

Informational characteristics of microbial biofilms formed by clinical strains of *Klebsiella pneumoniae* in vitro on the surface of the cover glass

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Purpose Obtaining quantitative and informational characteristics of biofilms formed by clinical strains of *Klebsiella pneumoniae* in vitro on the surface of a cover glass. **Materials and methods** In vitro biofilm formation on the surface of the cover glass was studied for clinical *Klebsiella pneumoniae* strains, isolated in a monoculture (ESBL +) (n = 3) and in associations with *Staphylococcus aureus* (n = 6) in 9 patients with chronic osteomyelitis of long bones harvested from fistulae in the preoperative period or from the infection focus during surgery. **Results** Monocultures of *K. pneumoniae* (BLRS +) differed by their lower adhesive ability when compared to strains of *K. pneumoniae* isolated from associations with *S. aureus*. The highest adhesive activity on the surface of the cover glass was observed in a mixed culture of *K. pneumoniae* + *S. aureus*. Informational characteristics depended on the type of biofilms formed. Common to biofilms was the absence of changes in the maximum possible structural diversity. Significant differences between the existing structural diversity of biofilms formed by monocultures of *K. pneumoniae*, *K. pneumoniae* isolated from associations and a mixed culture of *K. pneumoniae* + *S. aureus* were noted. **Conclusion** The absence of pronounced variability of information indicators during the experiment within each microbial community indicates the tendency of all systems of emerging biofilms to preserve stability.

Keywords: *Klebsiella pneumoniae*, biofilms, chronic osteomyelitis, information analysis

Bacterial biofilms play a big role in the pathogenesis of chronic osteomyelitis, influence the recurrence of the disease, its chronic nature and resistance to antimicrobial agents [1]. *Klebsiella pneumoniae* accounts for about 3 % in the etiological structure of this disease [2]. Pathogenicity of *K. pneumoniae* lies in its ability to produce extended-spectrum beta-lactamases (ESBL), which led to the emergence of multidrug-resistant strains and, as a consequence, the inclusion of this pathogen in the ESKAPE microorganism group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp.*) [3].

Like other bacteria, *K. pneumoniae* can form biofilms on biotic and abiotic surfaces. From 40

to 93.6 % of clinical isolates of *K. pneumoniae* from urine, blood, expectoration, wounds, have the ability to biofilm formation in vitro [4, 5]. There is an evidence of a higher biofilm capacity of clinical isolates that feature multidrug resistance [6, 7]. The highest biofilm-forming ability is characteristic of strains isolated from bone surface [7].

To date, a few works studied biofilm formation by strains isolated from an osteomyelitis focus. No studies on the informational state of biofilms have been found in the available literature. This fact determines the relevance of this study.

The purpose of our study is to obtain quantitative and informational characteristics of biofilms formed by clinical strains of *Klebsiella pneumoniae* in vitro on the surface of the cover glass.

MATERIAL AND METHODS

We used clinical *Klebsiella pneumoniae* strains as a monoculture (ESBL +) (n = 3), and in associations with *Staphylococcus aureus* (n = 6) in 9 patients with chronic osteomyelitis of long bones from the fistula in the preoperative period or from the infection focus during the surgery.

Isolation and identification of the strains was performed with standard methods.

Obtaining bacterial biofilms on the surface of a cover glass

A sterile cover glass 24 × 24 mm in size was placed on a sterile Petri glass dish with a diameter of 100 mm. On the surface of the cover glass, 200 µl of a 24-hour culture of the strain was carefully poured, with cell concentration of 5 × 10⁷ CFU / ml which corresponded to an optical density of 0.2 according to McFarland, followed by incubation in a thermostat

at 37° C for three hours. Next, pepted meat broth was added up to 2 ml and the culture was re-placed in a thermostat at 37° C for 24 and 48 hours. After incubation, the nutrient medium was decanted, and the glass surface was washed three times with 1.15 M phosphate buffer, fixed with ethyl alcohol of 96°, dried and stained with a solution of a gentian violet for 2 minutes at room temperature, then washed with phosphate buffer.

In vitro associative biofilm of *K. pneumoniae* + *S. aureus* was obtained by mixing 24-hour cultures of the strains in a ratio 1:1.

Axio Lab.A1 microscope, ZEN modular software (Carl Zeiss, Germany) and the ImageJ program (USA) were used to obtain digital images of the visual fields of the preparations of the biofilm and its quantitative characteristics.

