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AN EXAMINATION OF POTENTIAL DIFFERENCES IN BIOFILM PRODUCTION AMONG DIFFERENT GENOTYPES OF *PSEUDOMONAS AERUGINOSA*

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Abstract - In the present study, we have examined if there is any difference in biofilm production among different genotypes of *Pseudomonas aeruginosa*. The study investigated 526 non-duplicate *P. aeruginosa* isolated from clinical specimens and from a hospital environment. Isolates were grouped into thirty-five genotypes based on an identical ERIC2-band pattern. Biofilm formation was quantified by the microtiter plate test and all strains were classified into the following categories: no biofilm producers (0), weak (+), moderate (+), or strong (+++) biofilm producers. Only 2.45% of examined strains were not biofilm producers. Among biofilm producers, 39.26% were strong biofilm producers, 34.36% were moderate biofilm producers, while 23.93% were weak biofilm producers. Although the majority of strong biofilm producers were in genotype groups 2 and 3, the degree of *in vitro* biofilm formation in our study was not significantly affected by the genotype of *Pseudomonas aeruginosa*. In this study, we demonstrated that the degree of *in vitro* biofilm formation is not significantly affected by the genotype of *Pseudomonas aeruginosa*.

Key words: Biofilm, *Pseudomonas aeruginosa*, genotype

INTRODUCTION

Pseudomonas aeruginosa, which is predominantly an environmental bacterium, is an opportunistic pathogen that accounts for 10% of nosocomial infections. Infection generally depends on the host having a compromised immune system (Rutherford and Bassler, 2012). *Pseudomonas aeruginosa* is wonderfully adept at forming highly organized surface-associated communities encased within an exopolysaccharide and protein matrix, known as biofilms. (Hassett et al., 2009).

Bacterial biofilms are surface-associated, multicellular, morphologically complex microbial communities (Zhao et al., 2013). Biofilms consist of water, bacterial cells and a wide range of self-generated extracellular polymeric substances (EPS). Biofilm formation is shown to be coordinated by EPS production, cell migration, subpopulation differentiation and interactions (Yang et al., 2011).

While *Pseudomonas aeruginosa* isolates are known to have the ability to attach to a surface and form biofilms, it is not clear whether there is a difference in biofilm formation among genomically diverse environmental and clinical isolates of *Pseudomonas aeruginosa.* In this study, we have examined if there is any difference in biofilm production among different genotypes of *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Bacterial strains

The present study investigated 526 non-duplicate *P. aeruginosa* isolated in the tertiary care Mother and Child Health Institute of Serbia "Dr. Vukan Čupić" from November 2007 to November 2008. Most were collected from clinical specimens – respiratory tract (endotracheal aspirates, bronchoalveolar lavage fluid and sputa), urine, blood, various swabs etc. In addition, a number of isolates were recovered from the hospital environment (taps, drains, incubators and ventilator equipment). The identification of 526 isolates to the species level was carried out using API20NE (bioMerieux, Marcy-l'Etoile, France).

ERIC fingerprinting

Genomic bacterial DNA (0.1-1 μg) was used for the ERIC-PCR reactions using the sequence ERIC 2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) in a final volume of 25 μL as follows: an initial denaturation (95°C, 5 min), followed by 40 cycles of denaturation (94°C, 30 s), annealing (48°C, 30 s) and extension (72°C, 1 min) with a single final extension (72°C, 10 min). Isolates were grouped into genotypes based on an identical ERIC2-band pattern.

Biofilm production

Biofilm formation was tested in 96-well microtiter plates according to Stepanović et al. (2007). Briefly, one colony of the overnight cultures of bacterial strains was diluted in saline in order to adjust the turbidity of the bacterial suspension to 0.5 McFarland standard (approximately 10^8 CFU/mL). Wells of microplates were filled with 180 μL of trypticase soy broth (TSB, bioMerieux, Marcy-l'Etoile, France). 20 μL of previously prepared bacterial suspension was added to each well. Negative control wells contained broth only. The plates were incubated aerobically for 24 h at 35^0C. Thereafter, the content of each well was aspirated and the wells washed three times with 300 μl of sterile physiological saline. Biofilm was fixed with 200 μl of methanol per well, and after 20 min the plates were emptied and left to air dry. The plates were stained with 150 μl per well of Crystal violet used for Gram staining (Merck, Germany) for 5 min. After the plates were air dried, the dye bound to the adherent cells was resolubilized with 150 μl of 33% glacial acetic acid (Zorka Pharma, Šabac, Serbia) per well. The optical density of each well was measured at 570 nm by using an automated Multiscan EX reader (Labsystems, Helsinki, Finland).

