DOI 10.26724/2079-8334-2022-2-80-210-213
UDC 616.322-002.2:576.851.252-053.2-074

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BIOFILM-FORMING PROPERTIES OF PATHOGENIC MICROORGANISMS IN CHILDREN WITH RECURRENT TONSILLITIS

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Among the causes of chronic infection and resistance to antibiotic treatment are the formation of biofilms in the respiratory tract in respiratory infections. The purpose of the study is to identify the ability to film strains of S. aureus on the surface of the epithelial tissue of the oropharynx isolated from patients with recurrent tonsillitis. Clinical strains of Staphylococcus spp bacteria obtained from the oropharynx of 42 children with recurrent tonsillitis were investigated. The ability to form biofilms – when grown on 96-well plastic plates and on the surface of coverslips for three days. It was found that 12 (28.58 %) strains out of 42 had film-forming ability, among which 3 (7.14 %) strains showed strong film-forming, 8 (19.04 %) strains were moderate, one strain (2.38 %) – a moderate formation film and 30 strains (71.4 %) lacked this ability. The severity of staphylococcus film formation was determined by optical density at a wavelength of 550 nm. Conducted studies of the intensity of film formation over three days of cultivation, the rate of OD was 0.054 <OD<0.097 (0.075±0.013) (p<0.05) on the first day (24h), from 0.081<OD<0.198 (0.011±0.029); p<005) on the second (48h) and third (72h) 0.114 <OD<0.361 (0,204±0.084); p<0.05) showing an upward trend. Thus, the ability of isolated strains of bacteria to biofilm formation complicates the treatment process and causes relapses of infection.

Key words: biofilm, recurrent tonsillitis, degree of biofilm formation, Staphylococcus spp.

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ПЛІВКОУТВОРЮЮЧІ ВЛАСТИВОСТІ УМОВНО-ПАТОГЕННИХ МІКРООРГАНІЗМІВ У ДІТЕЙ, ХВОРИХ НА РЕКУРЕНТНИЙ ТОНЗИЛІТ

Однією з причин переходу інфекції у хронічний стан та резистентності їх до лікування антибіотиками є утворення біоплівок у дихальних шляхах при респіраторних інфекціях. Метою дослідження є виявлення здатності до плівкоутворення на поверхні епітеліальної тканини ротоглотки у штамів стафілококів, виділених від хворих з рецидивуючим тонзилітом. Було досліджено клінічні штами бактерій Staphylococcus spp., виділених з ротоглотки 42 дітей. Здатність до утворення біоплівок вивчали шляхом вирощування культури на 96-лункових пластикових планшетах та на поверхні покривних скелець протягом трьох діб. Встановлено, що12 (28,58 %) штамів із 42 мали здатність до плівкоутворення, серед яких 3 (7,14 %) штами проявляли сильне плівкоутворення, 8 (19,04 %) штамів –помірне, один штам (2,38 %) – слабке плівкоутворення та у 30 штамів (71,4 %) ця здатність була відсутня. Ступінь вираженості плівкоутворення стафілококів визначали за показником оптичної щільності при довжині хвилі 550 нм з подальшим фарбуванням генціанвіолетом. Проведені дослідження інтенсивності плівкоутворення протягом трьох діб культивування продемонстрували тенденцію до зростання показника ОЩ у лінійці: 0,054<ОЩ<0,097, p<0,05; (0,075±0,013), за першу добу (24 год); 0,081<ОЩ<0,198, p<0,05; (0,011±0,029), за другу (48 год) та за третю – 0,114<ОЩ<0,361, p<0,05; (0,204±0,084). Отже, виявлена здатність виділених штамів бактерій до плівкоутворення ускладнює процес лікування та спричиняє рецидиви інфекції.

Ключові слова: біоплівка, рецидивуючий тонзиліт, ступінь плівкоутворення, Staphylococcus spp.

The study is a fragment of the research project "Monitoring of antibiotic-resistant strains in somatic and infectious pathology in people of different ages", state registration No 0119U000168.

Tonsillitis is an inflammation of the lymphoid tissue of the tonsils of the pharyngeal ring Waldeyer. Acute tonsillitis is an infectious disease of the tonsils, caused by one or more types of bacteria that can cause also peritonsillar abscesses or viruses, fungi, and protozoa. Acute recurrent tonsillitis occurs when a person suffers from several cases of tonsillitis per year. Chronic tonsillitis is a disease characterized by a chronic inflammatory process, mainly in the tonsils. Both chronic and acute recurrent tonsillitis is associated with recurrent cases of tonsillitis, which can greatly affect the quality of life of the patient. [6, 9].

Among the reasons for the transition of the infection to a chronic state and resistance to antibiotic treatment is the formation of biofilms in the respiratory tract in respiratory infections.