The area of the visual field, the number and area occupied by single adherent cells and microcolonies were measured in the images. The number of single

adhered cells and microcolonies per unit area (1 mm²) and the portion occupied by them in the area of the visual field were assessed, taking into account the sizes of microcolonies: up to 10 µm², from 10 to 100 µm², from 100 to 1000 µm², from 1000 to 10,000 µm², > 10,000 µm². At least 20 random visual fields were introduced from each preparation, the results were averaged. To assess the informational state of the biofilm, the following integral criteria were calculated: maximum entropy (H_{max}, bit), current entropy (H, bit), relative entropy (h), redundancy indicators (R, %) and organizations (S, bit) [8].

Informational analysis and statistical data processing was performed using the spreadsheet editor Microsoft Excel 2010 and data analysis software AtteStat Version 13.0 [9]. Digital data are presented as medians (Me) and quartiles (Q₂₅-Q₇₅). To assess the statistical significance of differences between the groups, the Wilcoxon test was used. Differences between groups were considered significant at p < 0.05.

RESULTS

The results showed that the microorganisms featured the ability to biofilm formation on the surface of the cover glass. The analysis revealed similarities and differences in the structure of biofilms formed. Thus, all the tests showed the increase in the number of solitary adhered cells and microcolonies after 48 hours (**Fig. 1**). Microcolonies which size did not exceed 100 µm² prevailed in all the strains tested on the surface of the glass slip.

The differences consisted in the change of the ratio of microcolonies of various sizes. The formation of a biofilm by monoculture *K. pneumoniae* (ESBL +) occurred due to a uniform increase in the number of

microcolonies which were from 10 to 10,000 µm² in size; their share in the area of the visual field increased by 52–65 % after 48 hours. The size of the biofilm formed by the monoculture of *K. pneumoniae* isolated from the association of *K. pneumoniae* + *S. aureus* increased mainly due to microcolonies ranging in size from 100 to 1000 µm², the proportion of which increased by 42 %. The number of microcolonies which were over 10,000 µm² in size increased by 2.6 times during the growth of the mixed biofilm of *K. pneumoniae* + *S. aureus* that resulted in an increase of their portion by 62 % when compared with the previous experiment time-point.

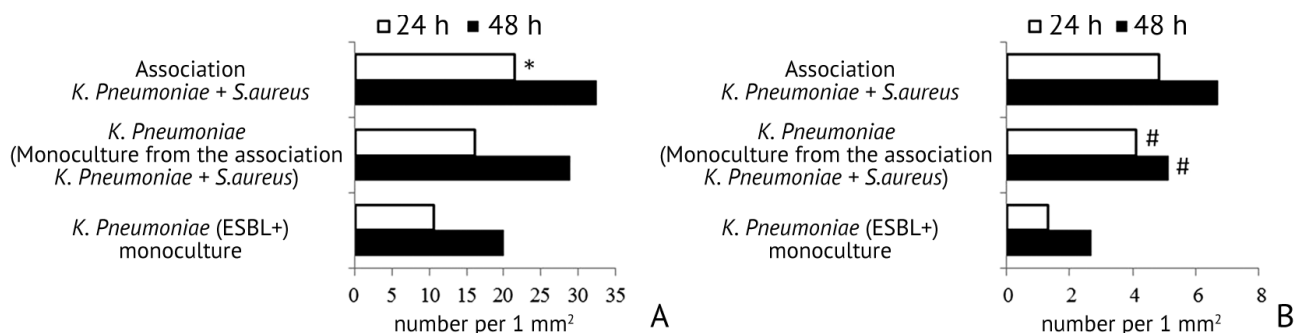


Fig. 1 Change in the number of adhered solitary cells (A) and microcolonies (B) on the surface of the cover glass during the experiment. Note: * – p < 0.05 – the differences are significant compared with the monoculture of *K. pneumoniae* isolated from the association of *K. pneumoniae* + *S. aureus* in the corresponding experimental period; # – p < 0.05 – the differences are significant compared with the monoculture of *K. pneumoniae* (ESBL +) in the corresponding experimental period

Significant differences were established between the number and portions of microcolonies ranging in size from 1000 to 10,000 μm^2 ($p = 0.04$) formed by the monoculture of *K. pneumoniae* isolated from the association of *K. Pneumoniae* + *S. aureus* and a mixed culture of *K. Pneumoniae* + *S. aureus* after 24 hours and the number of microcolonies ranging in size from 100 to 1000 μm^2 ($p = 0.04$) after 48 hours. It should be noted that regardless of the size, the number and proportion of microcolonies formed by monocultures in all periods of the experiment were lower than the number and proportion of microcolonies formed by the association of microorganisms.

