

Modifying ability for biofilm formation by clinical isolates of gram-negative microorganisms under negative pressure conditions *in vitro*

Modificirajuća sposobnost formiranja biofilma kliničkih izolata gram negativnih mikroorganizama u uvjetima negativnog tlaka *in vitro*

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Professional paper

Vacuum-assisted closure therapy has been utilized as a ubiquitous wound management resource. Current studies of the subject focus on the influence of negative pressure on the bacterial load of wound bed and the possibility to remove matured biofilm, however, its impact on the ability of microorganisms to activate or reduce the biofilm producing modality remains unexplored. The goal of the present study was to evaluate the potential effect of negative pressure on biofilm producing modality of gram-negative rods with regard of initial biofilm positive or biofilm negative phenotype. Biofilm formation was evaluated for the strains of *A. baumannii*, *P. aeruginosa*, *E. cloace*, *K. pneumonia* and *P. mirabilis* isolated from infected war wounds of the extremities. The changes of optical density of the biofilm produced by the same strain under normal and negative pressure conditions, as well as the number of strains, which modified their ability to biofilm production, were measured. The strains with initial biofilm negative phenotype under negative pressure switched to biofilm producing more vigorously than biofilm positive ones regardless of their taxonomical belonging. *Pseudomonas* strains demonstrate the highest rate of induction to biofilm producing under negative pressure conditions among all studied microorganisms. Thus, the activation of biofilm producing mechanisms under negative pressure could be considered as a protective strategy, which helps ensure persistence of microorganisms in the wound even in case the vacuum-assisted closure therapy is applied.

Stručni rad

Terapija negativnim tlakom ili vakuum terapija (engl. *vacuum-assisted closure* – VAC) univerzalna je potporna metoda liječenja rana. Novija istraživanja iz ovog područja fokusiraju se na utjecaj negativnog tlaka na bakterijsko opterećenje rane i mogućnost uklanjanja zrelog biofilma, no njegov utjecaj na sposobnost mikroorganizama da aktiviraju ili reduciraju modalitet formiranja biofilma ostaje neistražen. Cilj ovog rada bio je procijeniti potencijalni učinak negativnog tlaka na formiranje biofilma kod gram-negativnih bakterija s obzirom na početni pozitivni biofilm ili negativni biofilmski fenotip. Formiranje biofilma istraženo je za sojeve bakterija *A. baumannii*, *P. aeruginosa*, *E. cloace*, *K. pneumonia* i *P. mirabilis* izoliranih iz zaraženih ratnih rana ekstremiteta. Izmjerene su promjene optičke gustoće biofilma proizvedene istim bakterijskim sojem u uvjetima normalnih i negativnih tlakova, kao i broj sojeva koji su modificirali svoju sposobnost proizvodnje biofilma. Sojevi s početnim negativnim fenotipom biofilma su pod negativnim tlakom počeli snažnije proizvoditi biofilm od onih s pozitivnim biofilmom bez obzira na njihovu taksonomsku pripadnost. *Pseudomonas* sojevi pokazali su najveću stopu indukcije stvaranja biofilma pod negativnim tlakom od svih ispitanih mikroorganizmima. Zaključno, aktivacija mehanizama koji proizvode biofilm pod negativnim tlakom može se smatrati zaštitnom strategijom, koja osigurava perzistenciju mikroorganizama u rani čak i u slučaju primjene terapije negativnim tlakom.

Key words

biofilm formation
vacuum-assisted closure

Ključne riječi

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Introduction

It is proved that negative pressure wound therapy (NPWT) benefits wound healing and closure [1]. Withdrawal of toxins and inflammatory fluid from the wound bed is considered as the main mechanism of such positive impact [2]. As soon as bacterial contamination delays wound healing, the extraction of bacteria from the wound by aspiration of wound secretion fluid constitutes an additional positive effect of NPWT. This fact was proved in a comparative study of regular gauge and vacuum-assisted closure (VAC) [3]. However, further investigations reported that bacterial colonization of wound has significantly increased with NPWT [4]. Nowadays, the statement whether NPWT could reduce the bacterial load of wounds is still controversial. Microbiological examination of VAC foams contacting with wound bed revealed that all foams had a heavy microbial load. This load remained high during the healing despite the regular change of the foams. The study conducted among patients with uninfected wounds failed to demonstrate the differences in the culture of tissue swab of the wound before, during, and after NPWT therapy. Moreover, the quantity of infectious complications was higher in the group where NPWT has been applied [5].

