ORIGINAL ARTICLE

BIOFILM FORMING ACTIVITY OF NON-FERMENTING GRAM-NEGATIVE BACTERIA

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ABSTRACT

The aim: To study the influence of chemical, physical factors on the biofilm forming activity of *P. aeruginosa*, *A. baumannii*.

Materials and methods: Biofilm forming activity of *P. aeruginosa* (10 isolates) and *A. baumannii* (10 isolates) was studied in nutrient media of different composition. There was used the method in 96-well crystalline violet staining plates with spectrophotometry (STAT FAX®4300, wavelength of 620 nm).

Results: Results showed that in standard medium (trypto-soy broth), strains of P. aeruginosa (90%) and A. baumannii (60%) obtained high biofilm forming activity. *A. baumannii* formed biofilms even in sterile water. Biofilm forming activity of urease positive *P. aeruginosa* increased in the medium with 1.0% urea. Both *Acinetbacteria* and *Pseudomonas* intensively produced their biofilms in the presence of 5% serum or sub-bacteriostatic concentrations of levofloxacin in the media. High concentrations of sodium chloride inhibited their biofilm activity.

Conclusions: Isolates of *Acinetobacter* and *Pseudomonas* obtain the protective biofilm-forming ability under such adverse environmental conditions as insufficient nutrients, high osmotic pressure, the presence of antibiotics but at high concentrations sodium chloride biofilm-formation is stimulated only in the first bacteria and suppressed in the second one.

KEY WORDS: bacteria, biofilm, levofloxacin, *Acinetobacteria*, *Pseudomonas*

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INTRODUCTION

Far evolutionary and taxonomically different bacteria are united in a heterogeneous group of non-fermenting Gram-negative bacilli by a number of phenotypic features and are characterized by ubiquitous distribution. High ecological plasticity allows these bacteria to dominate in many ecological niches, as they are common inhabitants of natural reservoirs, found in soils of different geographical zones, sewage, capable of colonizing numerous plant substrates, skin and mucous membranes of animals and humans. In human surroundings, they can be found on the surface of sanitary equipment, in ventilation and humidification systems, surfaces of kitchen equipment and equipment for cleaning rooms, computer keyboard, soil of houseplants, etc. These bacteria have a selective advantage over other microflora, primarily because of their minimal nutrient requirements and natural resistance to chemical influences, including exposure to antibiotics and disinfectants [1, 2, 3].

In recent decades, the representatives of the genera *Pseudomonas* and *Acinetobacter*, which role in the etiological structure of opportunistic human infections is steadily increasing, attract special attention of specialists. Taking into account the selective benefits, favorable conditions for microorganisms of these genera exist in the hospital environment. The greatest problems of *Pseudomonas* and *Acinetbacteria* are created in the intensive care units, departments of combustological and surgical profile, where patients with secondary immunodeficiency due to severe underlying disease are concentrated. The ability of these microorganisms to easily colonize the metal and polymer surfaces of medical equipment and tools has expanded the list of entrance gates for them because of increasing the number of invasive manipulations in the process of improving medical technologies [4, 5, 6, 7].

Alarming statistics on the increase in the number of infectious lesions, associated with the provision of medical care due to antibiotic-resistant variants of *P. aeruginosa* and *A. baumannii*, have forced in February 2017, the World Health Organization to put two types of bacteria on the list of "priority pathogens", which pose the greatest threat to human health [8]. The most dangerous clinical characteristic of *Pseudomonas* and *Acinetbacter* is the high level of natural and acquired resistance to antibiotics. The presence of resistance to many antibiotics is determined even in the "wild" strains of these bacteria, isolated from the environment not contaminated with human activities [4, 9, 10].

Hospital strains are usually characterized by the simultaneous presence of many mechanisms of resistance to β-lactam antibiotics, aminoglycosides, fluoroquinolones, amphenicols, tetracyclines. Since the 1990s, strains of *Acinetobacter* and *Pseudomonas*, resistant to carbapenems and colistin have been recorded and the rate of isolation of such isolates has been steadily increasing. So in surgical hospitals of Ukraine for the

period from 2011 to 2015 the release rate of carbapenem-resistant *P.aeruginosa* strains increased by 67.4%. Similar trends are found in other countries. There are no effective methods of therapeutic management for patients with infectious processes caused by pan-resistant to antibiotics bacterial strains. This leads to high mortality rates and to significant socio-economic losses [9, 10, 11, 12].