Based on the optical densities of bacterial biofilms, all strains were classified into the following categories: no biofilm producers (0), weak (+), moderate (+), or strong (+++) biofilm producers, as previously described (Stepanović et al., 2007).

Correlation between the genotype and the degree of biofilm formation was examined by the Pearson chi-square test.

RESULTS

Analysis of the genetic similarity by ERIC2 rep-PCR revealed great clonal diversity among 163 isolates. Thirty-five different profiles were identified, of which three were dominant and comprised 49.1% of isolates (Fig. 1).

We examined and compared the biofilm formation of 163 *Pseudomonas aeruginosa* isolates in 96-well polystyrene microtiter plates. As shown in Table 1, the *Pseudomonas aeruginosa* isolates from different individuals as well as from the hospital environment expressed different levels of biofilm formation. Only 2.45% of examined strains were not biofilm producers. Among biofilm producers, 39.26%, were strong biofilm producers, 34.36% were moderate biofilm producers, while 23.93% were weak biofilm producers

Correlation between the genotype and the degree of biofilm formed is shown in Fig. 2. The majority of strong biofilm producers were in genotype groups 2 and 3. The degree of *in vitro* biofilm formation is not significantly affected by the genotype of *Pseudomonas aeruginosa* ($p<0.079$).

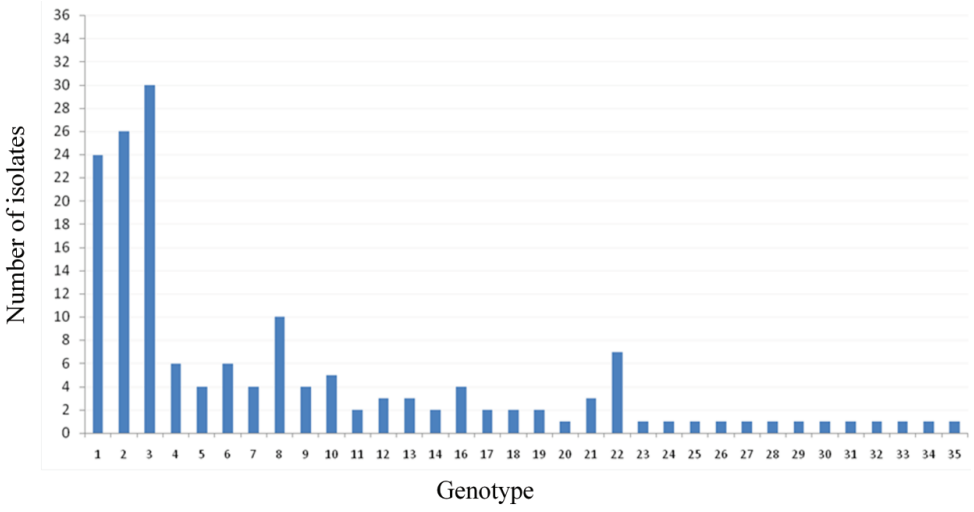

Fig. 1. Distribution of 163 isolates in 35 different profiles.

