content of the primer.

Six isolates did not show amplification bands with primer 208, while primer 1281 failed to amplify DNA of 18 isolates, three of which did not show amplification products with both primers and are referred as untypeable. In that context, Menon et al. (2003) found one untypeable strain among 15 *P. aeruginosa* strains recovered from endophthalmitis cases. In addition, Zulkifli et al. (2009) who studied the diversity of *Vibrio parahaemolyticus* isolated from cockles in Indonesia,

reported that, certain isolates failed to produce any products with the primers used.

Although, isolate number 6, 23, 27, 40, 38 and 46 were not discriminated in the dendrogram using primer 208, they were discriminated in the dendrogram using primer 1281, but were placed in sub-groups of higher similarities, which demonstrates the importance of using more than one primer in RAPD typing. In the dendrogram using primer 208, isolate number: 40, 27, 26, 24, 23 and 6 from different isolation sites were from the same hospital, and were placed in the same sub-groups which indicate common sources of infections in hospitals environment, regardless of the isolation source and virulence. On the other hand, the dendrogram using primer 1281, isolates grouped in cluster B were from the same hospital, except isolate number 45 and 44, which reinforces the observation of the common source of infection within the same hospital.

Differences in antibiotic resistance profile in tested strains were observed according to the isolation source. Isolates from sputum exhibited the highest resistance percentages to tested antibiotics, followed by isolates from urine (Figure 2).

Conclusion

This study shows a high rate of antibiotic resistance among *P. aeruginosa* isolates from different clinical

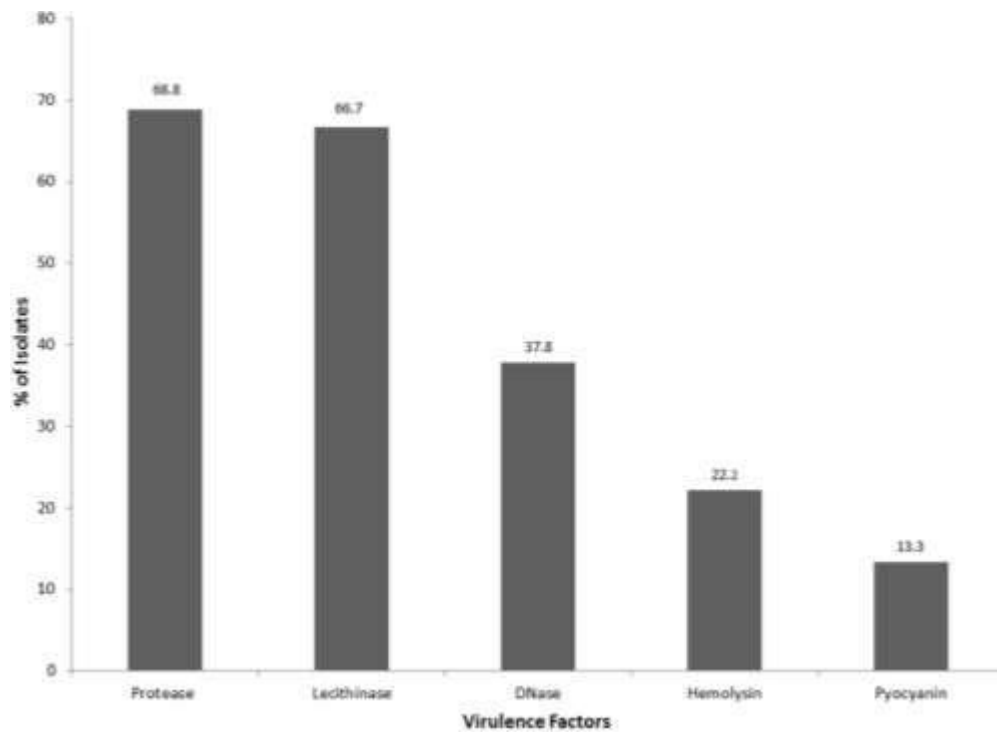


Figure 3. Virulence factors of *P. aeruginosa* isolates.