In *P. aeruginosa* and *A. baumannii* there is proved the ability to survive in the presence of high concentrations of antibiotics, antiseptics and disinfectants, due to the presence of enzymatic and efflux mechanisms of action on antibiotic molecules and formation of biofilms at the locus of lesions. After all, these types of bacteria are characterized by the highest level of biofilm forming activity, in comparison with other bacterial pathogens. Bacteria biofilms are a complex community of bacterial cells, that are fixed on any surface and united by an extracellular polymer matrix. Biofilms have ordered multilayered topography and cytoarchitectonics, but individual cells in their composition differ in metabolic activity and can be functionally differentiated. In fact, bacterial biofilms are a form of extracellular organization of bacteria, that enhances the population's ability to survive. As a part of biofilms, bacteria obtain the resistance to aggressive environmental factors [13, 14, 15].

The structure of bacterial biofilms and the dynamics of their formation have been studied in detail at the molecular level. Many studies are devoted to the comparative evaluation of the biological properties of bacterial cells in the composition of biofilms and planktonic forms. However, in the scientific literature there is insufficient data about the influence of external factors on the biofilm process [16].

Understanding of the factors, influencing the intensity of biofilm formation by *Acinetobacter* and *Pseudomonas*, will help reliably predict the likelihood of the formation of highly resistant forms of pathogens inflammatory processes in clinical settings, and make this important biological process manageable.

THE AIM

The aim – to study the effect of osmotic pressure and the presence of certain organic substances, antibiotics in a nutrient medium on the intensity of film formation of *P. aeruginosa*, *A. baumannii.*

MATERIALS AND METHODS

In the research there were used 10 clinical strains of *P. aeruginosa* and *A. baumannii*, isolated from patients, treated in burn and surgical departments of Vinnitsa medical establishments. Identification of microorganisms was performed taking into account morphological, tintorial, culture and biochemical properties. Biochemical typing of isolated strains was performed using diagnostic panels NEFERM test 24 (PLIVA – Lachema a. s. Brno, Czech Republic).

Determining the biofilm forming activity of studied strains was carried out using the standard method of analyzing bacterial biofilms in 96-well platelets with crystalline dye. The intensity of the film formation was estimated by the optical density of the studied samples of bacterial film. Cultivation of the microorganisms was carried out in tryptone soy broth (TSB) (GRASO Biotech, Poland) and "starvation" media based on sterile distilled water with the addition of inorganic and organic compounds. All spectrophotometric measurements were performed on a STAT FAX® 4300 spectrophotometer (Netherlands) at a wavelength of 620 nm. The optical density (OD) for each strain was determined in three replicates, the results averaged. A strain was considered to be positive for film-forming ability if its average OD value was greater than the meaning of optical density in negative control, which had increased by three standard deviations (SD): (OD negative control + (3 × SD negative control). The negative control OD was calculated for each tablet separately. The intensity of the film formation was evaluated by the value of the relative optical density [17].

The sensitivity of the tested strains of microorganisms to antibiotics was studied by the method of double serial dilutions of the drug in liquid nutrient medium (TSB). The artificial formation of resistance of microorganisms to antibiotics (meropenem, amikacin, levofloxacin) was performed *in vitro* by the method of passages of microorganisms in meat-peptone broth under the increasing antibiotic concentrations. For this purpose, a series of consecutive double dilutions of the antibiotic in tubes with meat-peptone broth was prepared. The test cultures of bacteria were introduced into test tubes and incubated for 24 hours at 37^0 C. After that, in a row there had been determined the tube with the maximum concentration of the antibiotic, in which there was found no bacteriostatic action of this antibiotic. The content from this tube was used as inoculum for the next passage.

RESULTS

Determination of the film-forming activity of clinical strains of genius *Pseudomonas* and *Acinetobacter* when cultured in TSB at 37^0 C demonstrated significant differences between strains. In general, the property studied was expressed more strongly in *P. aeruginosa*. Biofilm forming abilities of the tested microbial strains are presented in fig. 1. Accordingly, a black horizontal line indicates the lowest OD value above which the strain was considered positive for biofilm ability.

