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Full length Research Paper

Comparison of cell surface hydrophobicity and biofilm formation among ESBL-and non–ESBL-producing *Pseudomonas aeruginosa* clinical isolates

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Pseudomonas aeruginosa **is a major cause of nosocomial infections. Recently multidrug resistance and extended-spectrum ß-lactamase (ESBL)-producing *P. aeruginosa* isolates are emerging worldwide. These isolates are reported to be more virulent than the non multidrug resistance and non ESBL producing isolates. In order to find a correlation between ESBL production and virulence, we tested two cell surface factors involved in pathogenicity, hydrophobicity and biofilm formation in ESBL and non ESBL producing isolates. ESBL was determined phenotypically by combined disc method. Hydrophobicity was tested by microbial adhesion to hydrocarbon; biofilm formation was determined by microtiter plate. Mean of hydrophobicity in ESBL and non ESBL producing isolates was 38.85 and 30% respectively. Weak reaction in hydrophobicity was significantly higher in the non ESBL isolates (6%); while percent of moderate and strong reaction was higher in the ESBL producing isolates (94%). In biofilm formation mean of ESBL and non ESBL isolates were 0.182 and 0.136 respectively. Percent isolates producing moderate and strong biofilm was 72% for ESBL and 32% for non ESBL isolates (p value < 0.001). Our data demonstrate that hydrophobicity and biofilm formation was higher in the ESBL producing compared with non ESBL producing isolates. The properties can render the ESBL positive isolates more pathogenic.**

Key words: *Pseudomonas aeruginosa*, ESBL, hydrophobicity, biofilm formation, virulence.

INTRODUCTION

Pseudomonas aeruginosa is one of the most common pathogen causing nosocomial infections that affect mainly immunocompromised patients with severe underlying diseases (Cappello and Guglielmino, 2006). Infections cause by *P. aeruginosa* often are difficult to treat due to high level of resistance to multiple antibiotics as a result of both intrinsic and acquisition of resistance genes (Mesaros et al., 2007). In addition to constitutive low susceptibility of *P. aeruginosa* to antimicrobial agent emergence of new resistance mechanisms, such as ESBL belonging to different classes have been identified in these organisms. More studies found that antibiotic resistant phenotypic variants of *P. aeruginosa* have enhanced ability to form biofilm (Drenkard and Ausubel, 2002). Other studies demonstrate the relationship of PER-1-type ESBL-producing *Acinetobacter* spp. and *P. aeruginosa* with poor clinical outcome (Vahaboglu et al., 2001). ß-lactamases are mostly coded by plasmids and are transferable between different bacterial species; these enzymes confer resistance to penicillins, cephalosporins, and aztreonam (Philippon et al. 1994; Sturenburg and Mack, 2003). Infection caused by ESBL-producers are associated with severe adverse outcomes (Lee et al., 2006; Schwaber et al., 2006; Schwaber and Carmeli, 2007), infections caused by ESBL-producers may be related to increased virulence of these strains. Often due to delay in effective therapy and the failure to use antibiotic active against ESBL-producing isolates. Another possibility for the high rate of mortality considered with infections caused by ESBL-producers may be related to increased virulence of these strains. Indeed, several studies have reported an association

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between ESBL-production and the higher expression of pathogenicity factors (Sahly et al., 2008). *P. aeruginosa* is able to synthesize various surfaces and extracellular components as virulence factor (Stanley, 1983), cell surface properties result from the special chemical structure of the cell surface. The hydrophobicity of the microbial surface plays a critical role in the adherence of bacteria to the wide variety of surfaces (Rosenberg, 1981). Hydrophobicity is also a significant determinant of adhesion and biofilm formation on polystyrene surfaces (Pompilio et al., 2008). Biofilm formation is a well-known pathogenic mechanism in device-related nosocomial infection. Biofilm are communities of bacterial cells that are irreversibly attached to a substratum, an interface, or to one another (Donlan and Costerton, 2002). The ability of biofilm formation can increase survival of microorganism, because cells growing in biofilm are highly resistant to the components of the human immune system and many antimicrobial agents (Matsukawa and Greenberg, 2004; Leid et al., 2005).

Attachment of bacteria to and following invasion into host cells are regard an important step in the infectious process (Ofek et al., 2003). Such infections are extremely difficult to eradicate and the fact that cell surface hydrophobicity (CSH) and biofilm formation plays a significant role in a wide range of microbial infections, this study was performed to evaluate and compare the CSH and biofilm formation as two cell surface determinant involved in pathogenicity in ESBL and non ESBL producing clinical isolates of *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

A total of 50 ESBL-producing and 50 non-ESBL producing *P. aeruginosa* strains from nosocomial infections obtained from January, 2008 - June, 2009 were included in the study. The study population included the hospitalized patients of all age groups.