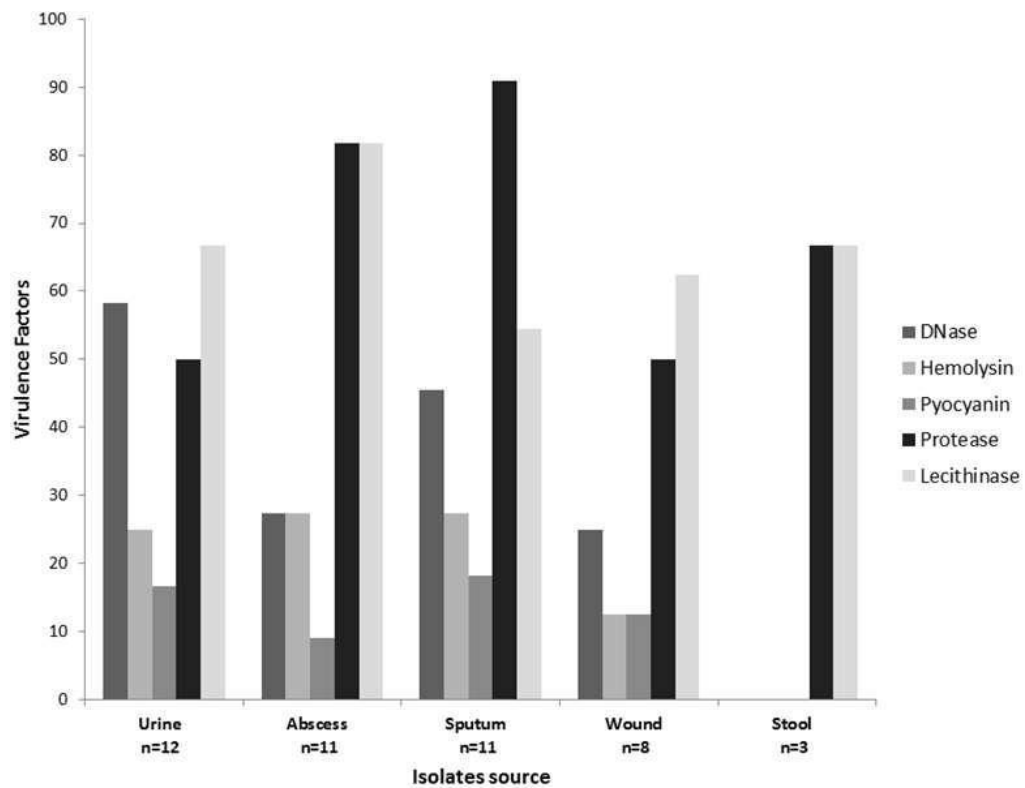


Figure 4. Distribution of different virulence factors in *P. aeruginosa* isolates according to the clinical sources.

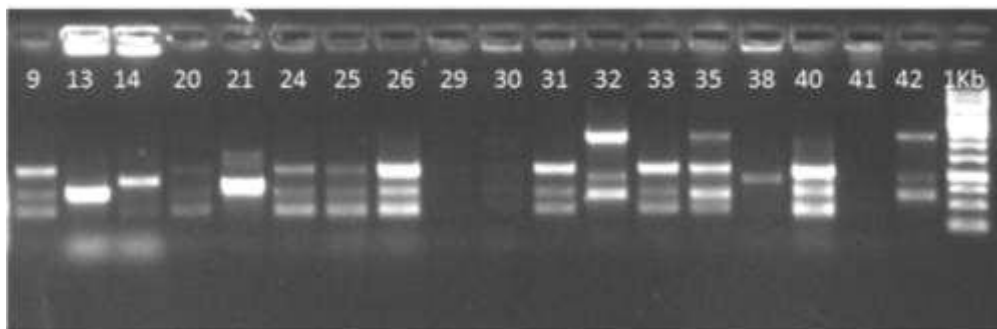


Figure 5. Representative DNA-banding profiles of *P. aeruginosa*-isolates by agarose gel electrophoresis following RAPD PCR using primer 208. Lane 1-18: *P. aeruginosa* isolates, lane 19: 1 Kb DNA ladder.