It is a well-known fact that under the challenging conditions microorganisms change their metabolic functions, growth intensity and gene expression. For example, negative pressure (NP) as a physical factor significantly alters the growth intensity of *Pseudomonas aeruginosa*, decreases the secretion of virulent substances, slows down the biofilm formation, decreases the biofilm thickness,

and causes its fragmentation [1]. The negative pressure influence on the gram-positive microorganisms is similar to the one mentioned above [6]. However, investigations of NPWT role in biofilm formation remain limited. Despite individual studies suggesting its compression effect on already established mature biofilms, the efficacy of NPWT for preventing the biofilm formation remains unclear [7]. The reasons why bacterial load of NPWT foams, which could serve as solid substances for biofilm formation, increases, are still unclear.

All current studies usually focus on the NPWT's influence on the matured biofilm. The question whether negative pressure environment triggers bacteria to biofilm production or reduces their ability to biofilm formation was not investigated before.

The present study is aimed at evaluating and validating the potential effect of NPWT onto the changes in biofilm producing process by gram-negative rods when applied on bacteria in planktonic form. The results may provide a better understanding of the therapeutic effect of negative pressure onto bacterial load of the wound.

Methods

The negative pressure effect on the biofilm formation ability was evaluated for the following microorganisms: 21 *Acinetobacter baumannii*, 22 *Pseudomonas aeruginosa*, 5 *Enterobacter cloacae* and *Klebsiella pneumoniae*, and 1 *Proteus mirabilis* strains respectively.

Bacterial strains were obtained from the collection of Department of Microbiology, Vinnytsia National Medical



Figure 1. Design of the negative pressure system. The air was sucked from the chamber by Heaco-NP32S Vacuum Pump Device. The Heaco-NP32S Vacuum Pump automatically produces and maintains the negative pressure at -125mmHg in the airtight chamber, which was used as the incubator. The 96-well flat-bottom polystyrene microtiter plate inoculated with the cultures of interest was allocated in this chamber. The chamber was placed into the thermostatic (38°C) conditions for 24 hours. The room air was inflated into the incubator every 15min in order to maintain the oxygen concentration of about 20%. In order to maintain humidity, an open vessel with water was placed inside the airtight chamber.

University, Ukraine. The microorganisms were isolated from the infected war wounds of the extremities, which were sustained in the military conflict in the East of Ukraine during 2014 – 2016 years. Based on microtiter biofilm formation assay, two groups were formed: one group containing biofilm positive isolates, and another group with biofilm negative isolates.

In order to assess the influence of negative pressure on the biofilm formation, bacteria in both groups were simultaneously being grown under the atmospheric and negative pressure, while the other conditions were equal. The

growth media used were Nutrient Agar and "Nutrient Broth, Farmaktyv LLC, Kyiv, Ukraine. The differences in the formed biofilm were evaluated by percentage of optical density changes of the same strain in different environment, and by the number of strains, which modified their ability to biofilm production.

Negative pressure conditions were created for bacterial growth, and an airtight chamber was used as the incubator. The air was sucked from the chamber with the vacuum pump device (Heaco-NP32S, "HEACO-Ltd", UK) provided by Military Medical Clinical Center of Central

