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The efficacy of bacteriophages in destroying biofilms on urinary catheters in experimental *in vitro* and *in vivo* models

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Abstract

Introduction. The formation of biofilms by healthcare-associated infection (HAI) pathogens on invasive medical devices is an increasingly urgent problem in clinical practice. Microbial biofilms contribute to persistent infections, complicate treatment, increase healthcare costs, and reduce the quality of patient care. The resistance of biofilm-embedded bacteria to antibiotics is a key factor in chronic and recurrent infections. In this context, bacteriophages may serve as a promising therapeutic agent against bacterial infections, including those caused by biofilm-forming microorganisms.

Objective. To assess the efficacy of lytic bacteriophages in disrupting microbial biofilms on urinary catheters using *in vitro* and *in vivo* experimental models.

Materials and methods. The study employed microbiological, morphological, and electron microscopy techniques. *In vitro*, biofilms were cultured on urinary catheter surfaces and subsequently treated with bacteriophages. For the *in vivo* model, catheter-associated urinary tract infection (CAUTI) was induced in mice, which were then divided into experimental and control groups. Bacteriophages were administered transurethrally.

Results. In the *in vitro* model, bacteriophages effectively disrupted biofilms, inducing bacterial cell lysis and degradation of the exopolysaccharide matrix. In the *in vivo* experiments, mice treated with bacteriophages exhibited regression of CAUTI, as confirmed by morphological and bacteriological analyses. Electron microscopy revealed biofilm destruction on 5 out of 6 catheters. In contrast, the positive control group showed progressive infection, while no biofilm formation was observed in the negative controls.

Conclusions. The findings from both *in vitro* and *in vivo* experiments demonstrate that bacteriophages are capable of degrading biofilms and may represent an effective therapeutic strategy against biofilm-associated HAIs.

Keywords: *bacteriophages; biofilms; biofilm forms of microorganisms; healthcare-associated infections; catheter-associated urinary tract infections; optical density; scanning electron microscopy*

Ethics approval. The authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with international and Russian standards and recommendations (GOST 33216 "Guidelines for the maintenance and care of laboratory animals. Rules for the maintenance and care of laboratory rodents and rabbits," Directive 2010/63/EU on the protection of animals used for scientific purposes, Appendix A to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123), SanPiN 3.3686-21 "Sanitary and epidemiological requirements for the prevention of infectious diseases," Sanitary Rules for the Construction, Equipment, and Maintenance of Experimental Biological Clinics (Vivaria) (approved by the Chief State Sanitary Doctor of the USSR on April 6, 1973, No. 1045-73), GOST R 51000.3-96 "General requirements for testing laboratories," GOST R 51000.4-96 "General requirements for the accreditation of testing laboratories," "Rules for conducting work using experimental animals" approved by the Ministry of Health of the USSR (1977), "Rules for the handling, maintenance, anesthesia, and euthanasia of experimental animals" approved by the Ministry of Health of the RSFSR (1977), Order of the Ministry of Health of the Russian Federation No. 267 dated June 19, 2003 (Rules of laboratory practice in the Russian Federation)). The research protocol was approved by the Local Ethics Committee of the North-Western State Medical University named after I.I. Mechnikov (protocol No. 3, March 3, 2024).

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Оригинальное исследование
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Эффективность применения бактериофагов для деструкции биоплёнок на мочевых катетерах в экспериментальных моделях *in vitro* и *in vivo*

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Аннотация

Введение. Проблема формирования биоплёнок возбудителями инфекций, связанных с оказанием медицинской помощи (ИСМП), на инвазивных устройствах, применяющихся в медицинской практике, в настоящее время приобретает высокую актуальность. Существование микроорганизмов — возбудителей инфекций в виде биоплёнок создаёт большие проблемы в медицинской практике, наносит экономический ущерб и снижает качество оказания медицинской помощи. Формированием биоплёнок объясняются причины длительно протекающих инфекционных заболеваний, не поддающихся антибиотикотерапии. В сложившихся условиях одним из эффективных компонентов борьбы с бактериальными инфекциями, в том числе вызванными биоплёночными формами микроорганизмов, может явиться использование бактериофагов.

Цель работы — оценить эффективность применения литических бактериофагов для деструкции микробных биоплёнок на мочевых катетерах в экспериментальных моделях *in vitro* и *in vivo*.

Материалы и методы. В работе применяли микробиологические, морфологические и электронно-микроскопические методы. *In vitro* моделировали биоплёнки на поверхности мочевых катетеров с последующим добавлением бактериофагов. *In vivo* моделировали катетер-ассоциированную инфекцию мочевыводящих путей (КА-ИМП) у мышей, разделённых на опытную и контрольные группы, с последующим трансуретральным введением бактериофагов.

Результаты. В экспериментальной модели *in vitro* бактериофаги разрушали биоплёнки, вызывая лизис клеток и деструкцию экзополисахаридного матрикса. *In vivo* у мышей опытной группы наблюдался регресс течения КА-ИМП, подтверждённый морфологическими и бактериологическими исследованиями. Электронная микроскопия выявила деструкцию биоплёнок на 5 из 6 катетеров. В группе положительного контроля инфекция прогрессировала, в отрицательном контроле биоплёнки отсутствовали.

Выводы. Результаты проведённых экспериментальных исследований *in vitro* и *in vivo* продемонстрировали, что бактериофаги способны разрушать биоплёнки и могут быть эффективным средством для борьбы с ИСМП, вызванными микроорганизмами, формирующими биоплёнки.