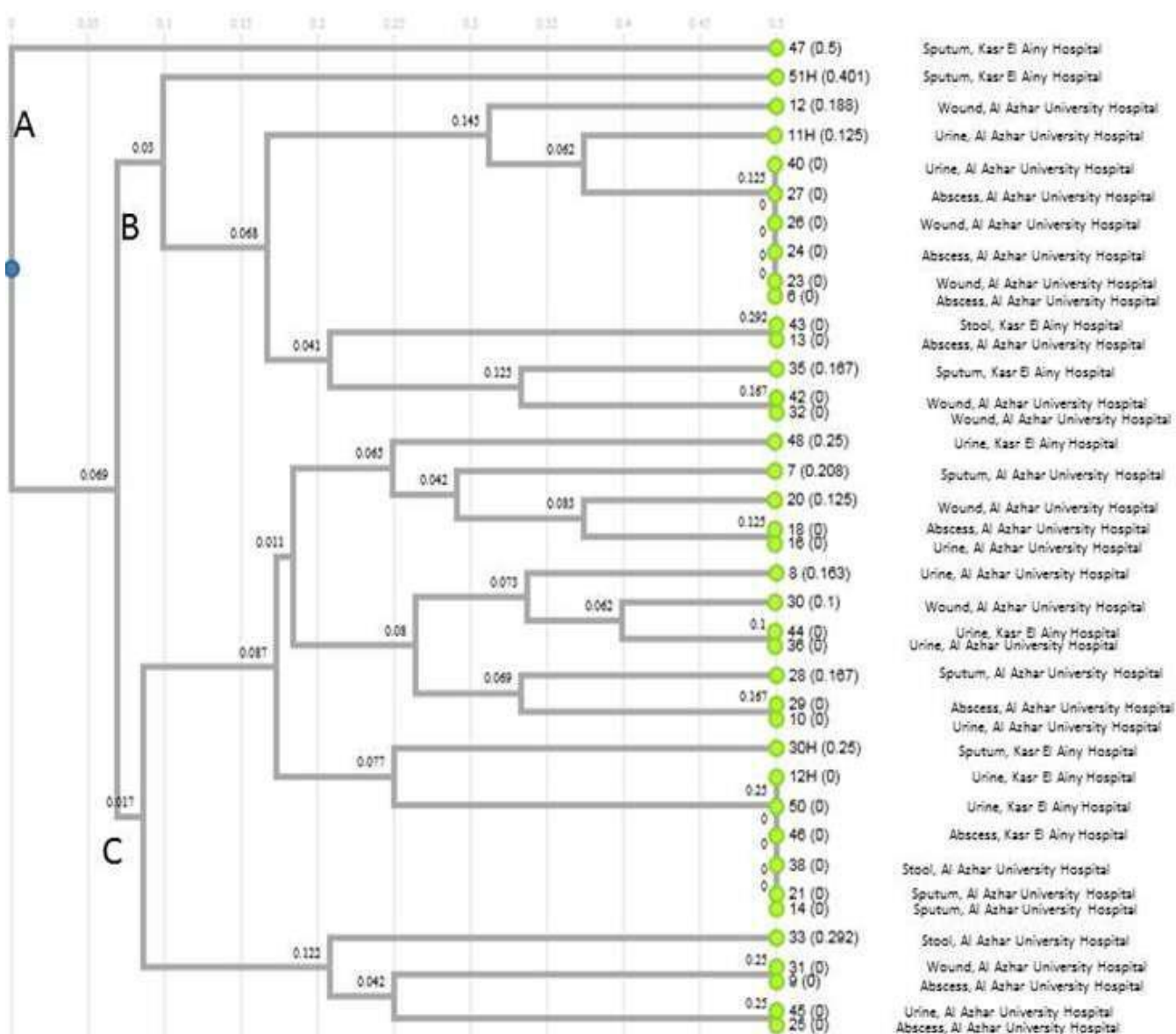


Figure 6. Phylogenetic tree using primer 208.