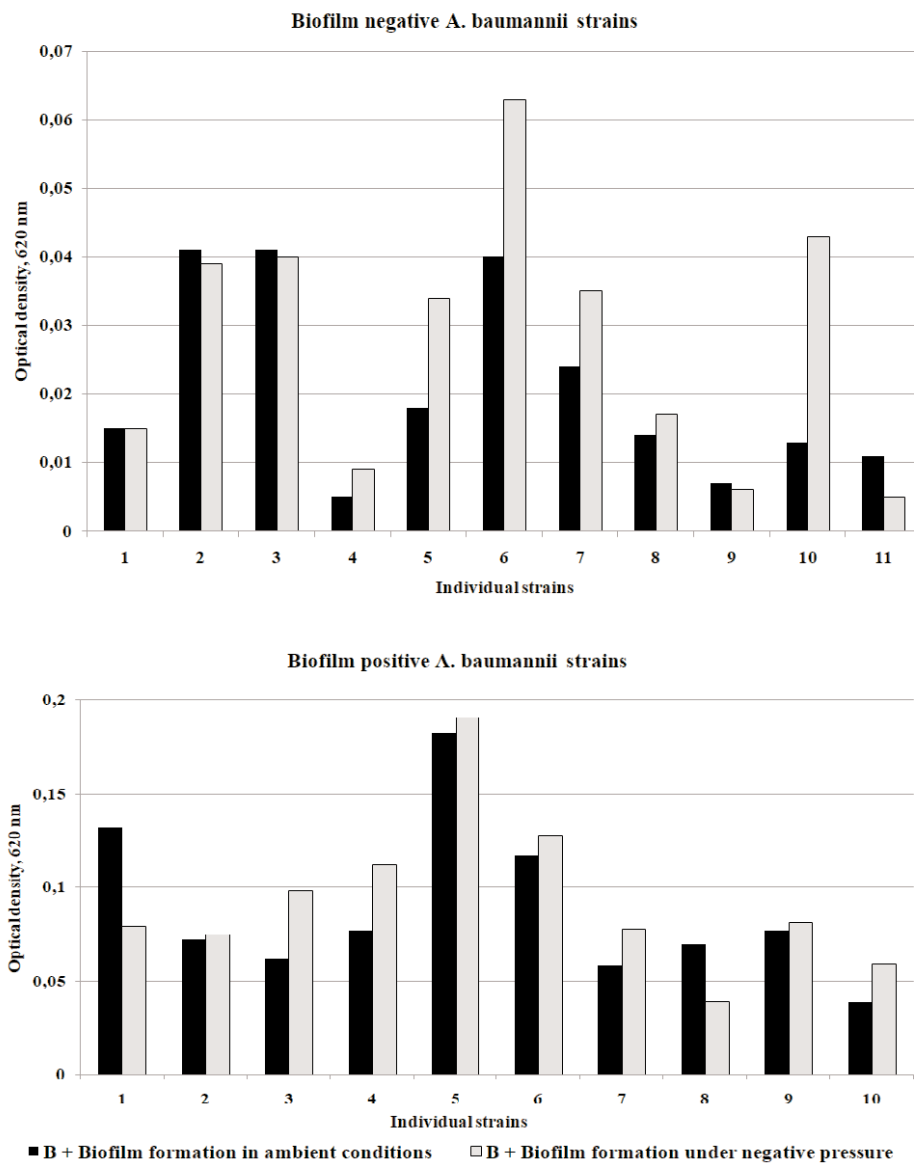


Figure 2. The optical density of biofilm produced by biofilm positive and biofilm negative strains of *A. baumannii* under different pressure conditions

Region, Vinnytsia, Ukraine, which could automatically produce and maintain the negative pressure at -125mmHg . The oxygen concentration was constantly maintained at 20%, as adequate amount of room air was poured into the incubator every 15min. In order to maintain humidity, an open vessel with water was placed inside the airtight chamber. The design of this negative pressure system is presented on Fig 1. The ability to form biofilm was evaluated by measuring the optical density of biofilm formed by individual strains grown under normal and negative pressure conditions. Bacteria were grown in individual wells of 96-well flat-bottom polystyrene microtiter plate (VWR Tissue culture plate, VWR International bvba, Leuven NL). The plate where strains were cultivated under negative pressure conditions was placed into thermostat (CT – 150C, RIVASTAL LLC, Kyiv, Ukraine) in the airtight chamber. In this chamber, the negative pressure was sustained as described above during the whole period of growth.