Ключевые слова: *бактериофаги; биоплёнки; биоплёночные формы микроорганизмов; инфекции, связанные с оказанием медицинской помощи; катетер-ассоциированные инфекции мочевыводящих путей; оптическая плотность; сканирующая электронная микроскопия*

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Introduction

The current global trend toward increasing bacterial resistance to antimicrobials significantly limits the arsenal of tools available to combat microorganisms [1]. The development of antibiotic resistance is becoming a serious threat in healthcare-associated infections (HAIs) associated with biofilm forms of microorganisms [2]. The formation of biofilms explains the causes of long-term infectious diseases that are resistant to antibiotic therapy [3]. Microorganisms that are part of the biofilm show significantly higher resistance to antimicrobials — up to 1000 times compared to planktonic cells [4]. This significantly complicates the fight against HAI pathogens associated with biofilm forms of microorganisms [5]. The problem of biofilm formation by HAI pathogens on invasive devices used in medical practice is of particular importance [6–8].

Continuing the current trend will negate all modern achievements and successes in the treatment of infectious diseases. In this regard, the World Health Organization and health authorities around the world are drawing attention to the urgent need to address the problem of drug resistance, calling for full support for efforts to develop new ways to combat infectious diseases amid a slowdown in the development of new antimicrobials by many pharmaceutical companies [9].

Under the current circumstances, one of the effective components in the fight against bacterial infections, including those caused by biofilm forms of microorganisms, may be the use of bacteriophages (BP) [10]. The application of BP for biofilm destruction is a promising direction and is of great interest to specialists in various fields, as evidenced by the increase in the number of scientific papers in recent years [11]. At the same time, the number of publications on this topic using scanning electron microscopy (SEM) is quite limited.

The aim of this study is to evaluate the effectiveness of lytic BP for the destruction of microbial biofilms on urinary catheters (UC) in experimental *in vitro* and *in vivo* models.

Materials and methods

Microbiological methods

The analysis of the effectiveness of lytic BP for assessing changes in the structure of biofilms on UC fragments was carried out using collection bacterial strains of HAI pathogens *Pseudomonas aeruginosa* ATCC 12903/ NCTC 27853 and *Escherichia coli* ATCC 25922/NCTC 12241. Biofilms were grown *in vitro* on the inner surfaces of silicone-coated UC fragments.

Electron microscopy methods

The assessment of biofilm formation by *P. aeruginosa* and *E. coli* on the surface of UC fragments and the identification of destructive changes under the influence of BP were carried out using SEM at the Scientific Park of St. Petersburg State University Interdisciplin-

ary Resource Center in the field of nanotechnology. The preparations were made by fixing the material in 25% glutaraldehyde solution for 12 hours at 4°C. After that, to ensure the drainage of excess charge during SEM studies, a thin (5–10 nm) conductive film was formed on the surface: carbon/silver was applied by ion (Ar^+) sputtering of the corresponding targets in the vacuum chamber of the Gatan PECS (Model 682) device, and gold was applied by magnetron sputtering in a VUP C156RS vacuum chamber (Scientific Technologies and Service LLC).

Biofilms were visualized using a Merlin SEM and an Auriga Laser dual-beam workstation (both from Carl Zeiss) with an Everhart-Thornley secondary electron detector. The imaging conditions varied depending on the sample to achieve maximum contrast and image quality: accelerating voltage — 5–20 kV, beam current — 500–700 pA.

BP were selected by spot testing, evaluating their lytic activity on a five-point scale (based on the number of crosses). The study used BP specific to the strains under investigation, Sextaphage and Intestiphage (Microgen) with high lytic activity (++++) (**Fig. 1**).

Bacterial biofilms of HAI pathogens were grown on 1 cm long fragments of Foley's UC, cut lengthwise for accessibility to the inner surface. Manipulations with UC were performed under sterile conditions. Bacterial suspensions of the studied strains of *P. aeruginosa* and *E. coli* were diluted in a sterile nutrient medium to a standard turbidity of 0.5 according to McFarland, then seeded in a continuous lawn in Petri dishes on the surface of Mueller–Hinton agar. Five UC fragments were placed on each Petri dish with the seeded lawn of the studied strains of HAI pathogens. Fifty microliters of liquid nutrient medium were applied to each UC fragment, and the Petri dishes were placed in a thermostat. Cultivation was carried out under thermostatic conditions at 37°C for 24 hours.

One day after incubation, the UC fragments were removed from the surface of the dense nutrient medium and placed in individual sterile 1.5 mL eppendorf tubes, where they were washed twice to remove planktonic cell forms. The washing procedure consisted of adding 1 mL of sterile water to the tubes, treating them in a Vortex device for 30 seconds at 1500 rpm, centrifuging for 30 seconds at 13,000 rpm, and then removing the supernatant. The UC fragments were transferred to new sterile eppendorf tubes without disturbing the sediment. Then, one of the five UC fragments underwent a control rapid assessment for the presence of biofilms using the method of G.A. O'Toole et al., based on the ability of gentian violet dye to bind to the exopolysaccharide matrix of biofilms [12]. Biofilm formation was assessed by measuring the optical density (OD, optical density units) on a Thermo Scientific Multiskan GO microplate spectrophotometer at a wavelength of 590 nm [13].

