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Original Article

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CorrelationBetween Antibiotic Resistance and Biofilm Formation Power of Pseudomonas Aeruginosa

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Abstract

P. aeruginosa has been mentioned as the major causative agents of nosocomial infections. Pseudomonas infections are often serious and show different resistance to treatment due to the distribution of antimicrobial resistance. Meanwhile, some strains are also able to form Biofilm during contamination, which helps bacteria to be even more persistent to treatment. We examined the antibiotic resistance pattern of P. aeruginosa clinical isolates against Gentamycin, Clarithromycin, Cephalosporin, Ciprofloxacin, Imipenem, Azithromycin, and Ceftazidime and the correlation of antibiotic resistance with Biofilm forming capability of isolates.

Seventy-two clinical specimens were screened for P. aeruginosa, by culturing on bacteriology standard medium. The isolates were confirmed by standard bacteriology tests. The antibiotic resistance pattern of isolates against antibiotics was achieved by standard method of Kirby–Bauer. Biofilm forming power of isolates was examined according to Microtiter Dish Biofilm Formation Assay standard method. The data were analyzed statistically to show the correlation between Biofilm forming and antibiotic resistance phenomena.

The resistance against tested antibiotics was observed by most of the clinical isolates. 54% of isolates showed resistance against Azithromycin and Clarithromycin, while, only 21% showed anti- Ciprofloxacin characteristic. 10% of isolates were able to form Biofilm (OD= 1.2), the Biofilm forming isolates showed resistance at least against one antibiotic. A high rate of resistance was seen against ciprofloxacin (29.2%), Azithromycin (75%), Cephepime (58.3%), Ceftazidime (40.3%), Clarithromycin (75%) Gentamycin (50%) and Imipenem (45.8%). In total, 93.1% of the isolates were characterized as MDRPA. Biofilm formation was seen in 91% of the isolates.

The high rate of MDRPA and its ability to produce Biofilm is an alarm for public health. The statistical analysis showed that Biofilm production in the MDRPA isolates was significantly higher than that in the non–MDRPA isolates ($P < 0.001$).

Keywords: P. aeruginosa, Biofilm forming, antibiotic resistance, correlation

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Introduction

P. aeruginosa is a major microorganism, a cause of nosocomial infections, particularly in burn patients (Adibi et al. 2013). The treatment of infections caused by P. aeruginosa is frequently complicated since the organism is intrinsically resistant to many drug classes and is able to acquire resistance to all the effective antibiotics (Hosseini Doust et al. 2012). In many studies, the term multidrug-resistant P. aeruginosa (MDRPA) has been used to report isolates with resistant to at least three different classes of antimicrobial agents, mostly aminoglycosides, carbapenems, antipseudomonal penicillins, quinolones, and cephalosporins (Sam Boasi et al. 2013). P. aeruginosa also possesses a large number of virulence factors such as exotoxin a, exoenzyme S, elastase and sialidase, which are powerfully regulated by cell-to-cell signaling systems (Bruchman et al. 2013). The major virulence factor produced by most of the P. aeruginosa isolates is exotoxin A (ETA), which plays a major role in the pathogenesis of the organism (Djerb et al. 2012). An extracellular neuraminidase is also thought to have a key role in the implantation of the bacterium, but the genetic basis of this process is still unknown (Guilherm et al. 2017).

Biofilm are sessile populations of microorganisms which are enclosed by the self-secreted extracellular poly¬saccharide matrix, or slime. Biofilm act as efficient bar¬riers against antimicrobial agents (Finlayson and Brown 2011). Despite the high incidence of P. aeruginosa infection in burn patients, there is currently little infor¬mation on the distribution of the virulence factors and the ability of Biofilm production among the isolates of P.aeruginosa. Biofilm formation power has been reported in various species of bacteria, including P. aeruginosa (Hosseini Doust et al. 2012).It was also reported that antibiotic can affect the emergence of Biofilm by some strains. P. aeruginosa is a major contributor to hospital infections and treatment of the infections caused by it is one of the major health problems (Bruchman et al. 2013). Antibiotic resistance in this bacterium is due to intrinsic resistance due to low permeation, the presence of a system of permeation, acquisition of resistance gene through the inherent plasmid due to low permeability, the presence of tracheal systems, acquisition of resistance gene through transpose plasmids, antigens and Biofilm production (Hirscha B. et al. 2016).