In order to grow biofilm, the aliquots (200 μL) of each strain in mid-exponential phase (adjusted to 0.5 McFarland absorbance) were added to 8 wells. The plates were covered and incubated for 48 h at 37 °C without shaking. The content of the wells was aspirated, and the wells were washed with 150 μL sterile normal saline with vigorous shaking. The content was aspirated, and each well was washed three times more with 150 μL sterile normal saline. In order to fix the remaining attached bacteria, 90 μL of ethanol (99 %) was added to the wells and left for 15 min, then removed by decanting. After the wells dried, a crystal violet (150 μL) was added to each well and left for 5 min, then discarded. The excess dye was rinsed off by washing the plate with the running water. The plates have been washed until the water became colourless, and then the plate was left to be air dried. In order to solubilize the dye bound to biofilms, 160 μL of glacial acetic acid 33 % v/v was added to the wells, and the plate was placed in microplate reader ("ChroMate 4300", Hague, Nether-land) to measure the absorbance at 620 nm. The biofilm forming ability was tested three times in 8 wells for each tested strain and the average results are presented below.

The gradation of biofilm producing was defined by the method used by Stepanovic et al. (2007) [8]. According to the method, the wells with sterile culture medium are used as a means of negative control to identify biofilm producing strains and categorize strains depending on the intensity of produced biofilm. One case of increased biofilm production was defined as the strain shifted from "no biofilm producer" or "weak biofilm producer" category to "moderate" or "strong biofilm producer" category. In reverse, one case of losing the biofilm producing capacity was defined as the strain shifted from "moderate" or "strong biofilm producer" category to "no biofilm producer" or "weak biofilm producer" category.

The data are presented in graphic form when applicable. The mean and \pm standard deviation was used to analyze the optical density of biofilm formed by individual strains. The association between the categorical characteristics such as the ability of strains (with biofilm positive and biofilm negative phenotype) to modify their biofilm producing capability under atmospheric and negative pressure was examined using chi-square test. The level of significance was set at $p < 0.05$.

Results

The results of biofilm producing by *A. baumannii* under different pressure conditions are presented on Fig2. Negative pressure had variable influence on the biofilm producing capability of *Acinetobacter* strains. The strains with initial biofilm negative phenotype switched to biofilm producing under negative pressure conditions, ($p < 0.05$), whereas only two of ten strains from biofilm positive group have lost this modality (see Table 1). The biofilm produced under negative pressure was slightly thicker than the one grown under atmospheric pressure regardless of the initial biofilm phenotype. The optical density OD_{620} of biofilms formed by biofilm negative strains of *A. baumannii* under the atmospheric and the negative pressure was observed as follows: $m = 0.023 \pm 0.02$ and $m = 0.028 \pm 0.02$ respectively. Turning to biofilm positive strains, the figures OD_{620} were: $m = 0.089 \pm 0.04$

Table 1. Shifts of biofilm formation modality within biofilm negative and biofilm positive group of *A. baumannii* strains under different pressure conditions

Groups with initially defined biofilm formation ability, n	Biofilm formation ability under normal pressure, n		Biofilm formation ability under negative pressure, n		χ^2
	B–	B+	B–	B+	
B–; (n=11)	11	0	5	6	6.471; $p < 0,05$
B+; (n=10)	0	10	2	8	2.222; $p > 0,05$

B– biofilm negative, B+ biofilm positive

Table 2. Shifts of biofilm formation modality within biofilm negative and biofilm positive group of *P. aeruginosa* strains under different pressure conditions

Groups with initially defined biofilm formation ability, n	Biofilm formation ability under normal pressure, n		Biofilm formation ability under negative pressure, n		χ^2
	B-	B+	B-	B+	
B-; (n=11)	11	0	7	4	10.267; p<0,01
B+; (n=11)	0	11	3	8	3.474; p>0,05