Another similar fragment of the UC was prepared using the standard fixation method for examination in SEM.

The remaining fragments of the UC were placed on Petri dishes with a dense Mueller-Hinton nutrient medium. One of the three remaining samples was inoculated with 50 µL of BP preparation specific to the bacterial strain under study, while the remaining two UC fragments were inoculated with 50 µL of sterile nutrient medium. The dishes were then placed in a thermostat at 37°C for another 24 hours for incubation.

On the third day of the experiment, a visual assessment of the growth of microbial colonies on the Petri dish was performed (**Fig. 2**). Then, the UC fragments were removed from the dense nutrient medium and, after separation from the suspension cells (see the procedure description above), the preparations were fixed using the standard method for SEM. At the same time, one of the fragments underwent a control rapid assessment for the presence of biofilms using the method of G.A. O'Toole et al. [12], followed by OD measurement.

In the part of the study devoted to evaluating the effectiveness of BP for the destruction of *P. aeruginosa* biofilms *in vivo*, using an experimental model of catheter-associated urinary tract infection (CAUTI) in mice, 4 mm fragments of Teflon 26G Polyflex catheters

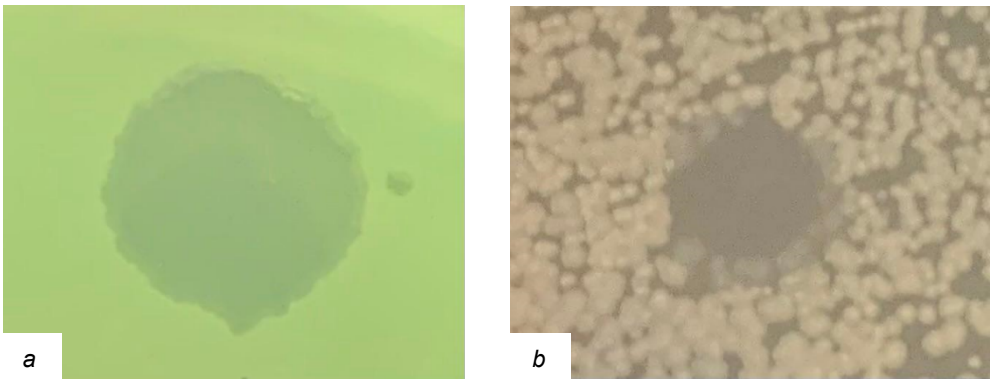

Fig. 1. Visual assessment of BP lytic activity.

a — complete lysis of *P. aeruginosa* culture at the drop site without secondary growth colonies (++++);
b — complete lysis of *E. coli* culture at the drop site without secondary growth colonies (++++).

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(Polymed) fragments that had been left in the bladder for a long time (5 days) were examined *in vivo*. BP selection was performed using spot testing, and the drug Sextaphage (Microgen), specific to the studied strains with high lytic activity (+ + + +), was used in the experiment. To assess the formation of *P. aeruginosa* biofilms on the surface of catheter fragments and to identify destructive changes under the influence of BP, the SEM method was used (according to the procedure described above).

The experiment used 18 sexually mature outbred female ICR (CD-1) laboratory mice aged 2–4 months, weighing 17–24 g, kept in identical conditions and divided into three groups: experimental, positive control, and negative control, with six animals in each group. The animals were randomized into groups using a random number generator.

The experimental group consisted of catheterized mice with simulated CAUTI associated with the formation of *P. aeruginosa* biofilms on an invasive device, receiving BP transurethrally. The positive control group consisted of catheterized mice with simulated CAUTI associated with the formation of *P. aeruginosa* biofilms on an invasive device that did not receive BP. The negative control group consisted of catheterized mice without simulated CAUTI that did not receive BP.

Inhalation anesthesia was used during bladder catheterization procedures. Isoflurane was used for anesthesia, and induction was performed using the hood method. Anesthesia was maintained using an extended nasal cone with cotton balls soaked in 0.5 mL of isoflurane, without the use of an evaporator. Anesthetized mice were placed on their backs on a sterile diaper. The periurethral area was wiped with a napkin containing 0.05% chlorhexidine bigluconate and treated with a 10% povidone-iodine solution using an applicator with a cotton tip. Then, through a peripheral 22 G Vasofix Certo catheter (BBraun) treated with sterile surgical lubricant, a 4 mm fragment of 26 G Polyflex Teflon catheter was inserted into the mouse's bladder using a guide needle and left in the bladder. After that, the 22 G peripheral catheter was removed from the urethra. This procedure was performed in each group of laboratory animals.

Modeling of CAUTI in laboratory animals in the experimental group and the positive control group was performed in accordance with the protocol of M.S. Conover et al. [14] using the collection bacterial strain *P. aeruginosa* ATCC® 12903/NCTC 27853. After catheterization of the bladder with an insulin syringe through a peripheral 26 G Polyflex catheter, mice were transurethrally injected with 50 µL of a bacterial suspension of *P. aeruginosa* with a turbidity of 0.5 according to McFarland (**Fig. 3**).

Subsequently, starting from 2 days after bladder catheterization and CAUTI modeling, mice in the experimental group were administered 50 µl of BP (Sextaphage, Microgen Scientific and Production Association) once a day for 3 days under anesthesia using the method described above.