Tracheal pumps can penetrate a wide range of microbial compounds without any similarity between their performance structures (Susy Hota et al. 2009). Antibiotics that are used to treat infections caused by this bacterium are recombinant compounds and their excretion from bacterial cells has a significant reduction in their therapeutic effect. P. aeruginosa is one of the major reasons for hospital infections like pneumonia, urinary infections and bacteremia (Ramakishna et al. 2017). These infections are especially seen in immunocompromized patients, such as neutropenic or cancerous patients. This organism is a common cause of death in hospitalized patients and those with the lack of immunization (Jefferis J et al. 2012). Based on epidemiological studies, it is shown that the prevalence of resistance patterns in P. aeruginosa varies within different regions of the world (Mahmoud AB et al. 2013). Treatment of Pseudomonas aeruginosa has become increasingly complex due to the inherent resistance to conventional antibiotics used in the hospital (Majtan J. et al. 2007). Not only are these bacteria inherently resistant to a wide range of antibiotics, but also they earn the ability to increase resistance when treated (Mohuz G. et al. 2017). The relationship between antibiotic resistance and Biofilm – formation ability is now under huge consideration (Kannan RD et al. 2016).

Materials and methods

Seventy-two clinical strains were isolated from various hospital specimens from Tehran Burn Rescue Hospital. The specimens were labeledand carried to the laboratory within the suitable carrier. Each specimen was cultured on routine media for

Pseudomonas growth. The suspicious colonies were screened by standard bacteriologic pathways and characterized as strains as P. aeruginosa and identified with standard bacteriological tests such as hot catalyzes test, oxidize test, sugar fermentation and pigment production, Simon Citrate test and MR / VP test, and OF test (Ramakishnan M et al. 2016).

Antimicrobial susceptibility was determined according to the Clinical and Laboratory Standards Institute (CLSI) guideline (Rodrigo et al. 2016), using the Kirby Bauer disk diffusion assay on Mueller-Hinton agar (Hina S. et al. 2015). The susceptibility profiles were determined for seven antibiotics including Azitromicin (AZ, 100 μg), Clarythromicin (CLR, 30 μg), Ceftazidime (CAZ, 30 μg), Gentamicin (GE N, 10 μg), Cefepime (SP, 30 μg), Ciprofloxacin (CIP, 5 μg), and Imipenem (IPM, 10 μg) (Mast Diagnostics, Mast Group Ltd, Merseyside, UK). P. aeruginosa ATCC 27835 was used as quality control in each antimicrobial susceptibility assay. The results were interpreted as susceptible or resistance according to the criteria recommended by the CLSI and the manufacture's protocols (Mast, UK).

The power of isolates to produce Biofilm was determined using microtiter dish Biofilm formation assay (Abdul Samad et al. 2017). Briefly, the P. aeruginosa isolated were grown overnight at 37˚C in Tryptic Soy Broth (TSB) contain¬ing 0.25% glucose. The cultures were diluted 1:100 in TSB me¬dium. Sterile flat-bottomed 96-well poly styrene microtiter plates were inoculated with 125 μl of the bacterial suspen¬sion and incubated for 24 hours at 37˚C without agitation. The wells were washed three times with 300 μl distilled water, dried in an inverted position at room temperature and finally stained with 125 μl of 0.1% crystal violet solution in water for about 10 - 15 minutes. After staining, the wells were washed three times with distilled water. The wells were distained with 125 μl of 30% acetic acid in water. A new sterile flat-bottomed 96-well poly styrene microtiter plate was inoculated with 125 μl distaining solution in each well. The absorbance of the distaining solution was measured at 570 nm using an ELISA reader (Stat Fax-2100) (Tarana S. et al. 2015). Statistical Package for Social Sciences (SPSS) software (SPSS Inc. No. 21) was used for statistical analyses. Fischer ex¬act test or $\chi 2$ test was used for the analysis of the categorical data. And SPSS test and P value < 0.05 was considered statistically significant (Liva V. et el. 2014). P. aeruginosa ATCC27853 was used as control strain in all experiments. All antibiotic sensitivity patterns were evaluated according to the CLSI tables.