Thus, the biofilm-forming ability of the association of microorganisms (*K. Pneumoniae* + *S. aureus*) was higher when compared to monocultures. The results

confirm the literature data that interactions between the types, changing the physiology and functions of the entire community, can lead to increased biofilm formation [10, 11].

It is known that there are informational systems at all levels of biological organization in which information is produced, stored, transmitted, processed and perceived [12]. Microorganisms in biofilms, obeying the laws of thermodynamics, enter into various symbiotic relationships, exchange substances and energy with each other, which determines their adaptation to the environment [13].

Informational analysis showed that the information capacity of the system (maximum entropy) in the formation of both mono- and mixed biofilms was the same and amounted to 2.322 bits (Table).

Table

Informational characteristics of in vitro biofilms (Me (Q25–Q75))

	Time	Information indicators				
		H, (bit)	H _{max} (bit)	h	R, %	S, bit
<i>K. pneumoniae</i> (ESBL+) monoculture (n = 3)	24 h	1.357 (1.215 – 1.725)	2.322	0.584 (0.523 – 0.743)	41.561 (25.694 – 47.672)	0.965 (0.597 – 1.107)
	48 h	1.463 (1.456 – 1.843)	2.322	0.630 (0.627 – 0.794)	36.977 (20.641 – 37.313)	0.859 (0.479 – 0.866)
<i>K. pneumoniae</i> (monoculture from association <i>K. pneumoniae</i> + <i>S. aureus</i>) (n = 6)	24 h	1.352 (1.061 – 1.558)	2.322	0.582 (0.457 – 0.671)	41.755 (32.893 – 54.313)	0.970 (0.764 – 1.261)
	48 h	1.405 (1.206 – 1.545)	2.322	0.605 (0.520 – 0.665)	39.506 (33.481 – 48.043)	0.917 (0.777 – 1.116)
Association <i>K. pneumoniae</i> + <i>S. aureus</i> (n = 6)	24 h	1.871* (1.429 – 1.906)	2.322	0.806* (0.615 – 0.821)	19.406* (17.925 – 38.475)	0.451* (0.416 – 0.893)
	48 h	1.825* (1.770 – 1.870)	2.322	0.786* (0.762 – 0.806)	21.397* (19.448 – 23.784)	0.497* (0.452 – 0.552)

Note Significant differences as compared with *K. pneumoniae* monoculture, isolated from the association *K. Pneumoniae* + *S. aureus* in a corresponding experiment time: * – $p < 0.05$.

After 48 hours, the biofilms formed by monocultures showed an increase in the current and relative entropy and a decrease in the organization of the system and the redundancy coefficient. Despite the lower adhesive activity of the biofilm formed by the *K. pneumoniae* monoculture (ESBL+) compared to the *K. pneumoniae* monoculture isolated from the association, their average information indicators were similar.

In biofilms formed by associations of clinical strains (*K. Pneumoniae* + *S. aureus*), on the contrary, there was a decrease in absolute and relative entropy and an increase in indicators of organization and redundancy.

The highest values of information entropy were found in the associative biofilm (*K. Pneumoniae* + *S. aureus*), which indicates a more diverse set of elements in the system and, accordingly, a greater heterogeneity. Lower entropy values of mono-type biofilms compared

with associative ones indicate a more homogeneous composition, as well as a lower grade of disorder.

Analysis of information characteristics showed that biofilms formed by monocultures are deterministic systems with a high margin of structural stability, as evidenced by the values of redundancy ratios that exceed 30 %. Associative biofilms are quasideterministic structures (values of the redundancy coefficients ranged from 10 to 30 %).

Thus, the obtained results show that all clinical strains had the ability to form a biofilm on the surface of the cover glass. The strains of *K. pneumoniae* (BLRS+) as a monoculture differed by a lower adhesive capacity compared to the monoculture of *K. pneumoniae* isolated from associations with *S. aureus*. The highest adhesive activity on the cover glass was observed in the mixed culture of *K. Pneumoniae* + *S. aureus*.

Information characteristics depended on the type of biofilms formed. Absence of changes in the maximum possible structural diversity was common for the biofilms. At the same time, significant differences were noted between the existing structural diversity of the biofilms formed by monocultures of *K. pneumonia*, isolated

from associations and the mixed culture of *K. Pneumoniae* + *S. aureus*.

The absence of a pronounced variability of information indicators during the experiment within each microbial community indicates the tendency of all the systems of the emerging biofilms to preserve stability.

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Received: 18.09.2017

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