B- biofilm negative, B+ biofilm positive

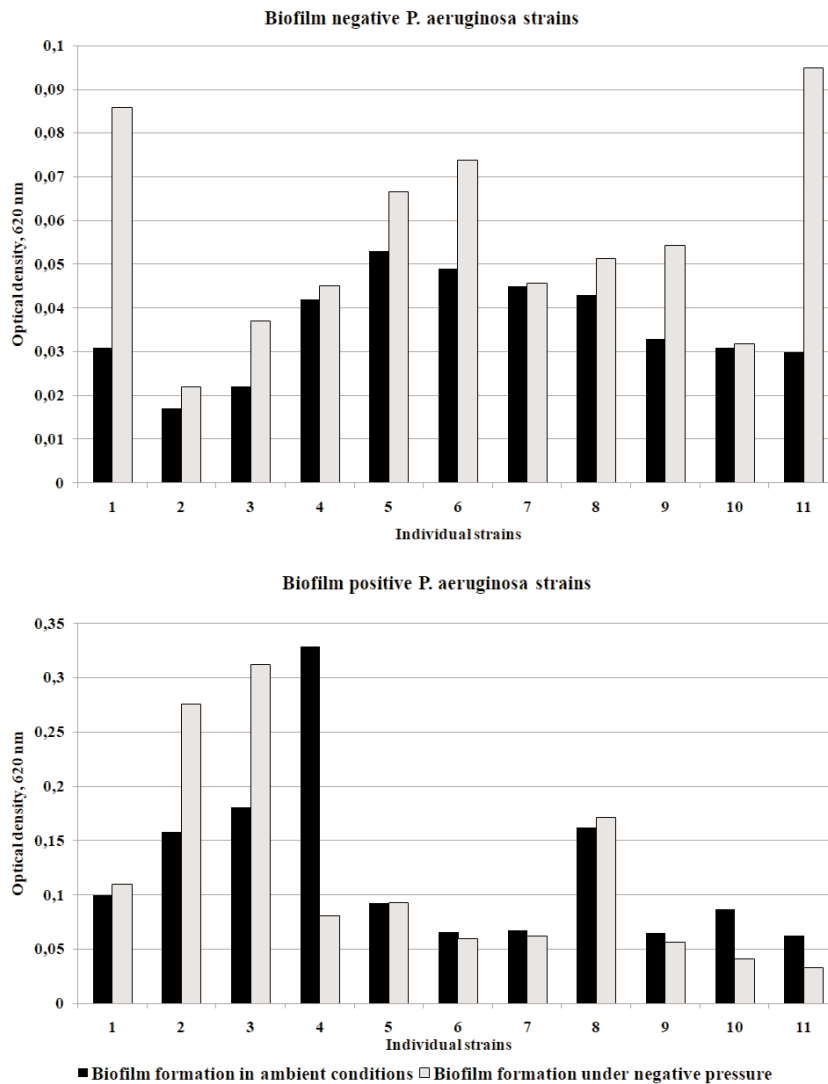


Figure 3. The optical density of biofilm produced by biofilm positive and biofilm negative strains of *P. aeruginosa* under different pressure conditions

and $m=0.094\pm 0.04$. The observation stated above wasn't confirmed statistically for both phenotype groups.

The results of biofilm producing by *P. aeruginosa* under different pressure conditions are presented on Fig 3. Negative pressure had variable influence on the ability to produce biofilm by *Pseudomonas* strains. The strains with the initial biofilm negative phenotype vigorously switched to biofilm producing under negative pressure conditions, ($p < 0.01$), see Table 2. In this group biofilm produced under negative pressure was thicker than the one grown under atmospheric pressure, and OD_{620} were $m=0.055\pm 0.006$ and $m=0.036\pm 0.003$ respectively ($p < 0.05$). The strains of biofilm positive group lost this modality in minor quantity, along with the diminishing of biofilm thickness, however, this observation was statistically insignificant. The optical density OD_{620} of biofilms formed under the atmospheric and negative pressure was observed as follows: $m=0.125\pm 0.008$ and $m=0.112\pm 0.094$ respectively.

The results of biofilm producing by *Enterobacteriaceae* under different pressure conditions are presented in the Table 3. Similarly to *P. aeruginosa* strains of *E. cloace*

and *K. pneumonia* actively started producing biofilm under the influence of negative pressure. The optical density of biofilm formed under negative pressure was higher compared to the atmospheric conditions.

Discussion

NPWT has been utilized as a ubiquitous wound management resource and is widely used for acute open wounds, chronic wounds, as well as infected wounds. The previous studies have investigated the impact of negative pressure on matured biofilms [9]. In vitro results indicated that the application of NP may be an effective approach to influence the biofilm morphometric parameters, reduction in bacterial counts [9]. However, it was not clear why bacterial load remained so high during the wound treatment. The goals of our study were to evaluate the potential effect of NPWT on biofilm producing modality of gram-negative rods. In this study, the negative pressure value (-125 mmHg) was applied to the planktonic cells and their ability to transform into the biofilm form, taking into account the initial biofilm positive or biofilm negative phenotype that was evaluated.