To date, the role of microbial biofilms in the etiology and pathogenesis of many bacterial infections: urinary tract [14], middle ear [12], as well as chronic hyperplastic tonsillitis [13], prosthetic valves [3], catheter-associated infections [5], dental diseases (caries, periodontitis, gingivitis) [8, 11]. In general, 63–78 % of clinical isolates of Staphylococcus aureus are capable of forming biofilms [13].

Biofilm infections respond poorly to standard antibiotic therapy, and their treatment is a serious problem in clinical practice [10].

Information about the biofilm-forming ability of certain bacteria is important for choosing the best ways to treat and prevent disease.

The purpose of the study was to identify the ability of biofilm-forming S. aureus strains on the surface of the epithelial tissue of the oropharynx isolated from patients with recurrent tonsillitis.

Materials and methods. Clinical strains of Staphylococcus sp. were obtained from the oropharynx of 42 children with recurrent tonsillitis. The material was collected with a sterile cotton swab, followed by

seeding on elective and selective nutrient media, which were incubated at 37° C for 24–48 hours. Identified microbes according to Bergie's classification. In some cases, the identification was performed using a semi-automatic microbiological analyzer "Vitek–2". The study of strains began with the study of the adhesive ability of the latter on the surface of the cover glasses (18×18mm), located in Petri dishes – 40. Visual study of the features of bacterial adhesion and biofilm formation was carried out on slides with subsequent detection for 1, 2, 3 days.

Daily plankton culture was removed, the biofilm slides were washed twice with 10 ml of phosphate buffer (pH7.2), stained with 0.1 % crystal violet solution, followed by fixation with 90 % ethyl alcohol.

Qualitative indicators were evaluated on a four-point scale (from 0 to 3) after examination under a MICROmed light microscope with a CCD video eyepiece, video camera (5.0MPix).

At three-day cultivation on 96-well plastic plates. In six wells of each row of the plate was made 100 µl of inoculum of a certain strain of staphylococci containing 10^7 CFU/ml, after which the plates were incubated for 24 hours in a thermostat (37° C). A day later, planktonic culture was removed from all wells, followed by processing of biofilms according to conventional methods.

The degree of ability to film staphylococci was determined by the optical density (OD) on Multiskan FC at a wavelength of 550 nm, followed by staining with crystal violet solution. The optical density (OD) of each strain was obtained by comparing the arithmetic mean of the absorption of light from six wells and the mean of the absorption of light of the negative control (OD_{nc}). To determine the severity of the film, the following classification was used: no biofilm production ($OD<OD_{nc}$), weak production – ($OD_{nc}<OD<2\times OD_{nc}$), moderate production – ($2\times OD_{nc}<OD<4\times OD_{nc}$) and strong biofilm production – ($4\times OD_{nc}<OD$) [4].

Statistical analysis was performed using Excel (Microsoft Corp., Redmond, WA, USA) using the nonparametric Mann-Whitney test, differences between different exposure times of the studied strains were considered statistically significant at $p<0.05$. All studies were conducted after signing the informed consent of the parents of patients, which was approved by the Bioethics Commission of I. Horbachevsky TNMU. The research was conducted in accordance with the ethical norms and moral and legal requirements of the order of the Ministry of Health of Ukraine №281 dated November 1, 2000.

Results of the study and their discussion. In a microbiological study of biomaterial obtained from patients with recurrent tonsillitis, 42 strains of Staphylococcus aureus of the genus Staphylococcus were isolated and identified. The mean value of the age group was 7.32±1.23 years.

Bacteria of the species Staphylococcus aureus are often used as an object to study the patterns of functioning of microorganisms in the biofilm. The rate of biofilm formation of the clinical strains of this species of bacteria isolated by us was studied on the surface of the cover glasses and microtiter plates *in vitro*. The analysis of adhesiveness revealed both individual groups of bacterial cells and their conglomerates. Microscopic control of biofilm formation began after 12 hours of cultivation on the surface of the cover slides, when single bacterial conglomerates were observed, the latter were irregularly shaped, covering almost the entire surface of the cover glass. Most conglomerates are separated, in some loci there is a fusion of elements with a pronounced tendency to form compacted islands, the number of which at this stage of biofilm formation is insignificant. On the second day of cultivation, a decrease in small isolated elements was observed. A significant area of the surface of the cover glass was covered with merging conglomerates irregularly shaped, grouped by two, three or four elements. At the end of the third day of cultivation, an almost homogeneous structure formed by a large number of conglomerates and separately located elements with insignificant gaps between individual fragments of the biofilm was observed.

Of the 42 studied clinical strains, 12 (28.58 %) were able to form a biofilm, while in 30 (71.42 %) isolates the ability to form a biofilm was not detected. Among the twelve isolates in 3 (7.14 %) strains, strong film formation was detected – more than 2 points; for 8 (19.04%) moderate film formation – up to 2 points; one strain (2.38 %) – weak film formation and 30 strains (71.4 %) - this ability is absent (non-adherent) (fig. 1).

