Saudi Journal of Biological Sciences 26 (2019) 1731–1736

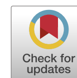
Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Molecular characterization of clinical and environmental *Pseudomonas aeruginosa* isolated in a burn center



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ARTICLE INFO

Article history:
Received 21 May 2018
Revised 30 June 2018
Accepted 19 July 2018
Available online 20 July 2018

Keywords:
P. aeruginosa
MDR
Biofilm formation
Antibiotic resistance
PFGE

ABSTRACT

In burn centers, *Pseudomonas aeruginosa* acts as a major cause of nosocomial infections. Therefore, this study aimed to characterize molecularly *P. aeruginosa* isolates collected from environmental samples and burn patients. A total of 78 strains (including 58 clinical and 20 environmental isolates) of the *P. aeruginosa* were collected from Beasat hospital of Hamadan, west of Iran, and was identified using API 20NE. The disk diffusion method according to the CLSI was applied for determination of the antimicrobial resistance. Moreover, the microtiter plate test was used for the quantification of Biofilm formation. The genomic features of the isolated strains was evaluated using Pulsed Field Gel Electrophoresis (PFGE). We found that 94.8% of clinical and 80% environmental isolates were capable of forming biofilm. The rate of MDR in clinical and environmental isolates was 51.7% and 40%, respectively. A significant relationship was observed between biofilm formation capability and multiple drug resistance ($p < 0.05$). PFGE typing showed 11 different clusters with two major clusters A with 30 (38.5%) and B with 14 (17.9%) members, containing up to 56.4% of all isolates. There was no relationship between biofilm formation ability and antibiotic resistance patterns with PFGE patterns. According to the results, the clonal spread of environmental *P. aeruginosa* isolates is associated with clinical isolates, and both environmental and clinical isolates are attributed to a high prevalence of the antibiotic resistance and biofilm formation ability. This study highlighted that the prevention programs should be implemented in the hospital environment to control the spread of *P. aeruginosa* in burn units.

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1. Introduction

Pseudomonas aeruginosa as an opportunistic Gram-negative pathogen causes severe infections in cancer patients undergoing chemotherapy, patients with cystic fibrosis (CF), severely burned patients, and the patients who are immunocompromised or vulnerable. *P. aeruginosa* infection is often associated with high morbidity and mortality in the mentioned patients (Maroui et al., 2017; Schaber et al., 2007; Alikhani et al., 2017).

Increasing resistance of *P. aeruginosa* to different class of antibiotics is a major concern worldwide. Following the overuse of broad-spectrum antibiotics in the burn ward and Intensive Care Units (ICUs) by creating a selective pressure on bacteria likely led to the emergence of multidrug-resistant (MDR) strains. Nowadays MDR *P. aeruginosa* is responsible for 4–60% of nosocomial infections around the world (Safaei et al., 2017). Various mechanisms are displayed by *P. aeruginosa* for the resistance of multiple classes of antibiotics. In one of the main resistance mechanism; the production of several enzymes deactivates beta-lactams and carbapenems including extended spectrum beta lactamases (ESBLs)

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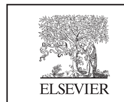
Peer review under responsibility of King Saud University.



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https://doi.org/10.1016/j.sjbs.2018.07.009
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and metallo-β-lactamases (MBLs) (Hassuna et al., 2015; Karami et al., 2017).

The severity *P. aeruginosa*-induced infections is dependent on the production of several virulence factors affecting various aspects of *P. aeruginosa* pathogenesis (Schaber et al., 2007; Alikhani et al., 2014; Safari et al., 2014). In addition, the ability of *P. aeruginosa* to form biofilms under different conditions reduces the efficiency of the antibiotic treatments and subsequently increases chronic infectious diseases (Rasamiravaka et al., 2015a). Biofilm helps microorganism attachment to surfaces and protects them against drying, host defense, and both chemical or physical biocides and antibiotics (Rasamiravaka et al., 2015b).

P. aeruginosa is a ubiquitous microorganism that has the ability to grow under various conditions such as a media with high humidity and simple nutrients. Due to its high resistance to antibiotics and disinfectants, this bacteria are frequently found in various areas of the hospital, including physician's and nursing staff's clothing, sinks, food, computer keyboards, drains, taps, drinking water, pharmacy preparations, and contaminated medical equipment (Davane et al., 2014; de Abreu et al., 2014).