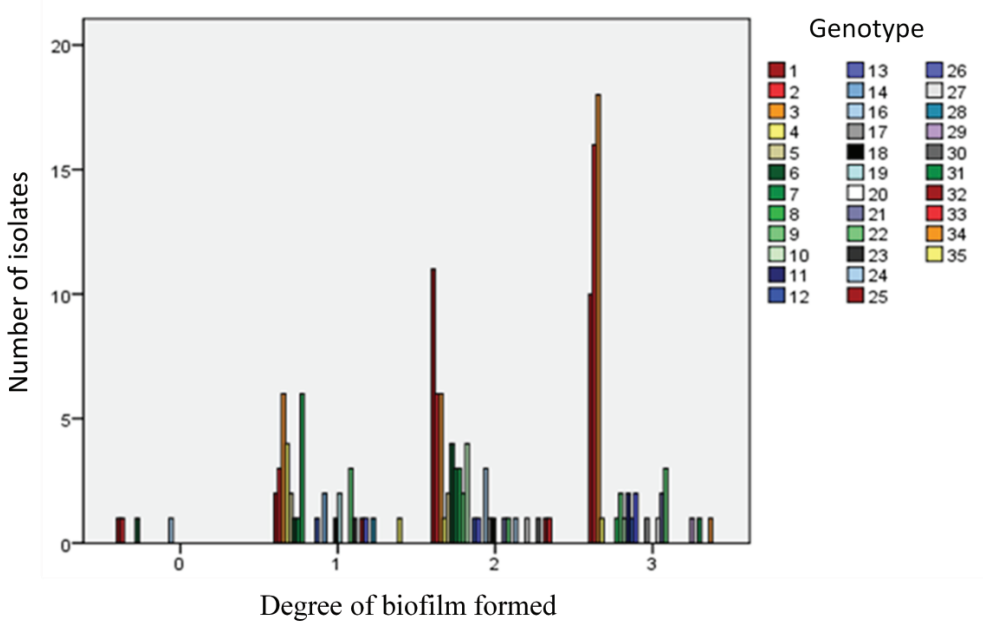

Fig. 2. Correlation between the genotype and the degree of biofilm formed. $p<0.079$

DISCUSSION

Biofilms are communities of bacteria that form in response to environmental stimuli. These environmental signals lead to global regulatory changes within each bacterium, resulting in binding to a surface, aggregation and production of exopolysaccharides. Over the last couple of decades, much evidence has supported the hypothesis that biofilms play a large role in the pathogenesis of chronic infections (Moreau-Marquis et al, 2010).

Bacterial biofilm formation has been studied with increasing intensity during the last 5 to 10 years, and it is now generally recognized that bacterial life on surfaces is often the dominant lifestyle.

Table 1. Distribution of four categories of biofilm producers.

Categories of biofilm producers	Number of isolates	Percent of isolates
No biofilm producers (0)	4	2.45
Weak biofilm producers (+)	39	23.93
Moderate biofilm producers (++)	56	34.36
Strong biofilm producers (+++)	64	39.26
Total	163	100

Pseudomonas aeruginosa has attracted particular interest as a model system for biofilm development, partly because it is a frequent pathogen in humans and partly because it is a well-characterized organism with a very versatile capacity to persist and proliferate in many different environments (Lee et al., 2005).

Pseudomonas aeruginosa is a serious opportunistic pathogen in certain compromised hosts, such as those with cystic fibrosis, thermal burns and cancer. It also causes less severe noninvasive disease, such as external otitis and hot tub folliculitis, in normal hosts. *Pseudomonas aeruginosa* is phenotypically very unstable, particularly in patients with chronic infection. Phenotypic typing techniques are useful for understanding the epidemiology of acute infections, but they are limited by their discriminatory power and by their inability to group isolates that are phenotypically unrelated but genetically homologous. Molecular typing techniques, developed over the past decade, are highly discriminatory and useful for typing strains from patients with chronic infection where the bacterial phenotype is unstable; this is particularly true in cystic fibrosis, where patients are often infected with the same strain for several decades, but the bacteria undergo phenotypic alteration (Speert, 2002).

Biofilm formation is an important phenotype associated with chronic *Pseudomonas aeruginosa* pulmonary infections in cystic fibrosis. There is evidence to suggest that almost all strains of *P. aeruginosa* have the genetic capacity to synthesize alginate, a main matrix of biofilms (Kobayashi, 2005).

In this study, we evaluated the existence of possible difference in biofilm production among different genotypes of *Pseudomonas aeruginosa.*

Analysis of the genetic similarity by ERIC2 rep-PCR revealed great clonal diversity among 163 isolates. Thirty-five different profiles were identified, of which three were dominant and comprised 49.1% of isolates.

The results obtained in this study show that the degree of biofilm production is high in the examined isolates of *Pseudomonas aeruginosa*. Only 2.45% of examined strains were not biofilm producers. Among biofilm producers, the majority of isolates (39.26%) were strong biofilm producers.

Although the majority of strong biofilm producers were in genotype groups 2 and 3, the degree of *in vitro* biofilm formation in our study was not significantly affected by the genotype of *Pseudomonas aeruginosa*.

Genomic comparison between clinical and environmental isolates can yield useful information on the inherent virulence properties (Head and Yu, 2004). Head and Yu (2004) reported that various *P. aeruginosa* strains have different capacities of *in vitro* biofilm formation.

Understanding the route of biofilm development and its control may constitute a platform for the design of strategies that can be used to combat and eradicate the infection (Lee et al., 2005). Testing for various parameters that influence biofilm forma-

tion should contribute to a better understanding of the actual correlation between pseudomonas biofilm formation *in vitro* and the biofilm production in the human body.

In this study, we demonstrated that the degree of *in vitro* biofilm formation is not significantly affected by the genotype of *Pseudomonas aeruginosa*.

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