The dynamics of CAUTI in laboratory animals were assessed using microbiological urine tests before the start of the experiment and on days 2 and 5 in mice of all groups. Urine was collected in sterile 1.5 mL tubes by mechanically pressing on the blad-

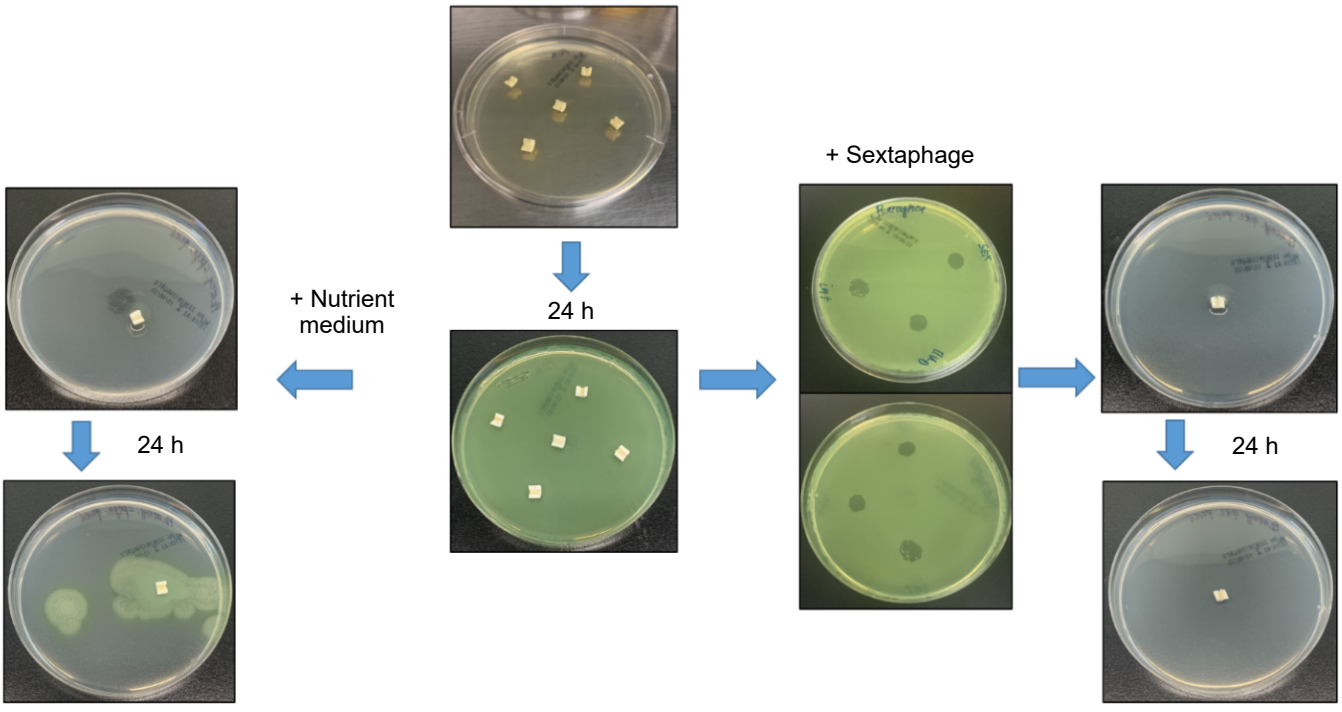

Fig. 2. Modeling of *P. aeruginosa* and *E. coli* biofilms on Foley's UC fragments followed by the addition of nutrient medium or BP (using *P. aeruginosa* as an example).

der area while holding the mouse over the tube. Then, a standard Gold urine culture was performed on Petri dishes with a dense UTI nutrient medium. The plates with cultures were incubated under thermostatic conditions for 48 hours at 37°C. The growth of microorganisms was assessed according to the specified methodology with verification of microorganisms by time-of-flight mass spectrometry (MALDI-TOF).

During the experiment, clinical examination of the mice was performed before each administration, assessing the following signs: the presence of a forced (hunched) posture, signs of exhaustion and dehydration, changes in the condition of the skin, the presence of hyperemia in the area of the external genital organs and changes in activity.

Euthanasia was performed by killing the animals with an overdose of isoflurane followed by cervical dislocation.

Furthermore, to assess the severity and activity of the inflammatory process in the urinary system in laboratory animals at the end of the experiment, a morphological method of examination was used.

Morphological method

Light microscopy was performed in the pathological anatomy department No. 2 of the N.I. Pirogov Clinic of High Medical Technologies Saint-Petersburg State University.

The material was fixed in sufficient volume in a 10% solution of buffered neutral formalin for 24 hours. Standard histological processing was performed using the HISTO-TEK VP1 device (Sakura), then the kidney and bladder material was poured into paraffin and serially sectioned using the Epredia HM 340E microtome (Thermo Fisher Scientific). The resulting histological sections were stained with hematoxylin and eosin according to the standard protocol. Morphological examination was performed using a Leica DM 1000 light microscope (Leica). Microphotographs were obtained using a Leica Flexacam C3 camera (Leica) with LAS X software. Morphometric analysis of the images was performed using FIJI software and included analysis of inflammatory infiltrate in the mucosa and lamina propria of the bladder, renal pelvis, and renal parenchyma. Morphological changes in the urothelium were also assessed, including the presence of bacterial colonies with determination of their percentage of the total surface area of the mucosa in the specimen, and microscopic features of the lamina propria of the mucosa, the presence and severity of necrosis and neutrophilic casts in the renal tubules, as well as their percentage relative to the preserved renal parenchyma. Morphometric analysis of the parameters was performed on an area of 0.2 mm^2 in the areas with the most pronounced changes (hot spots).