Due to the wide distribution of this bacterium in different environments, it is necessary to evaluate and identify its resources. Its distribution has been reported as cross-transmission by contaminated equipment or environmental sources. Because of a number of drawbacks in traditional typing methods, molecular typing has been known as a powerful tool in epidemiological and outbreak studies of human infections caused by *P. aeruginosa*. The analysis of macro-restriction fragment patterns with PFGE is a robust discriminating method for typing *P. aeruginosa* (Johnson et al., 2007; Maroui et al., 2017).

The patient environment in hospital environment may become contaminated through infected patients, hospital staff, and visitors. Furthermore, the hospital areas can harbor the stains of MDR *P. aeruginosa* and consequently circulate within the hospital. These contaminated areas could act as a source for cross-colonization and outbreaks (Chemaly et al., 2014; de Oliveira and Damasceno, 2010). Promoting awareness about the environmental reservoirs, modes of dissemination, and monitoring the infection sources of *P. aeruginosa* within healthcare facilities can finally enable us to control the bacterial load and reduce the exposure to the infectious agent, and subsequently reduce the risk of infection. The objective of this study was to characterize antibiotic resistance profiles, biofilm formation capacity, and clonal relatedness of *P. aeruginosa* strains isolated from burn patients and burn unit environment.

2. Materials and methods

2.1. Isolation and identification of P. aeruginosa

This cross-sectional study was conducted in the Microbiology Department, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan, west of Iran. Wound swabs were taken from 493 patients suffering from moderate to severe burn wounds with clinical symptoms and signs of burn wound infection attending the Burn unit at Beasat General Hospital between July 2016 and April 2017. Swab specimens were inoculated within two hours onto Tryptic Soy Broth (TSB) and the initial isolation was conducted on McConkey and blood agar. The standard bacteriological methods were used for identification of *P. aeruginosa* isolated from burn wounds which included: Gram staining, colony morphology, pyocyanin pigment production, growth at 44 °C, catalase, oxidase and Triple Sugar Iron (TSI) fermentation tests. All isolates were confirmed by analytical profile index (API) 20NE (BioMérieux Co, France) test.

2.2. Antimicrobial susceptibility

The disk diffusion method was applied in accordance with the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI) to determine the antimicrobial sensitivity or susceptibility (Hassuna et al., 2015). The antibiotic disks (Mast Group Co, UK) used in this study included cefepime (30 μg), colistin (10 μg), piperacillin-tazobactam (100/10 μg), meropenem (10 μg), imipenem (10 μg), aztreonam (30 μg), tigecycline (30 μg), piperacilin (100 μg), and ciprofloxacin (5 μg). *Pseudomonas aeruginosa* ATCC 27853 was used as control strain for this test. The isolates with resistance to at least 3 additional antibiotic classes were selected as MDR *P. aeruginosa*, as already explained (Azizi et al., 2015).

2.3. Biofilm formation

A colorimetric microtiter plate-based assay was used for evaluation of the formation of biofilm by as previously described in detailed (Bardbari et al., 2018). Briefly, *P. aeruginosa* isolates were grown at 37 °C in TSB supplied with 1% glucose for 24 h. Then, a new TSB medium was used for dilution of the bacterial suspensions (1:100). Next, 100 μL of the obtained dilution was added to each well flat-bottomed 96-well polystyrene microtiter plates, and then incubated at 37 °C for 24 h. Following overnight incubation, phosphate buffered saline (PBS) was applied for washing wells Washing operation was repeated with PBS three times, and then 100 μL of methanol was used to fix the solution. After 10 min, the wells were stained with crystal violet (CV) 1% (w/v) for 5 min.

Afterwards, 100 μL of absolute ethanol was added to the washed wells and the biofilm formation was quantified by measuring optical density (OD) at 595 nm. For each isolate tested, biofilm assays were performed in triplicate and the mean biofilm absorbance value was determined. TSB without bacteria was used as negative control.