RESULTS

Clinical isolates of P. aeruginosa were cultured from blood (12.5%), chips (33.3%), wounds (19.4%), sputum (27.8%), and urine (9 / 6%) (Figure 1-1).

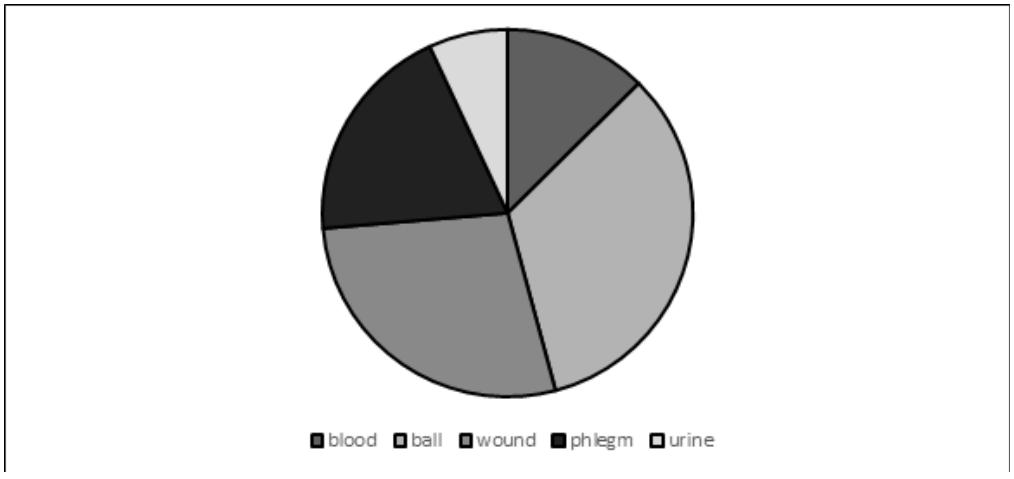

Figure 1: Distribution of clinical specimens

The resistance pattern of P. aeruginosa isolates related to antibiotics were reported as follows: the highest percentage of resistance was against Azithromycin and Claritromycin (95% each), and the lowest was for Ciproforoxacin and Ceftazidime (29.2% and 40.3% respectively); The reference P. aeruginosa showed to Azithromycin and Clarithromycin and sensitive to Cefepime and Gentamycin, Ciprofloxacin, Imipenem, and inhibiting the formation of Biofilm at presence of Ciprofloxacin had the most effects on Biofilm formation.

Table 1: Correlation of Biofilm formation capacity of P. Aeruginosa with antimicrobial resistance patterns

Resistance phonotype	N (%)	OD 660nm
Non_MDR	22 (31)	0.001- 0.399
MDR	12 (17)	0.499- 0.799
XMDR	38 (52)	0.800-1.20