Of the 10 *P.aeruginosa* isolates studied, only one strain did not meet the chosen criterion. Among the tested strains of *A. baumannii*, four of them did not show any film-forming activity. In this case, the parameters of intensity (M±SD) of the formed biofilm in *P. aeruginosa* was 0.53±0.23, and it was significantly lower in *A. baumannii* (0.26±0.17). Interestingly, in the content of the studied strains of microorganisms in the absence of any nutrients (sterile water for injection), *Acinetobacter* showed higher level of biofilm forming activity, in comparison with *Pseudomonas.* The average rate of *P. aeruginosa* biofilm formation under these conditions did not differ from the negative control, and in the tested strains of *A. baumannii* was 0.094 ± 0.037. In culture media containing one of the carbohydrates, which were able to be cleaved by tested strains (accordingly to their verified enzymatic activity: glucose or galactose), significant interspecies differences were detected at 1%.

Strains with initial high rates of biofilm forming activity in the presence of carbohydrates did not undergo signif-

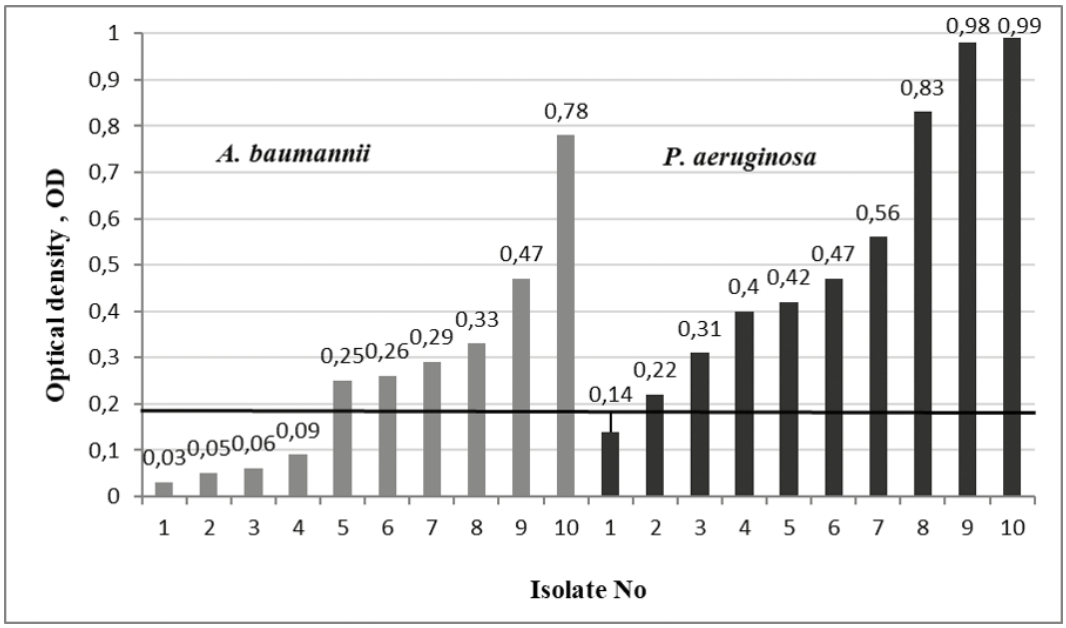

Fig. 1. *P. aeruginosa* and *A. baumannii* film-forming activity in TSB cultivation.

icant changes in this indicator. Strains without biofilm forming activity in "hungry" conditions, significantly have activated this process in the presence of carbohydrates. The intensity of biofilm formation in such strains has increased by 34 – 38% in comparison with the initial level. A similar pattern was extended to representatives of both species of non-fermenting bacteria studied. Addition of 1% of any single amino acid (lysine, leucine, arginine) into the "hungry" media did not affect the biofilm forming activity of *Pseudomonas* and *Acinetobacter* in the fasting environment. The same pattern was also proved in strains of *Pseudomonas*, which had showed the ability to cleave amino acids in the NEFERM test. As for, *Acinetobacter*, they did not alter film-forming activity in the presence of 1.0% urea in nutrient solution. Urease positive strains of *Pseudomonas* increased the film-forming intensity by an average of 52.0% to baseline. Biofilm forming activity of bacteria of both species significantly increased in the presence of 5.0 % of normal equine serum. In *Pseudomonas*, the average increase in the intensity of biofilm formation was 21.0%, and in *Acinetobacter*, it reached 67.0%.