2.4. Pulsed-field gel electrophoresis typing

PFGE analysis of genomic DNA of *P. aeruginosa* from the collected isolates was performed as already described (Rasamiravaka et al., 2015b). The plugs were digested with the restriction enzyme *Xba*I for 1 h at 37 °C. The *Salmonella braenderup* was used as a molecular size marker. A pulsed-field electrophoresis system (Chef Mapper; Bio-Rad Laboratories, Hercules, CA, USA) was applied for electrophoresis process. The electrophoresis procedure was conducted by programming two states under the following conditions: voltage 6 V/cm, temperature 14 °C, switch angle 120°, switch ramp 2.2–35 s for 19 h. Then, ethidium bromide (1 μg/mL) was used for gel staining under ultraviolet light for 30 min.

2.5. Cluster analysis

The obtained gel images were normalized with *Salmonella braenderup* and patterns of the macrorestriction DNA fragments were analyzed using BioNumerics software version 7.5 (Applied Maths, StMartens-Latem, and Belgium). Similarity clustering analyses was estimated using the unweighted pair group method with mathematical averaging (UPGMA), and Dice correlation coefficient with 1.5% optimization and a 1.5% tolerance setting. Clusters were considered at an 80% similarity cut-off and classify of isolates performed with more than 80% similarity in clusters.

2.6. Statistical analysis

SPSS software (version 23.0, Chicago, IL, USA) was used for data analysis. The frequency of parameters such as antibiotic susceptibility, biofilm formation category were determined in both clinical and environmental samples. In order to compare the qualitative variables, chi-square and Fisher exact tests were used. The association between biofilm formation and the antibiotic resistant with PFGE type were made using chi-square tests. All statistical values were considered significant at a *P*-value less than 0.05.

3. Results

3.1. Bacterial isolates and epidemiological data

A total of 78 isolates of *P. aeruginosa* (non-duplicate) were collected, including 58 (11.8%) clinical isolates from 493 patients and 20 (18.5%) isolates from 108 environmental samples. The mean age of patients was in the range 14–63 years old (32.5 ± 1.7); 34 (58.6%) patients were male and 24 (41.3%) were female. Of the 108 environmental samples, 20 (18.5%) generated *P. aeruginosa* isolates. Floor samples showed a high level of contamination (3/5 = 60%) compared to the medical ventilator samples taken from ICU (4/10 = 40%).

3.2. Antimicrobial susceptibility test

Table 1 shows the patterns of antibiotic susceptibility of *P. aeruginosa* isolated from clinical and environmental specimens. The highest resistance was to ticarcillin-clavulanate (75.9% and 55% in clinical and environmental isolates respectively). The lowest resistance was to amikacin (46.6% and 45%), meropenem (51.7% and 40%), and imipenem (53.5% and 50%) in clinical and environmental samples, respectively. There was no statistically significant difference in resistance rates between the clinical and environmental isolates except for aztreonam ($p < 0.05$). Results indicated that there was sensitivity to colistin and tigecycline in all environmental and clinical isolates. Moreover, there was a severe antibiotic resistance among the 58 isolates of *P. aeruginosa* collected from patients hospitalized in the burn ICU in comparison with environmental isolates. The MDR rate in clinical and environmental isolates was 30 (51.7%) and 8 (40%), respectively and no significant difference was seen between them.

3.3. Ability to form biofilm

Biofilm formation was quantified using a microtiter plate assay for all clinical and environmental isolates. The mean optical density of environmental and clinical isolates was 0.831 ± 0.287 (in the range 0.189–1.631) and 0.679 ± 0.280 (in the range 0.169–1.387) respectively. In the present study, we found that 18 (80%) environmental isolates and 55(94.8%) of clinical isolates had the ability to form biofilm, and all of the isolates were MDR (38 isolates, 48.7%) were also biofilm producer. Consequently, statistical analysis showed that there was a significant relationship between multiple drug resistance and biofilm formation capability ($p < 0.05$).

3.4. PFGE typing

The genetic diversity detection of 58 clinical isolates of *P. aeruginosa* and 20 environmental isolates were performed using PFGE method. The analysis of PFGE banding patterns for clinical and environmental isolates are presented in Fig. 1. Eleven distinct genetic profiles (A, B, C, D, E, F, G, H, I, G, and K) were detected among clinical and environmental of *P. aeruginosa* isolates. The pattern A (with 30 isolates, 24 clinical and 6 environmental isolates) and B (with 14 isolates, 11 clinical and 3 environmental isolates) were two major PFGE group which comprised 56.4% of the isolates. Other profiles comprising patterns C, D, E, F, G, H, I, G and K have 6 (7.7%), 5 (6.4%), 3 (3.8%), 3 (3.8%), 3 (3.8%), 2 (2.6), 4 (5.1%), 4 (5.1%) and 4 (5.1%) isolates, respectively. Clinical and environmental isolates showed 11 and 9 patterns, respectively. Analysis of the patterns showed that there was a similarity between the genotypes of clinical and environmental isolates.