The severity of inflammation was assessed semi-quantitatively. Based on the composition of inflammatory infiltrate cells in 1 field of view (0.2 mm^2) at ×400 magnification ("+" — up to 30 cells, "++" — 31–79 cells, "+++" — ≥ 80 cells, "–" — no inflammatory infiltrate).

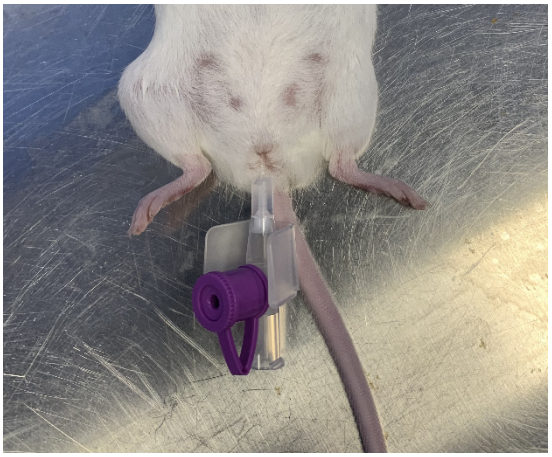

Fig. 3. Transurethral access for the introduction of *P. aeruginosa* bacterial suspension through a 26 G Polyflex peripheral catheter

Statistical analysis of the data was performed using R software (version 4.4.3). Data on OD indicators are presented as the arithmetic mean and standard deviation ($M \pm SD$). To compare the OD indicators of biofilms formed by different strains of microorganisms with the negative control, the nonparametric Mann–Whitney U test was used, since the distribution of values in the groups was not tested for normality due to the small sample size. The statistical significance of differences in microbial load (CFU/mL) between groups of animals in the *in vivo* experiment was assessed using the Mann–Whitney test for independent samples. Differences were considered statistically significant at a significance level of $p < 0.05$.

Results

During modeling and subsequent visualization of daily biofilms on fragments of siliconized Foley UC, average OD values were obtained for various bacterial strains: *P. aeruginosa*, *E. coli* and negative control (0.103 ± 0.002 OD units). The results obtained were classified according to their biofilm-forming potential using the following methodology [15].

Comparison of the obtained OD values with those of the negative control showed that the cells of the studied strains of *P. aeruginosa* (0.168 ± 0.003 OD units) and *E. coli* (0.154 ± 0.005 OD units) showed a moderate tendency to form biofilms. The average OD values of the studied strains exceeded the OD values of the negative control by approximately 1.5 times ($p < 0.05$). At the same time, during the rapid indication of two-day biofilms on fragments of siliconized Foley UC, the average OD values were 0.215 ± 0.008 for *P. aeruginosa* and 0.203 ± 0.005 units OD, which indicated that the

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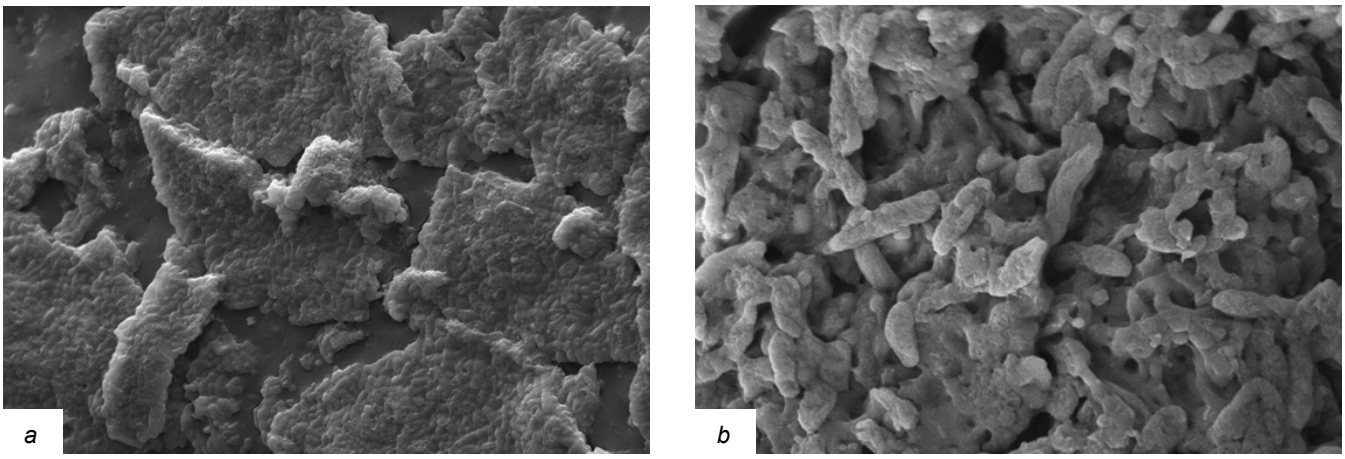

Fig. 4. SEM image of the inner surface of a Foley urinary catheter fragment with a 24-hour biofilm.
a — *P. aeruginosa* bacterial biofilm (24 h), × 5000; *b* — *E. coli* bacterial biofilm (24 h), × 20,000.

OD values exceeded those of daily biofilms and negative controls ($p < 0.05$).