The study of the effect of osmotic pressure on the biofilm forming activity of Gram-negative non-fermenting bacteria was performed in conditions with an media in which glucose at a concentration of 1.0% was the only source of energy and carbon. Osmotic pressure was created by adding sodium chloride in various concentrations to the medium. The average values of the intensity of biofilm formation by *Acinetobacter* and *Pseudomonas* under conditions of different osmotic pressure are illustrated in fig. 2.

There was found, that in 0.9% sodium chloride solution (P=7.5 atm), *Acinetobacter* formed a biofilm less intensively than *Pseudomonas*. When the osmotic pressure was increased to 27.4 atm, corresponding to a 3.0 % solution of sodium chloride, the intensity of film formation of bacteria of both species increased. The relative growth rate of optical density of biofilms, formed by *Pseudomonas*, reached about 20.0%, in comparison with optical density of biofilms, produced in conditions similar to blood plasma. In *Acinetobacter* the same criteria increased no more than 7.0%. Further increase in osmotic pressure in the environment has differently influenced the film-forming activity of *Acinetobacter* and *Pseudomonas*.

There was found that, in 6.0 % sodium chloride solution (P=52.14 atm), the intensity of film formation in *Acinetobacter* continued to increase, the optical density of the biofilms increased more than 30.0%, compared to films formed under isotonic conditions. Under these conditions the ability to biofilm production in *P.aeruginosa* was suppressed, since the optical density of biofilms dropped to values less than in isotonic conditions. There was proved, that a further increase in osmotic pressure in the culture medium (P=79.66; 9.0% sodium chloride solution) stimulated the activity of *A.baumannii* biofilm formation and further suppressed the film formation process in *Pseudomonas*.

It well known, that for treatment of patients with inflammatory processes, caused by *P. aeruginosa* and *A. baumannii*, such antibiotics as fluoroquinolones, aminoglycosides or carbapenems are often used. Determination of the biofilm forming activity in studied strains of the non-fermenting bacteria under the presence of levofloxacin, amikacin or meropenem in the nutrient medium demonstrated that meropenem and amikacin at sub-bacteriostatic concentrations did not significantly alter the intensity of their biofilm formation ($p>0.05$). In the presence of sub-bacteriostatic concentrations of levofloxacin, the activity of biofilm forming bacteria of both species increased significantly. The optical density of biofilms in the presence of the antibiotic was on average 25.0% higher, in comparison with the control (cultivation of bacteria in a medium without antibiotic).

In our research there were investigated the patterns of formation of resistance to levofloxacin, amikacin, mero-

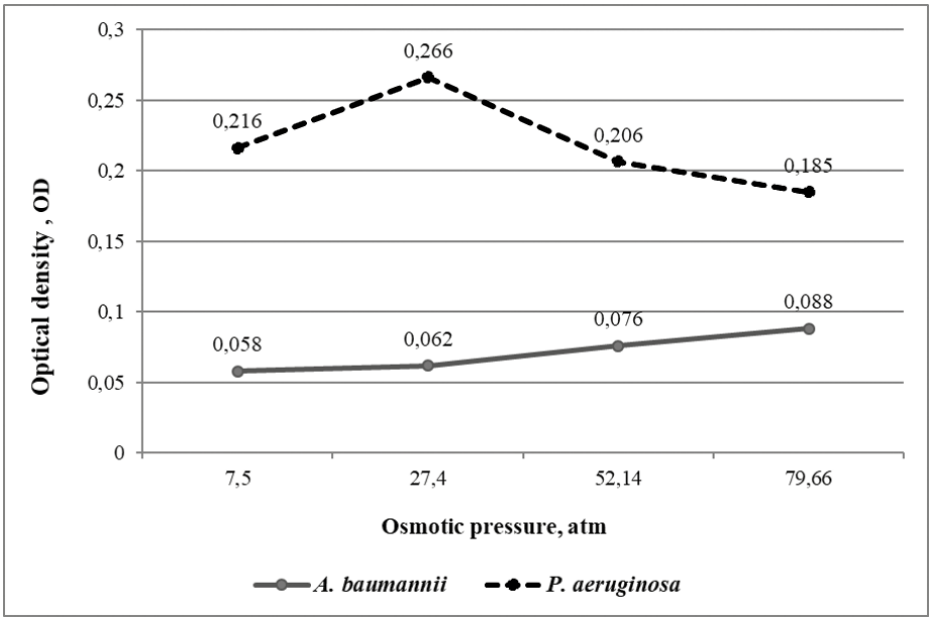

Fig. 2. The intensity biofilm forming activity of *P. aeruginosa* and *A. baumannii* in solutions with different osmotic pressure.

penem during the cultivation of non-fermenting bacteria in a nutrient medium with increasing concentrations of antibiotics [16]. And the artificially induced formation of resistance in these types of bacteria occured fairly quickly. By the tenth passage, the minimum bactericidal concentration of the drugs for most of the tested strains increased 16-fold, and to the 40th passage – almost 1000 times.