4. Discussion

P. aeruginosa infections are commonly implicated as a cause of health care acquired infections with high mortality and morbidity rates (Hassuna et al., 2015). It has been reported that this pathogen as a main colonizer of burn wound could cause an increase in the risk of infections in patient's burn (de Almeida Silva et al., 2017). The potential of *P. aeruginosa* to biofilm formation and intrinsic high resistance to multiple antibiotics, which allow its survival under harsh condition for instance, subsequent to antibiotic treatment or environmental conditions (de Almeida Silva et al., 2017; Taylor et al., 2014). Cross-transmission of this bacteria from inanimate surfaces or healthcare workers' hands may have a key role in its colonization and infections in burn units and the insufficient and inappropriate control of these infections leads to the spread of antibiotic resistance (Russotto et al., 2015; Arabestani et al., 2014). The role of environmental contamination for managing infection has been identified through molecular epidemiological of bacterial strains responsible for cross-transmission and/or nosocomial infection. In this study, clonal lineage, biofilm formation

Table 1
Antibiotic susceptibility patterns of clinical and environmental *P. aeruginosa* isolates.

Antibiotics	Clinical isolates No = 58		Environmental isolates No = 20		Total No = 78	
	S No (%)	R No (%)	S No (%)	R No (%)	S No (%)	R No (%)
Cefepime	27(46.5)	31(53.5)	10(50)	10(50)	37(47.4)	41(52.6)
Azteronam	17(29.3)	41(70.7)	9(45)	11(55)	26(33.3)	52(66.7)
Amikacin	31(53.4)	27(46.6)	11(55)	9(45)	42(53.8)	36(46.2)
Piperacilin	25(43.1)	33(56.9)	11(55)	9(45)	36(46.2)	23(53.8)
Ciprofloxacin	20(34.5)	38(65.5)	10(50)	10(50)	30(38.5)	48(61.5)
Imipenem	27(46.6)	31(53.5)	10(50)	10(50)	37(47.4)	41(52.6)
Meropenem	28(48.3)	30(51.7)	12(60)	8(40)	40(51.3)	38(48.7)
Ticarcillin - clavulanate	14(24.1)	44(75.9)	9(45)	11(55)	23(29.5)	55(70.5)
Tigecycline	0	0	0	0	0	0
Colistin	0	0	0	0	0	0

R: resistant; S: susceptible.