To assess dependence of Biofilm formation capacity and specific antibiotic resistances, Biofilm and planktonic state of isolates, we measured the MIC of isolates. The trange of MIC were from 0.5 to 1024 μg/ml. A high rate of resistance were seen against ciprofloxacin (29.2%), Azitromicin (75%), Sephepim (58.3%), ceftazidime (40.3%), Claritromicin (75%) Gentamicin (50%) and imipenem (45.8%). We first analyzed the correlation between MIC and IZ values of seven antibiotics and the biofilm-forming capacities of the72 strain and found negative correlation between the two qualities ($rs = –0.713$, $P < 0.001$), further indicating the inverse relationship between biofilmformation and resistance to even one antibiotic. Subsequently, by assessing the relationship betweenMICvalues, we detected a positive correlation between antibiotic resistance (MICvalues) and biofilm-specific resistance (ZI values) for the three antibiotics ($rs = 0.844$, $P < 0.001$). As depicted, there was a similar level of enhanced resistance after Biofilm formation among the majority of isolates. A high rate of resistance wer seen against biofilm formation and Ciprofloxacin($rs = –0.709$, $P < 0.001$), the relationship between biofilm formation and Azitromicin ($rs = -0.511$, $P < 0.001$),the relationship between biofilm formation and Sephepim ($rs = –0.653$, $P < 0.001$), the relationship between biofilm formation and ceftazidime ($rs = –0.735$, $P < 0.001$), the relationship between biofilm formation and Claritromicin ($rs = –0.0.621$, $P < 0.001$),the relationship between biofilm formation and Gentamicin ($rs = –0.654$ $P < 0.001$), and the relationship between biofilm formation and imipenem ($rs = –0.674$, $P < 0.001$). We first analyzed the correlation between MIC and IZ values of Spearman's rank correlation analyses indicated that for each of the three antibiotics, this enhance mention resistance occurred independent of the level of biomass produced ($rs = -0.735$, $P < 0.001$).

Discussion

Tracheal pumps can penetrate a wide range of microbial compounds without any similarity between their performance structures. Antibiotics that are used to treat infections caused by this bacterium are recombinant compounds and their excretion from bacterial cells has a significant reduction in their therapeutic effect (Sayyad G. et al. 2017). Pseudomonas aeruginosa is one of the major reasons for hospital infections like pneumonia, urinary infections and bacteremia.

Table 2: Relationship between Antibiotic Resistance Pattern and Biofilm formation capacity

Antimicrobial category	Antimicrobial agent	OD 660nm			rs	P.Value
		S	I	R		
Aminoglycosides	Gentamicin	0.19 , 0.27	0.323 , 0.48	0.5 , 0.9	-0.654	<0.001
Carbapenems	Impanel	0.090 , 0.200	0.29 , 0.350	0.41 , 0.69	-0.674	<0.001
Fluoroquinolon	Ciprofloxacin	0.001 .0.07	0.100 , 0.22	0.38 , 0,666	-0.709	<0.001
Cephems	Ceftazidim	0.033 , 0.30	0.32 , 0.40	0.410 , 0.71	-0.735	<0.001
	Cefepime	0.111 , 0.199	0.28 , 0.389	0.49 , 0.82	-0.653	<0.001
Macrolid	Azitromycin	0.280 , 0.30	0.35 , 0.46	0.55 , 0.91	-0.511	<0.001
	Claritromycin	0.25 , 0.36	0.47 , 0.69	0.700 , 0.98	-0.621	<0.001

S: Sensitive R: Resistant

I: Intermediate 4H: OD after 4 hrs incubation at 37° C, 24H: OD after 24 hrs incubation at 37° C

SPSS version 21 was used for statistical analysis of the data.The Data were analyzed by Student t test and chi-square test (or Fisher exact test). A P-value below 0.05 was considered as statistically significant

These infections are especially seen in immunocompromized patients such as neutropenic or cancerous patients (Snyder LA et al. 2013). This organism is a common cause of death in hospitalized patients and those with the lack of immunization. Based on epidemiological studies, it has been proven throughout the world that the prevalence of different drug resistance patterns in Pseudomonas aeruginosa varies from one country to another, from different geographical regions and even between different hospitals of the same geographical area. Despite the large gains in hospital care systems and the introduction of a wide range of antimicrobial agents, this bacterium is still a common cause of infection in hospitalized patients in different parts of the hospital (Webber & Piddock 2003). Antibiotic resistance and the cause are now very important, and in many cases, the treatment of patients has failed. Due to the increasing resistance of this bacterium to antibacterial drugs, and especially to β-lactam compounds, the importance of its resistance increases (Maike N. et al. 2012). Treatment of Pseudomonas aeruginosa has become increasingly complex due to the inherent resistance to conventional antibiotics used in the hospital. Not only are these bacteria inherently resistant to a wide range of antibiotics, but also they earn the ability to increase resistance when treated (Hosseini Doust et al. 2012).