In general, studies have shown an increase in the size of microbial complexes on the surface of the cover slides during three days of cultivation.

To determine the quantitative accumulation of biofilm biomass of isolated in microtiter plate systems (96-well plates) for three days of the experiment, we evaluated the optical density (OD) at a wavelength of 550 nm (fig. 2).

According to the results of a quantitative study of the dynamics of the formation of biofilms of clinical strains of this type of bacteria in the wells of microtiter plates for 24 hours of incubation, reached $0.054<OD<0.097$, $p<0.05$; (0.075±0.013). A similar trend persisted during the second day of cultivation. After 48 hours of cultivation on the surface of the microplates, the number of Staphylococcus aureus bacteria increased with increasing optical density: $0.081<OD<0.198$, $p<0.05$; (0.011±0.029). During the third day of bacterial cultivation, there was an increase in the number of OD microorganisms by 1–1.3 times in comparison with the previous day and an increase in the optical density of $0.114<OD<0.361$, $p<0.05$; (0.204±0.084). As we can see from the figure 2.A. biofilm tends to thicken from a few microns to several

millimeters. It should be noted that the dynamics of biofilm formation of the studied clinical strains resembles the growth curve of planktonic forms of microbial populations. It repeats the main phases of growth of the periodic culture of bacteria, namely: lag phase, exponential, stationary. Our research has confirmed the general model of biofilm formation and accumulation, the formation of which is a natural process and has the form of a sigmoid curve, which is the result of a balance between different physical, chemical and biological processes occurring simultaneously. In general, the ability to produce biofilms of isolated Staphylococcus aureus isolates in96-well plates during the three days of the experiment showed a tendency to increase the OD. In the studied strains of Staphylococcus aureus, the greatest increase in the number of cells in the biofilm is observed during the third day of bacterial cultivation, which confirms the OD (0.054<OD<0.361, p<0.05).

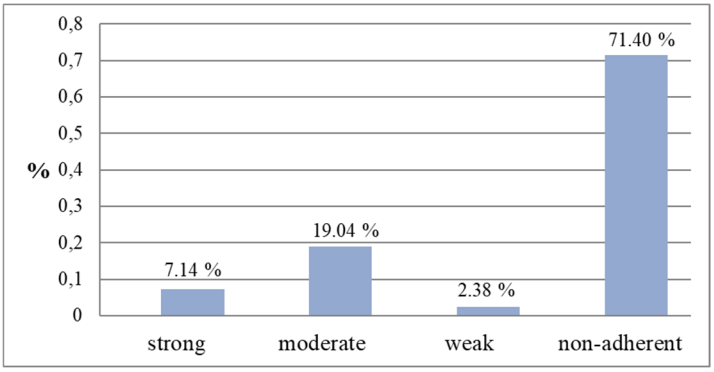
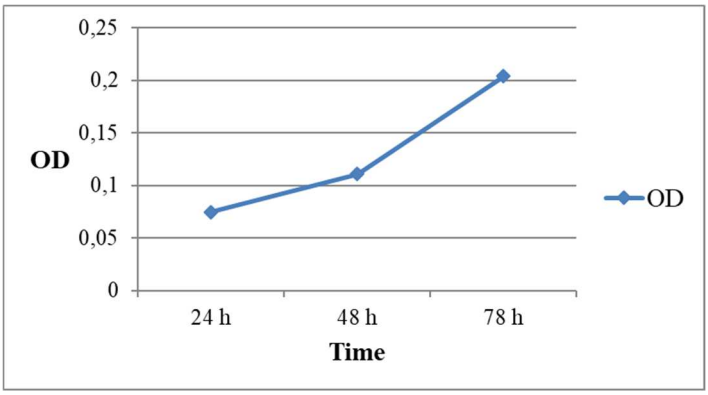
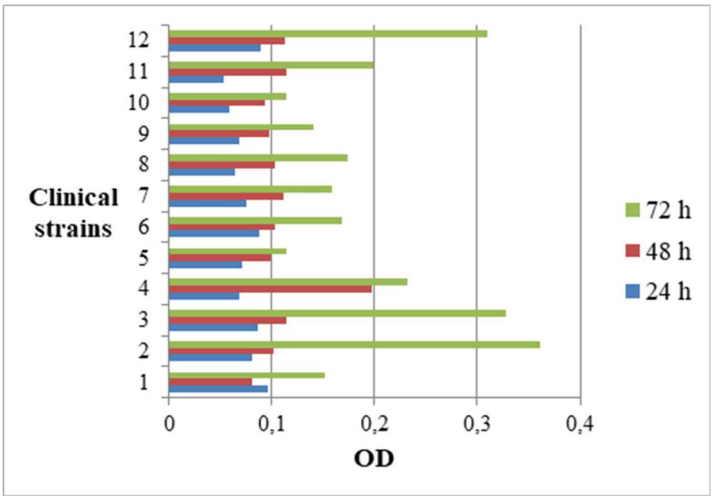


Fig.1. Intensity of biofilm formation by Staphylococcus aureus strains



A



B

Fig.2. Ability to film formation of clinical strains of Staphylococcus aureus: A – curve of biofilm growth dynamics; B – formation of biofilm by clinical strains

The growth dynamics of 12 clinical strains of Staphylococcus aureus isolated from patients with reticular tonsillitis can be seen in fig. 2.B.