On the UC scans after culturing the bacterial strains *P. aeruginosa* and *E. coli* for 24 hours without BP treatment, the presence of microbial communities in the form of formed biofilms was noted, while numerous dividing cells were detected in the thickness of the intercellular matrix that unites bacteria into a single biofilm structure (**Fig. 4**).

After 48 hours of incubation of UC with bacterial strains without the addition of BP intensively developed multilayer biofilms of *P. aeruginosa* and *E. coli* were observed (**Fig. 5**).

Electron microscopic analysis of the surface of the UC with a daily model biofilm of *P. aeruginosa* and *E. coli* after a single exposure to BP for 24 hours showed pronounced signs of destruction of the microbial community. The destructive effect of BP was manifested both on the vegetative forms of microorganisms and on their biofilm communities. First of all, a change in the morphology of bacterial cells included in the preserved fragments of biofilms was noted, which manifested itself in the deformation of the cell wall surface and its destruction. Disruptions in the structure of the cell wall were accompanied by destructive changes in the shape of bacterial cells. Ultimately, the effect of BP led to the lysis of most bacterial cells due to the destruction of cell walls. The destruction of a significant portion of the cells in the biofilms was evidenced by the discovery of areas of cell debris accumulation. At the same time, there was a marked decrease in the number of bacteria on the surface of the substrate that retained the morphological properties characteristic of viable bacterial cells (**Fig. 6**). At the level of the biofilm community of microorganisms, the destruction of the intercellular exopolysaccharide matrix, up to its complete disappearance in some areas of the microbial community, indicated a change in the structure of *P. aeruginosa* and *E. coli* biofilms as a result of BP exposure.

In the part of the study devoted to evaluating the effectiveness of BP for the destruction of *P. aeruginosa* biofilms *in vivo* in an experimental model of CAUTI

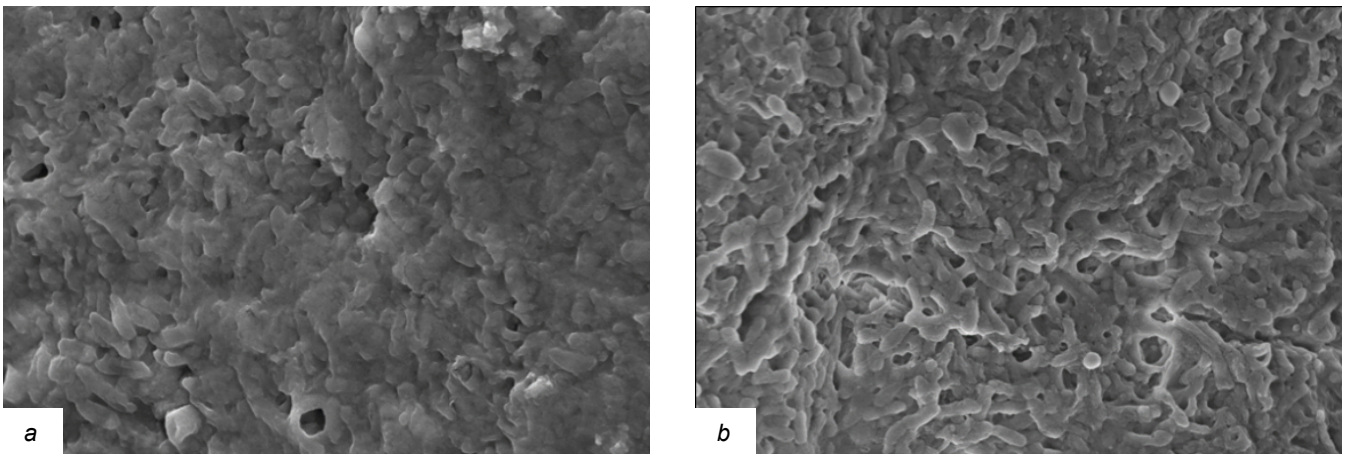

Fig. 5. SEM image of the inner surface of a Foley urinary catheter fragment with a 48-hour biofilm.
a — *P. aeruginosa* bacterial biofilm (48 h), ×10,000; *b* — *E. coli* bacterial biofilm (48 h), ×10,000.