To study the intensity of formation of biofilms there were used isolates of bacteria, which had undergone the cultivation in an environment with increasing concentrations of antibiotics, and acquired the ability to grow in the presence of at least 16000 μg/ml of each of antibiotics, was used in the research. The biofilm forming activity of the original bacterial strains (not adapted to antibiotics) and the same strains after artificial adaptation to a medium with a high concentration of antibiotics was compared. According to the received data we found, that no statistically significant changes in the intensity of biofilm formation by the microorganisms of the genus *Acinetobacter* and *Pseudomonas* occurred after the procedure for adaptation to antibiotics. However, in most strains, a tendency to a slight decrease in biofilm forming activity was observed in antibiotic-adapted strains when grown in a non-antibiotic medium. This tendency was particularly stable in strains of *Acinetobacter* artificially adapted to meropenem.

DISCUSSION

The ability to rapid fixation on solid surfaces and formation of biofilms is an essential property of the Gram-negative non-enzymatic bacteria of the genus *Acinetobacter* and *Pseudomonas*, which helps them to survive in poorly favorable conditions and to inhabit numerous ecological niches [15, 16, 18]. It is difficult to explain the established ability of *Acinetobacter* to produce biofilms rich in organic matter in the absence of any sources of energy and plastic material in sterile water for injection. This only confirms the availability of great adaptive capacity in bacteria of this kind. According to the level of biofilm forming activity, non-fermenting bacteria exhibit heterogeneity between strains: some strains are capable of forming biofilms even under "hungry" conditions; others require the presence of nutrients in the environment. The stimulation the process of biofilm formation may occur in the environment of such simple organic substrates as glucose and urea. The additional administration of individual amino acids to the culture medium in our experiments did not enhance the biofilm formation by *Acinetobacter* and *Pseudomonas*, whereas in the presence of whole animal proteins in the form of animal serum, this process was significantly enhanced.

Pseudomonas were proved to exhibit a higher level of film-forming activity compared to *Acinetobacter* in an environment with osmotic pressure isotonic blood plasma (0.9% NaCl solution). Increasing osmotic pressure in the environment within certain limits stimulated the biofilm forming activity of bacteria of both species. However, *Acinetobacter* and *Pseudomonas* have different activity of biofilm formation under hypertonic conditions. There was determined the decrease of biofilm forming activity of the *Pseudomonas* isolates in conditions of the medium, containing 6.0 % sodium chloride. And this ability of *Acinetobacter* continued to increase, even in 9.0% salt solution. This phenomenon, along with the ability to form biofilms in distilled water, may indicate a higher environmental plasticity of *Acinetobacter* compared to *Pseudomonas*.

The presence of antibiotics in the environment is considered to be an unfavorable factor for bacteria as it should stimulate the processes of biofilm formation. However, we did not observe a similar effect against antibiotics of aminoglycoside and carbapenem series in our study. Fluoroquinolone antimicrobial levofloxacin at subbacteriostatic concentrations activated the biofilm formation of most

studied *Pseudomonas* and *Acinetobacter* strains. There is understandable the reduction of the biofilm forming activity of *Acinetobacter*, which have long been adapting to meropenem, in a non-antiobiotic environment. Obviously, the elimination of «antibiotic pressing» reduces the activity of the protective mechanism, which is biofilm formation.

CONCLUSIONS

1. In Gram-negative non-fermenting bacteria of the genus of *Acinetobacter* and *Pseudomonas* the ability to form biofilms, is a developed protective response under such adverse environmental effects as insufficient nutrients, increased osmotic pressure and the presence of antibiotic-containing substances in the environment.
2. Sodium chloride at high concentrations (6.0% or more) is able to suppress the activity of biofilm forming by *Pseudomonas*, at the same time stimulate this process in *Acinetobacter*.

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The Authors declare no conflict of interest.

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