In three (7.14 %) of the twelve isolates, the optical density on the first day for these three strains ranged from 0.087<OD<0.09, after a day of cultivation, the OD showed a tendency to increase 0.102<OD<0.115, on the third the day of cultivation of clinical isolates marked significant accumulation of biomass in the formation of biofilm 0.328<OD<0.361, p<0.05. A similar pattern was observed in the cultivation of biofilms on the surface of the cover glass. For 8 (19.04 %) clinical strains isolated from patients, the quantitative indicator of biofilm formation and increase in bacterial biomass during the three days of cultivation also showed a tendency to increase. If during the first day of cultivation the indicator fluctuated within 0.64<OD<0.088, p<0.05, on the second day of biofilm formation reached 0.81<OD<0.198, p<0.05, then in 72 hours for eight the studied strains was 0.115<OD<0.232, p<0.05. Moderate dynamics of film formation was confirmed by the method of cover glasses. One of the studied strains (2.38 %) showed a slight accumulation of biomass during the formation of the biofilm. During three days of cultivation, the OD also showed an increase (from 0.059; 0.093 to 0.114, p<0.05), which was confirmed by a slight formation of conglomerates on the surface of the integumentary glass and was characterized by weak film formation. In general, the research methods used in the process (namely: the method of cultivation on the surface of the cover slides with examination of the results under a light microscope and the culture method on 96-well plastic plates) proved to be equally effective in monitoring bacterial growth in biofilm. However, the influence on the results of quantification of bacterial biomass of the state of aggregation of microorganisms on different surfaces for cultivation, as well as the spectrum of light scattering during the indication in the spectrophotometer cannot be ignored.

Both methods of cultivating biofilms have demonstrated similar dynamics of its formation, which begins with the phase of adhesion, phase, then the growth and accumulation of biomass in the form of small

conglomerates, which later turned into large solid groups of cells. The results show that in patients on the epithelial surface of the tonsils and adenoids, in whom antimicrobial drugs caused temporary relief, bacteria capable of forming biofilms were found, which could complicate the treatment process and cause recurrence of infection.

Confirmation of the effectiveness of the use of cultivation methods on glass and in 96-well plates in order to monitor the formation of biofilms is found in the works of scientists [1, 14]. Because the effectiveness of treatment depends on the rate of indication of film-forming bacteria in patients, the search for rapid tests to detect film-forming bacteria is not only to cultivate the latter in 96-well plates, but also to find less complex methods of growing biofilms, in particular cover glasses.

In our opinion, for the diagnosis of biofilm-forming bacteria in patients should use rapid methods of diagnosis, namely: the method of detecting biofilm-forming bacteria by elevated levels of exhaled nitric oxide [2], using different types of histological microscopy, including light and electron [1] and using a rapid urease test [7]. Given that the main cause of recurrent tonsillitis in both children and adults is microbial biofilms, the use of affordable and rapid testing methods is important for choosing a treatment strategy. The fact that treatments should be based on the individual potential impact of biofilms on disease development cannot be ignored.

Physicians should ensure that they have all the information about the effectiveness of selected diagnostic methods and treatments, such as local remedies, physical removal of biofilms, or other innovative treatments, instead of using more potent antimicrobials.

Conclusion

Thus, the ability of bacterial cells to form biofilms and their adhesive activity provides a selective advantage over planktonic forms and is an important factor in virulence that ensures the ability to survive in the human body for a long time. The degree of adhesive activity of isolated strains of S. aureus bacteria should be considered an unfavourable prognostic factor in the course of recurrent tonsillitis. It was found that of the 42 isolated strains identified as S. aureus, 12 (28.58 %) are biofilm-forming. Among them, 3 (7.14 %) with strong biofilm-forming ability, 8 (19.04 %) moderate; one strain (2.38 %) is a weak biofilm-forming agent, while 30 strains (71.4 %) lack this ability (non-adherent). The degree of expression of the biofilm-forming ability of the studied strains of staphylococcus showed an increase in optical density (OD) during three days of cultivation, while it ranges from $0.054<OD<0.361$, $p<0.05$.

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Стаття надійшла 23.05.2021 р.