Table 3. Changes of optical density and biofilm forming capability for *Enterobacteriaceae* under normal and negative pressure conditions

<i>Enterobacteriaceae</i> strains	Biofilm formation ability under normal pressure	Shifts of biofilm formation modality under negative pressure conditions	Optical density (OD_{620}) of biofilm produced under normal pressure	Optical density (OD_{620}) of biofilm produced under negative pressure
<i>E. cloace</i>				
1.	+	+	0.084	0.097
2.	-	+	0.022	0.038
3.	-	+	0.032	0.041
4.	-	-	0.026	0.024
5.	-	+	0.010	0.016
Total	1	4	$m=0.035\pm 0.03$	$m=0.043\pm 0.03$
$\chi^2 = 7.639; p < 0.01$				
<i>K. pneumonia</i>				
1.	+	+	0.045	0.046
2.	-	+	0.043	0.051
3.	-	+	0.033	0.054
4.	-	+	0.031	0.031
5.	-	-	0.030	0.095
Total	1	4	$m=0.364\pm 0.01$	$m=0.056\pm 0.02$
$\chi^2 = 4,412; p < 0,05$				
<i>P. mirabilis</i>	1	0	0.092	0.060

+ biofilm positive, - biofilm negative

Investigating the influence of negative pressure on biofilm, G. Wang and co-authors [1] assessed the structure of biofilm formed by the single strain of *P. aeruginosa*. They found out that the strain preserved the ability to form biofilm under negative pressure, however the biofilm had less extracellular components compared to that formed under normal conditions. This observation can be explained by the inhibition of proliferation of cells, as the fewer number of cells produce less glycocalyx mass. By contrast, the present study was focused on the changes of bacterial behavior in the hostile environment, but not on the structure or thickness of produced biofilm.

The variety of physiological and biological adaptation strategies, which are used by bacteria to survive in aggressive environment, impresses and remains unexplored. It covers the changes in an ultrastructure of ribosomal membranes, changes in the enzymes activity, spore and capsule formation by certain species [10]. The universal way for survival among prokaryotes is biofilm formation. The advantages of this strategy are well shown under the influence of sub-inhibitory concentrations of antibiotics [11]. Thus, the activation of biofilm producing mechanisms under negative pressure could be considered as a protective measure, which helps ensure persistence of microorganisms in the wound even if NPWT is applied. The foam, which drains wound fluid, serves as a solid substrate for cell adhesion and biofilm formation. The phenomenon of negative pressure activating the biofilm producing among the gram-negative rods could clarify why the bacterial load of VAC foams increases over time, and explain controversial results of foam bioburden examinations [4]. The boosted biofilm formation potentially impairs the wound healing, especially when the bones or orthopedics constructions are a part of the wound bed. In such cases, the colonized foam could be the source of microorganisms. The beneficial effects of NPWT were described in the other studies related to the destructive influence of negative pressure on the matured biofilms and their components. The clinical studies identifying the relationship between the initiation of biofilm producing and response to therapy will help establish the clinical relevance of this phenomenon. A better understanding of this process may help guide the wound therapy and lead to the development of novel co-therapeutic agents that would suppress the biofilm induction response.

It is worth mentioning that the present study has some limitations. The modification of bacterial ability to form biofilm under the negative pressure was examined only for gram-negative rods. These microorganisms are the predominant cause of war wounds infection, which are in focus of the present investigation. Our study is exclusively a laboratory experiment, so it does not cover all aspects of the host and microbes relations, which determine the microbial burden of the wound.

Conclusion

1. The strains with the initial biofilm negative phenotype under negative pressure switched to biofilm producing more vigorously than biofilm positive ones regardless of their taxonomical belonging.

2. *P. aeruginosa* strains demonstrated the highest rate of induction to biofilm producing under the negative pressure conditions among all studied microorganisms.

3. Evidently, the negative pressure is recognized by bacteria as an unfavorable harsh influence, which triggers a collective defense mechanism, such as biofilm formation.

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Conflict of Interest

The authors declare that they have no competing interests. No one involved in the publication process has a financial or other beneficial interest in the concepts mentioned in a submitted manuscript.

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