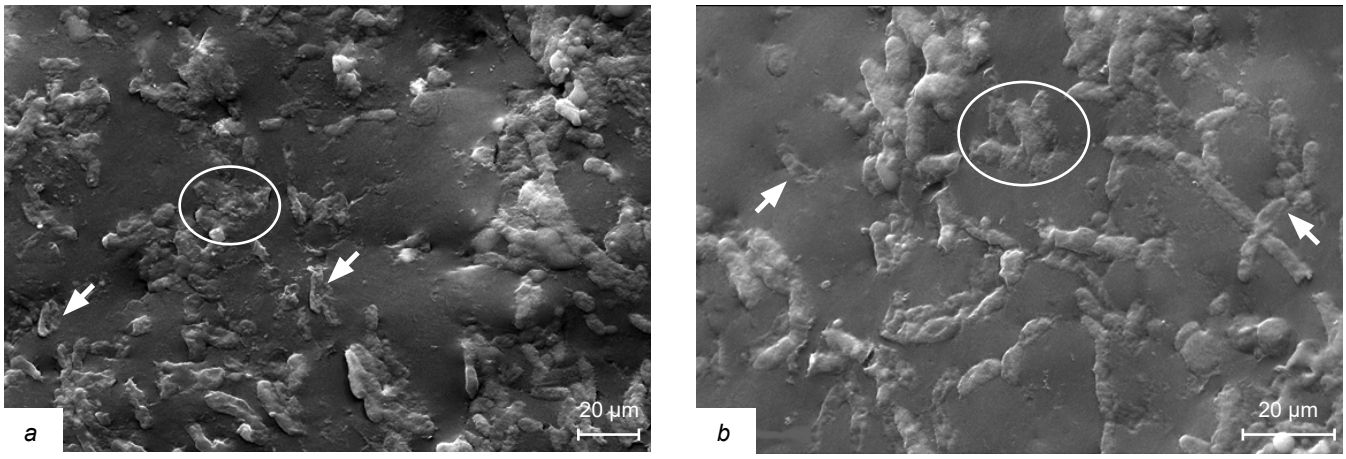

Fig. 6. SEM image of destructive changes in the daily biofilm on the surface of a Foley urinary catheter fragment after a single exposure to BP.

The oval marks areas of cell debris accumulation. The arrow indicates destructive changes in cell walls.
a — destruction of the *P. aeruginosa* biofilm after exposure to sextaphage, × 10,000;
b — destruction of the *E. coli* biofilm after exposure to intestiphage, ×15,400.

in mice, the results showed that during the experiment in the positive control group (animals with established catheter, after administration of *P. aeruginosa* and without BP instillations), 5 out of 6 animals showed progression of CAUTI. During clinical examinations, starting from the second day of the experiment and until its completion, hyperemia was recorded in the external genital organs of mice.

During bacteriological urine tests at reference points (days 2 and 5), this group showed a tendency toward a significant increase in the microbial load of *P. aeruginosa* from 10^1 to 10^3 CFU/mL. During bacteriological urine testing before the start of the experiment, one mouse in this group was found to have *Aerococcus viridans* growth at a titer of < 100 CFU/mL, which could be due to contamination of the biomaterial during sampling or endogenous colonization of this locus. *A. viridans* is a well-known Gram-positive opportunistic pathogen that can infect various animals, including mice, especially those with immunodeficiency [16]. However, bacteriological examination at subsequent reference points did not detect *A. viridans* in this animal.

According to electron microscopic analysis of invasive devices, microbial communities of microorganisms were observed in the form of *P. aeruginosa* biofilms (**Fig. 7**).

In morphological studies of these mice (5 out of 6) in the positive control group, the severity of the inflammatory process in the tissues corresponded to subacute active cystitis and subacute active pyelitis, respectively (**Fig. 8**).

In 1 of 6 animals in this group, CAUTI did not develop during modeling, which was confirmed by clinical examination data (absence of hyperemia of the external genital organs, signs of exhaustion and dehydration, etc.), as well as by the results of bacteriological, morphological, and electron microscopic examination methods.

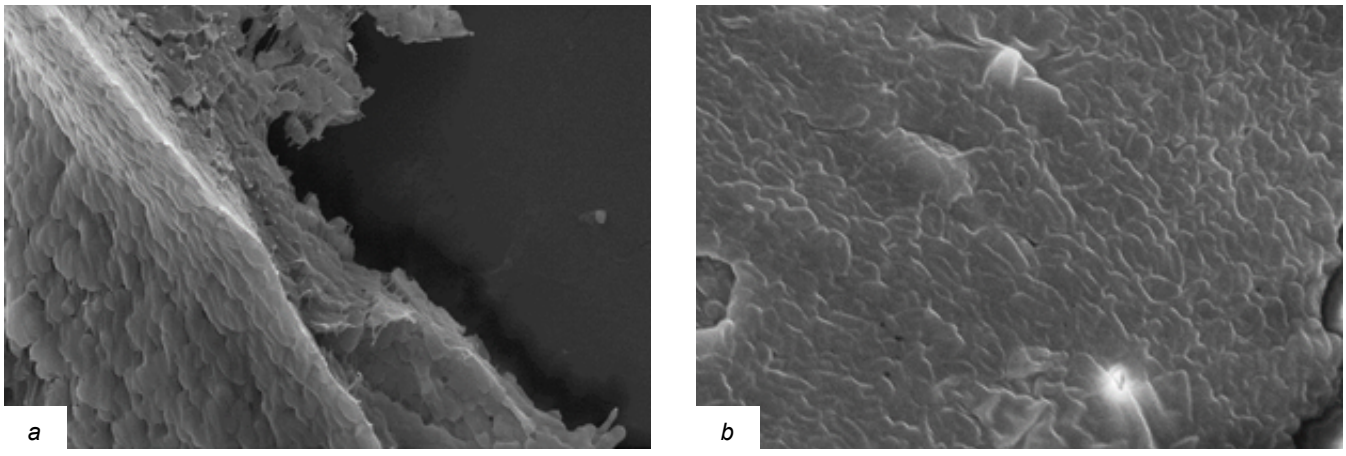

Fig. 7. SEM image of the surface of removed fragments of the catheter from the bladder of animals.

a — fragment of the catheter with a formed biofilm of *P. aeruginosa*, × 8590; *b* — fragment of the catheter with a formed biofilm of *P. aeruginosa*, × 10,000.