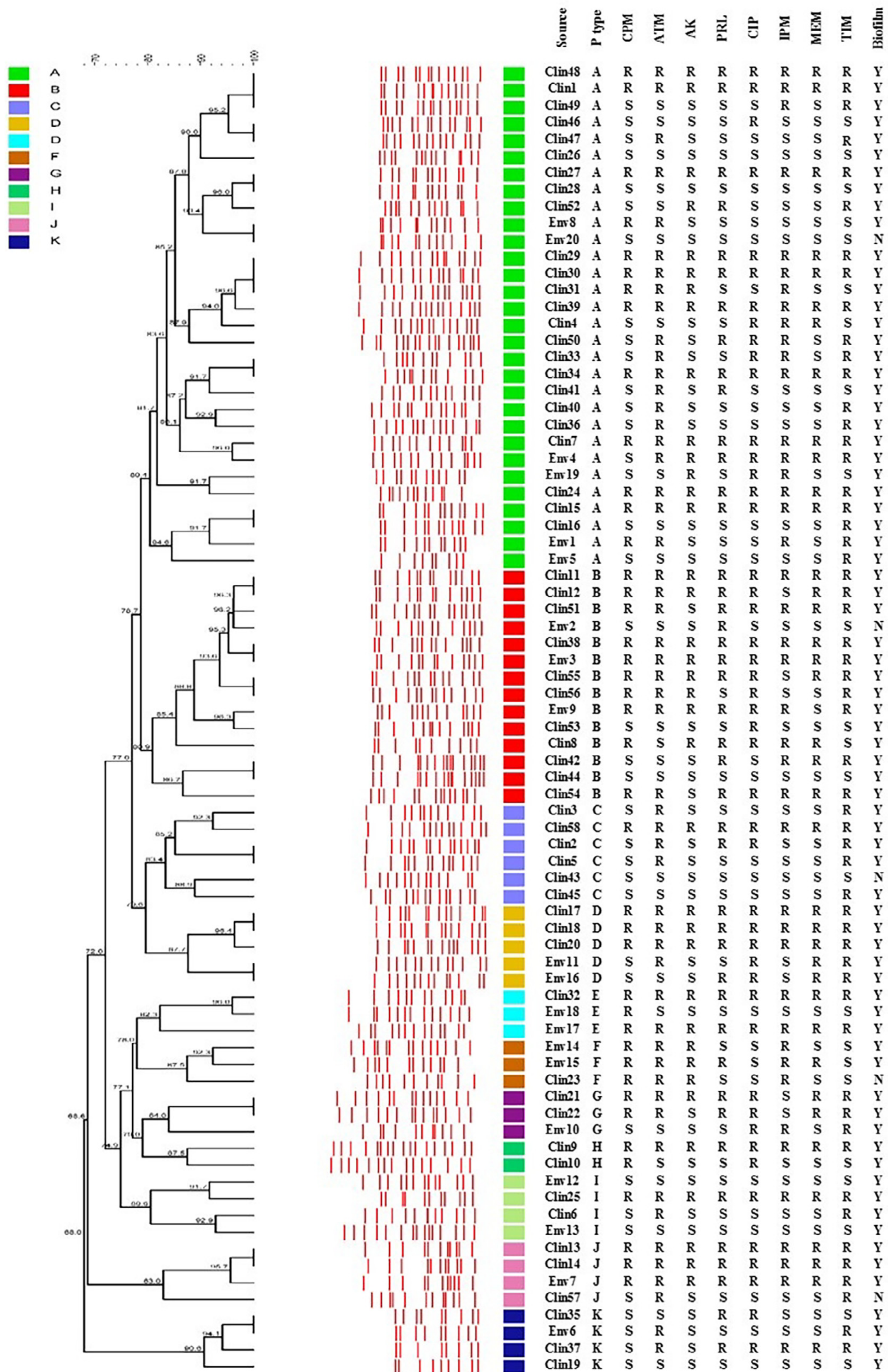

Fig. 1. Dendrogram cluster analysis of PFGE data for 58 clinical and 20 environmental *P. aeruginosa* isolates with resistance patterns, source of infection, and biofilm formation. Clin; clinical, Env; environmental, ATM; Azteronam, AK; amikacin, CIP; ciprofloxacin, MEM; meropenem, IPM; imipenem, CPM; cefepime, TIM; Ticarcillin - clavulanate, PRL; Piperacilin R; resistant, I; intermediate, and S; susceptible, Y; yes, N; no.

capability, and susceptibility patterns of clinical and environmental *P. aeruginosa* isolates were evaluated. The findings could be useful for finding an effective strategy to eliminate infections.

Among 272 positive cultures collected from burn wounds samples, 58 (21.3%) *P. aeruginosa* were isolated. Our finding is consistent with the prior research works. *P. aeruginosa* was the most predominant bacteria associated with the burn wounds (Forson et al., 2017; Pirnay et al., 2003).

The results obtained indicated that the prevalence of *P. aeruginosa* isolated from environmental samples was 20 (18.5%) which is less than that reported in other studies; (39.1%) (Okon et al., 2010) and 28% (Ndip et al., 2005). While in the study of Phoon et al., the incidence of *P. aeruginosa* in the environmental isolates was 5.1% (Phoon et al., 2018). This difference in prevalence rate among several studies can be attributed to differences in hygienic strategies and geographical location.

In the present study, MDR *P. aeruginosa* rate was determined to be 48.7% (38/78). A study performed by de Almeida Silva et al. (2017) in Brazil reported rates of MDR, which were as high as 71.4%, whereas study done by Biswal et al., in India reported lower rates of MDR (36.2%) (Biswal et al., 2014). However, the rates of our study are comparable to a study done in Iran, where Saderi et al., observed 54.7% MDR *P. aeruginosa* (Saderi and Owlia, 2015).