The specimens were collected from urin, blood, wound, burn and other sites of infection. The isolates were identified based on standard bacteriological methods. The standard strain including *P. aeruginosa* PAO1, *P. aeruginosa* ATCC 27853, *Klebsiella pneumonia ATCC* 700603 and *Escherichia coli* ATCC 25922 were used as the control of experiments. The isolated bacteria were stored in trypticas soy broth (TSB) with 40% glycerol at -70°C until used.

Detection of extended spectrum β-lactamases

Resistance to cefotaxime, ceftazidime and ceftizoxime was determined by agar dilution method. Any bacterial species which showed resistance to any of third generation cephalosporin was tested for ESBL production. Combined disc diffusion method was used to confirm ESBL production by *P. aeruginosa* strains. Ceftazidime, cefotaxime or cephpdoxime discs (30 µg) alone or in combination with clavulanic acid (10µg) were placed on mueller hinton agar plate inoculated with test organisms and incubated overnight at 37°C. Regardless of zone diameters, a ≥ 5 mm increase in a zone diameter of an antimicrobial agent tested in combination with clavulanic acid vs. its zone size when tested alone, indicated ESBL production (Bradford, 2001).

Microbial adhesion to hydrocarbons

Microbial surface hydrophobicity was assessed with xylene (Merck) according to Rosenberg and Gutnick (1980). All isolates including standard strain were grown into nutrient broth (50 ml) in a 250 ml erlenmeyer flask with shaking in 200 rpm. Cells were harvested by centrifugation (10000 × g, 15 min), washed twice in sterile phosphate-buffered saline (pH 7.1) and suspended in the same buffer to an initial optical density (OD) of about 1.0 (A0) at 600 nm. Next, 300 µl of xylene was added to 3 ml of microbial suspension and vortex for 2 min. After 10 min the OD of the aqueous-phase was measured (A1) at 600 nm. The degree of hydrophobicity was calculated as $[1 - (A1 / A0)]100[\%]$.

Static biofilm assay

Biofilm formation was measured as described by O'Toole and Kolter (1998). Overnight cultures of all isolates and standard species (*P. aeruginosa* strain PAO1 and *P. aeruginosa* ATCC 27853) were diluted to an OD at 600 nm of 0.1 in fresh Luria Bretani (LB) broth; 100 µl culture aliquots were added into the wells of sterile 96-well polystyrene microtiter plate (Falcon, USA) and incubated for 24 h at 30°C. After three times wash with water, biofilm formation was visualized by staining with 1% (wt/vol) crystal violet (CV) fallowed by three times rinse with water. The amount of biofilm formed was determined at OD595 after addition of 200 µl of 95% ethanol that solubilizing retained CV. *P. aeruginosa* strain PAO1 was used as a positive control and negative control wells contained uninoculated culture media.

Statistical analysis

All the statistical analysis was carried out with SPSS17 software. Independent Sample T Test was used to check the comparison of CSH between ESBL- and non–ESBL-producing isolates. Mann-Whitney Test was used to compare biofilm formation of clinical isolates with or without ESBL. Data were expressed as mean values ± standard deviation (SD), and P value ≤ 0.05 was considered to be statistically significant.

RESULTS

Cell surface hydrophobicity

In ESBL producing strains of *P. aeruginosa* (n = 50) CSH values ranged from 10.68 - 66.35% CSH with an average of 38.85% (Table 1). In strains with non ESBL phenotype the values ranged from 3.88 - 65.22% with an average of 30%. In whole the difference between the ESBL positive and negative isolates was significant (P = 0.002). CSH was also grouped in 3 categories from weak < 20%, to moderate 21 - 50% and strong > 50%. In ESBL positive isolates the weak reaction was low (6%), while the majority of the isolates were in the moderate (78%) or strong group (16%). The difference between the weak and moderate in the isolates from ESBL positive and negative was significant (P Value = 0.002).

Table 1. Percent cell surface hydrophobicity in ESBL- and non-ESBL clinical isolates of *P. aeruginosa*.

ESBL production	Mean ± SD of CSH	CSH* No (%) in each group		
		Weak 0 - 20%	Moderate 21 - 50%	Strong > 50%
Positive (n = 50)	38.85 ± 11.68	3 (6)	39 (78)	8 (16)
Negative (n = 50)	30 ± 16.02	16 (32)	27 (54)	7 (14)

*CSH was determined based on the difference of the OD of bacterial before and after adsorption to hydrocarbon × 100, weak (0-20%), moderate (21 - 50%) and strong CSH > 50%. Mean ± SD of CSH for *P. aeruginosa* strain PAO1 run in triplicate was 41.16 ± 1.93, and for *P. aeruginosa* strain 27853 was 18.48 ± 0.97. CSH of ESBL and non-ESBL isolates was statistically significant (P value = 0.002).