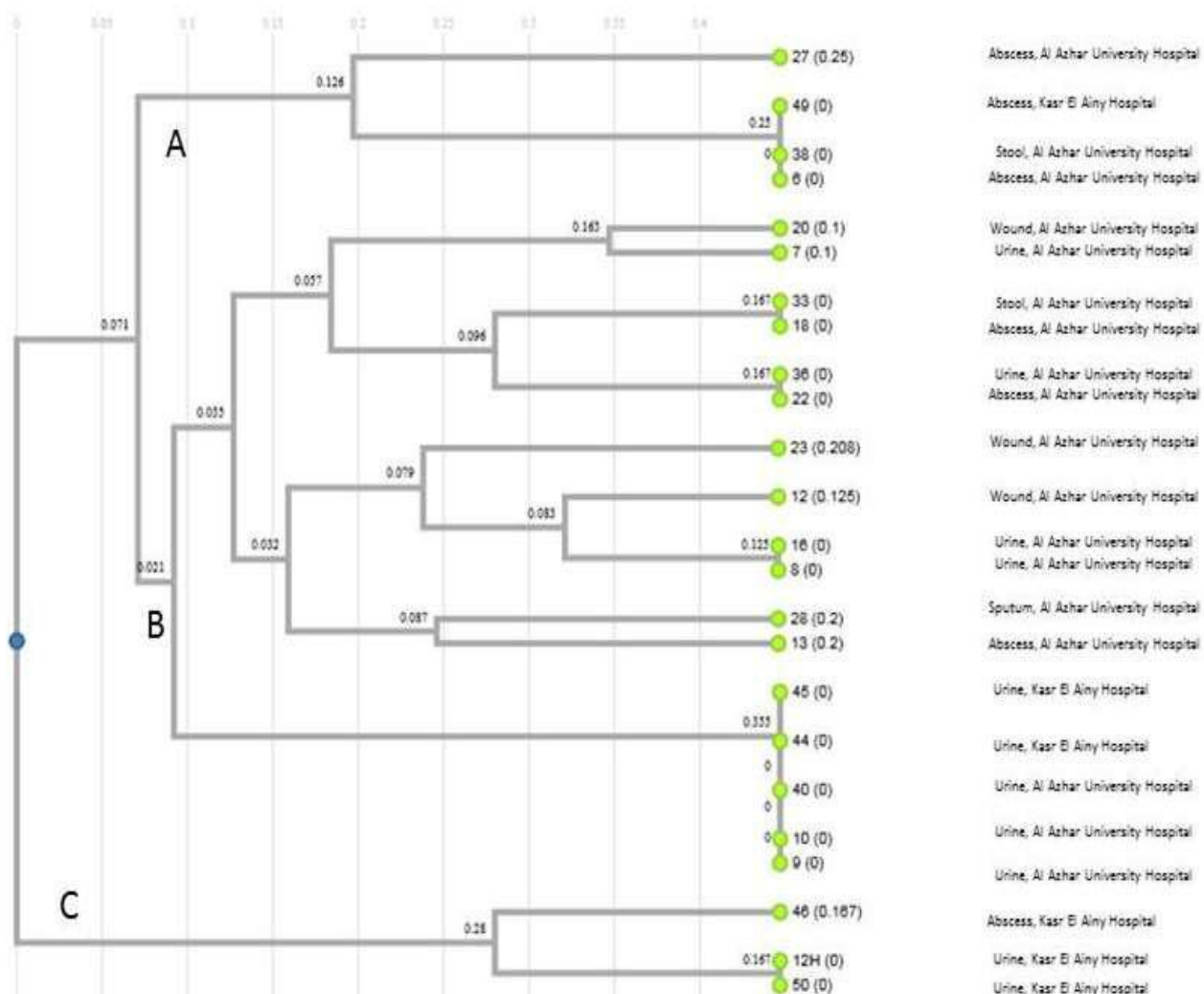


Figure 7. Phylogenetic tree using primer 1281.

sources. It also reveals that protease and lecithinase are the most common virulence factors produced by all isolates regardless of the isolation source. The common sources of infection in hospitals environment are obvious from the similar genotypic pattern of a large number of isolates. Further studies on a larger number of isolates are required to confirm our observations. We suggest applying strict disinfection policy in hospitals environment to prevent spreading of infections, in addition, new classes of antibiotics should replace traditional antibiotic treatment to prevent emergence of resistant strains of *P. aeruginosa*.

Conflict of Interests

The authors have not declared any conflict of interests.

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