In this study, the trend of other scientist's studies and research shows the overall increase in resistance to antibiotics. But this increase is fluctuating, including imipenem. Considering that all of the studies have used the publication of the disc method, the cause of these fluctuations can be due to the difference in resistance in different areas of the infection or the type of disc used. But in general, this is a reflection and should be explored further. In the present study, the rate of resistance is significantly increased, which can be due to the fact that hospitalization of patients in a particular area can indicate a prevalence of resistance among P. aeruginosa strains (Guilherm F. et al. 2017).

Results of this study, Pseudomonas aeruginosa isolates resistance pattern against antibiotics, the

highest antibiotic resistance level is Azithromycin and Claritromycin 75%, and the lowest is related to Ciprofloxacin with 29.2% and Imipenem with 45.8%; these percentages were resistant to 3 classes of used antibiotics which were considered as drug-resistant isolates. According to the CLSI standard table, in the study of Biofilm formation, we examined the isolates of Pseudomonas aeruginosa bacteria for two hours by incubation for 4 hours and 24 by microtiter plate and determining the optical absorption with ELISA (Jefferis J. et al. 2012).

The results showed that the highest optical absorption was 1.2. While in other studies performed by Nikookar et al., resistance to Imipenem (22.3%), Gentamicin (37.2%) Ciprofloxacin (63.3%) was reported (32). In present report, resistance to Ciprofloxacin (21%) and Gentamycin (33%), compared to Narten et al. that report the resistance to ciprofloxacin 59% and Imipenem 11% (Majtan J. et al. 2007).

In this study, resistance to ciprofloxacin was 21% and resistance of 33%, but in a study conducted by Kianpour et al. In Isfahan in 1989, resistance to ciprofloxacin and Imipenem was reported to be 42.85% and14.8%, respectively (Abdul Samad et al. 2017). In this research, resistance to ciproforoxacin was 29.2%, but also a comparison between Pseudomonas aeruginosa resistances for Imipenem in Japan in 2001 was 8.3%, in Canada 12%, Russia 13.4%, France 18.5%, and Spain 14%. The reported resistance rate to ciprofloxacin was reported by Imani in the year 89 in Ardabil was 20.9% (Susy Hota et al. 2009).

There have also been many studies in Iran in this regard. In this study, the resistance to Ceftazidime, Gentamicin and ciprofloxacin and Imipenem antibiotics was 29%, 36%, 21% and 33%, respectively. But in a study conducted by Nahayi et al. in Tabriz in 2006, the resistance to Ceftazidime antibiotics and gentamicin and ciprofloxacin and Imipenem were 69%, 51%, 22%, 15%, and 2%, respectively (Rodrigo et al. 2016). In this study, the aim was to investigate antibiotic resistance and its relationship with its potential for Biofilm production, and studies on Biofilm, which are Biofilm generators of Pseudomonas aeruginosa resistant antibiotics that synthesized nickel and its associates (Hosseini Doust et al. 2012) to form Biofilm Pseudomonas aeruginosa on the Catheter's surfaces were illustrated by electron microscope.

In this study, a study on antibiotic resistance and its relationship with the Biofilm production potential was studied, but in agreement with the results and studies conducted by Suzanne et al., there was a direct association between the increase in Biofilm production and optical absorption. Strain 214, which produced more mucoid colony on the surface of the plate than other strains, created a thicker Biofilm. This is more in confirmation of the relationship between the mucoid colony and the creation of Biofilm (Finlayson & Brown 2011). In this study, according to studies and researches of other scientists, we concluded that the strain of Pseudomonas aeruginosa, due to the uncontrolled and arbitrary use of antibiotics, increased resistance to antibiotics, which also had a direct relationship with the production of Biofilm. It has been concluded that strains that increase resistance to antibiotics, produce a thicker Biofilm (Guilherm F. et al. 2017). Biofilm are formed by the strains of P. aeruginosa, which causes the transmission of this bacterium to patients body, which causes a hospital infection, difficult to cure due to antibiotic resistance (Mahmoud AB et al. 2013).

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