Biofilm formation

Fifty isolates of *P. aeruginosa* producing ESBL and 50 non ESBL producing isolates were compared for their ability to form biofilm. Striking difference was observed among ESBL positive and ESBL negative isolates with regard to the biofilm formation ability based on the OD values. The mean OD of ESBL positive isolates were 0.182 and ranged from 0.07 - 0.50 while for non ESBL isolates OD ranged from 0.07 - 0.79, and a mean of 0.136 (Table 2) P values < 0.001. Biofilm formation of samples were Classified in three groups according to the cutoff OD. The cutoff OD (ODc) for the microtiter plate test was defined as three standard deviations above the mean OD of the negative control (ODc is 0.07 in our experiment). Isolates were classified according to Stepanovi et al. (2000) to three groups as follows: OD ≤ ODc = non-adherent, ODc < OD ≤ (2 × ODc) = weakly adherent; (2×ODc) < OD ≤ (4 × ODc) = moderately adherent and (4 × ODc) < OD= strongly adherent (Stepanovi et al., 2000).

Grouping the biofilm formation to weak (OD Weak group: 0.05 - 0.130), moderate (0.131 - 0.274) and Strong group (>0.274) showed that lower number of isolates with ESBL positive group (>0.274) had weak reaction (28%), in comparison with the ESBL negative group (68%) and the difference in the biofilm formation in all three group was significant.

DISCUSSION

P. aeruginosa is an important cause of nosocomial infection. Resistance to multidrug and production of extended-spectrum β-lactamases (ESBLs) are related to an increased length of stay, with a higher mortality rate and cost (Rodrguez-Bao and Pascual, 2008). Multi drug resistance (MDR) isolates are reported to have more virulent factors compared to sensitive isolates (Baquero et al., 1998). However, Deptuła et al. (2010) illustrate that MDR strains of P. *aeruginosa* strains have impaired virulence in vitro, specially had slow growth and reduced expression of some exoenzymes and pyocyanin when compared with sensitive strains (Deptu and Gospodarek, 2010).

Drug resistance in bacteria is usually plasmid mediated and the acquisition of extra gene can affect various other phenotypic characters of the bacteria. Both gaining (Sahly et al., 2008) or losing virulence properties (Deptu and Gospodarek, 2010) are reported in MDR bacterial isolates.

The first step in bacterial infection is adherence and colonization of the bacteria to the surface. CSH is an important for adherence and colonization (Absolom, 1988; Costa et al., 2006) CSH is a complex interaction between component of the surface of the bacteria and the surrounding environment (Courtney et al., 2009). Pompilio et al. (2008) found a positive correlation between CSH and biofilm formation in *Stenotrophomonas maltophilia* (Pompilio et al., 2008).

Resistance to ß-lactamase may change the cell wall of bacteria and renders the cell more adhesive (Lazar, 2004). CSH may change as a function of the bacterial physiology (Rodrguez-Bao and Pascual, 2008). In this study we found a significant difference in the CSH of the ESBL producing *P. aeruginosa* clinical isolates in comparsion with non ESBL producing isolates. This in accordance with Sharma et al. (2007) that shows a significant association between multiple virulence factors and ESBL production in extraintestinal *E. coli* (Sharma et al., 2007). Biofilm formation is an important virulence factor in many nosocomial infections, so that in 65% of nosocomial infections biofilm are formed (Potera, 1999). A positive correlation between biofilm formation and presence of ESBL *bla* $_{PER-1}$ among the *Acinetobacter baumannii* has been reported by Lee et al. (2008). A correlation between ESBL type PER in *Acinetobacter* species and *P. aeruginosa* with poor clinical outcome has been reported by Vahaboglu et al. (2001). In this study we found that ESBL producing isolates had a higher

Table 2. Biofilm formation by 100 clinical isolates of *P. aeruginosa*.

ESBL production	Mean ± SD of biofilm formation	Biofilm formation* No (%) in each group		
		Weak 0.05 - 0.130	Moderate 0.131 - 0.274	Strong > 0.274
Positive (n = 50)	0.182 ± 0.086	14 (28)	29 (58)	7 (14)
Negative (n = 50)	0.136 ± 0.108	34 (68)	14 (28)	2 (4)

Mean ± SD of CSH for *P. aeruginosa* strain PAO1 run in triplicate was 0.145 ± 0.012, and for *P. aeruginosa* strain 27853 was 0.101 ± 0.016. Biofilm formation of ESBL and non-ESBL isolates was statistically significant (P value < 0.001).

ability to form biofilm in comparison with non ESBL producing isolates, Also ESBL producing isolates had a significantly higher CSH; both of them are among the important virulent factors associated with cell surface. It has been suggested that a number of chromosomal gene rearrangement occurs upon acquisition of the ESBL-plasmid. It is possible that higher mortality and severity of infection caused by ESBL producing isolates is due to the expression of several virulence genes simultaneously, rather than gaining new virulence genes (Sahly et al., 2008).

As far as we know this is the first report on CSH and biofilm formation in clinical isolates of *P. aeruginosa* producing and non producing ESBLs. However, study on the gene expression is necessary to compare the pathogenicity of ESBL and non ESBL isolates. Due to highly emerged isolates producing different types of ESBL and the importance of ESBL producing *P. aeruginosa*, study on other virulence factors both genetically and phenotypically is recommended.

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