The ability of *P. aeruginosa* to form biofilm is a major factor for bacterial virulence, which causes bacterial survival under various environments such as burn wounds, and consequently results in chronic infections (de Almeida Silva et al., 2017). The current study proved that 73 (93.6%) of clinical and environmental isolates had the ability to generate biofilm that is consistent with the data published in previous studies (31–100% of biofilm positive isolates) (Saleh and Saleh, 2015; de Almeida Silva et al., 2017). Previous studies demonstrated that there was a correlation between MDR phenotype of *P. aeruginosa* and biofilm formation capability (Gurung et al., 2013; Yekani et al., 2017). In regard with the previous studies, our findings indicated that there was a significant difference between the MDR frequency and biofilm production ($P < 0.05$).

Because of the plasticity of the phenotypic characteristics, molecular techniques including multilocus sequence typing (MLST), amplified fragment length polymorphism (AFLP), repetitive-sequence-based PCR (rep-PCR), and PFGE have been developed popularity for strain differentiation and epidemiological studies of many organisms (Sabat et al., 2013). Among molecular typing methods, PFGE has been known as the 'gold standard' and useful tool for assessing outbreak investigations of a variety of pathogens such as *P. aeruginosa* (Maroui et al., 2017). It is essential to monitor the routes of colonization and infection for developing effective strategies in infectious disease prevention.

Genetic analysis of genome by PFGE revealed that there was a similarity between clinical and environmental isolates. This genetic similarity was observed at 70/78 = 89.7% of all isolates. All isolates were classified into 11 clusters (A, B, C, D, E, F, G, H, I, G and K). In line with previous related studies, our findings demonstrated that the cross transmission from patient to patient, patient to health staff, patient to the environment and vice versa have an obvious effect on the epidemiology of nosocomial colonization and infection caused by *P. aeruginosa* (Maroui et al., 2017; Salimi et al., 2010). The results proved there was a dynamic transmission of *P. aeruginosa* isolates between patients and hospital environment. In the present study, we could not detect 8 clinical isolates in clusters of H and C in the environmental isolates. This may be attributed to the cross transmission through direct contact from infected/colonized patient to another patient or caused by environmental reservoirs that we could not identify them.

By comparing the frequency of biofilm formation capability, it was found that there was no significant correlation between clinical and environmental isolates regarding the clusters. Wroblewska et al. proved that there was no connection between genotypes and biofilm formation ability.

Some published studies reported that there was a correlation between drug resistance and PFGE types (Salimi et al., 2010). By contrast, in the current study, *P. aeruginosa* isolates were detected with same genotypes and distinct antibiotypes, and others with same antibiograms and distinct genotypes.

Therefore, there was no correlation between PFGE types and antibiotic resistance profile. In the studies of Maroui et al. (2017) as well as Selim et al. (2015) were shown a single PFGE pulsotype contain different resistance profiles, and a given same resistance profile can be found in different PFGE types. This issue indicated that, phenotypic methods as like as antibiogram lack of discriminatory power for typing and highlights the need for genotypic methods such as PFGE to discriminate isolates with similar phenotypes but distinct genetic relatedness.

Understanding the route of biofilm development and its control may establish a platform for the design of strategies that can be used to struggle and eradicate the infection (Vasiljević et al., 2014). In certain studies, it was determined that there was a relationship between the ability of biofilm formation and genotype (Coban et al., 2009; Saffari et al., 2016). In the current study, in accordance with previous studies (Wroblewska et al., 2008; Vasiljević et al., 2014) no connection between genotypes and the ability to form biofilm was seen.

5. Conclusion

In this study, there was a significant correlation in clonal spread of *P. aeruginosa* between environmental and clinical isolates. Moreover, a high prevalence of the antibiotic resistance and biofilm formation ability were observed in both environmental and clinical isolates. There was no relationship between biofilm formation ability and antibiotic resistance patterns with genetic diversity of the isolates. This study highlighted that the prevention programs in the hospital environments are essential to control the transmission of *P. aeruginosa* in these burn units.

Acknowledgments

This investigation approved and financially supported by the Vice-chancellor of research and technology of Hamadan university of Medical Scienses, Hamddan, Iran.

Conflict of interest

None of the authors have any conflicts of interest to this article.

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