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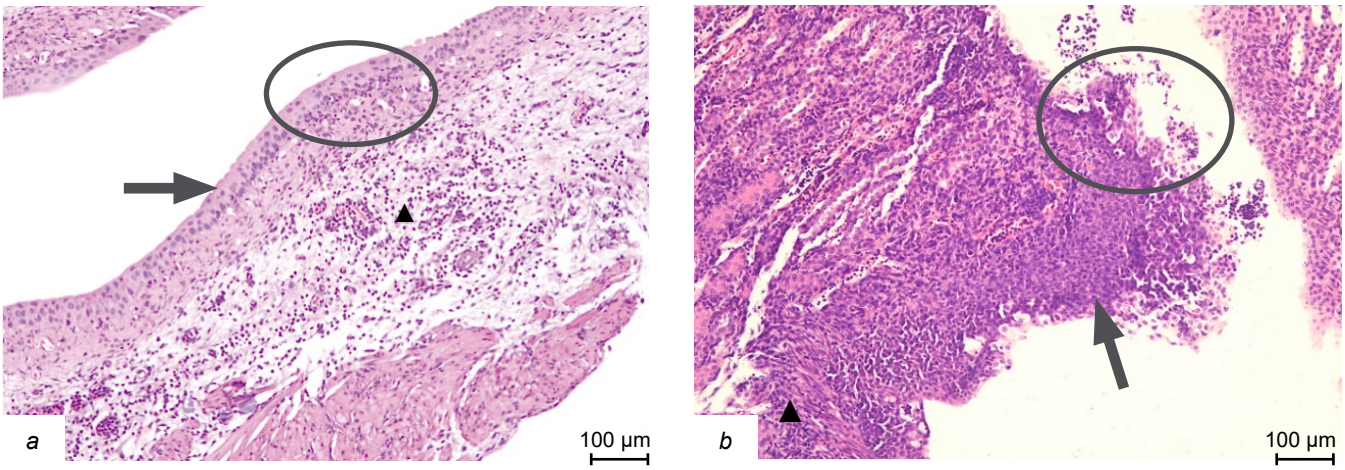

Fig. 8. Inflammatory process in the tissues of mice in the positive control group. Electron microscopy, stained with hematoxylin and eosin, ×100.

a — subacute active cystitis. The black arrow indicates the urothelium lining the bladder mucosa. The black oval marks the area of intraepithelial spread of a small number of neutrophilic granulocytes and lymphocytes into the urothelium. The black triangle marks a moderate lymphocytic inflammatory infiltrate of the lamina propria of the mucosa with an admixture of histiocytes and neutrophilic granulocytes, as well as moderate edema of the stroma and hyperemia of the vessels;
b — subacute active pyelitis. The black arrow indicates the urothelium lining the mucosa of the renal pelvis, with reactive changes in the form of an increase in the number of cell layers, their dystrophy, and local polarity disruption. The black oval indicates the area of intraepithelial spread of neutrophilic granulocytes into the urothelium. The black triangle marks a moderate lymphocytic inflammatory infiltrate with an admixture of histiocytes and plasmocytes in the renal stroma.

In the experimental group (animals with established catheter, after administration of *P. aeruginosa* followed by instillations of BP), 3 out of 6 mice showed regression of CAUTI and positive dynamics, as indicated by the results of bacteriological and morphological studies, clinical examinations, and SEM data.

According to bacteriological urine tests, no growth of flora was detected in any mouse in this group before the start of the experiment. Furthermore, when modeling CAUTI on the second day of the experiment, before the administration of BP, each mouse developed bacteriuria caused by *P. aeruginosa*, with a microbial load of 10^1–10^3 CFU/mL. Then, after BP instillations into the bladder, no flora growth was detected in the urine of 4 out of 6 animals on the 5th day of the experiment. In 2 of 6 mice, *P. aeruginosa* growth of 10^1 and 10^4 CFU/mL was detected in the urine, with one of them showing fulminant UTI, which was also confirmed by clinical examinations (hyperemia of the external genital organs, weight loss, forced (hunched) posture, and changes in activity) and confirmed by microscopic examination (purulent abscessing pyelitis and interstitial nephritis, purulent necrotic papillitis).

Electron microscopic analysis of removed fragments of the catheter extracted from the bladder of animals with negative bacteriological test results on the 5th day of the experiment, which underwent transurethral instillation of lytic BP, showed destruction of the exopolysaccharide matrix of biofilms and lysis of *P. aeruginosa* cells (**Fig. 9**).

Microscopic examination of mice in the experimental group with regression of CAUTI and observed positive dynamics (3 out of 6) revealed no morphological signs of inflammation in the studied material (**Fig. 10**).

Among the three remaining mice in the experimental group, morphological examination revealed that in two mice the severity of the inflammatory process in the tissues corresponded to subacute active cystitis and subacute active pyelitis, in one mouse, *P. aeruginosa* biofilm was detected on the surface of the catheter, in another, under the influence of BP, the formed biofilm was destroyed according to SEM data; in the third mouse, the severity of the inflammatory process in the tissues corresponded to purulent abscessing pyelitis, interstitial nephritis and purulent necrotic papillitis, with destruction of the formed biofilm also observed.

It is important to note that for the animal with the most pronounced inflammation and fulminant form of UTI, the infectious process was localized in the upper urinary tract, while in the lower urinary tract it was absent at the end of the experiment. In our opinion, this can be explained by the action of the phage in the lower urinary tract and insufficient concentration in the upper urinary tract.

The negative control group (animals with established catheter, without *P. aeruginosa* and without BP instillations) had no significant features and was characterized by the absence of UTI development, with the exception of 2 mice, in whose urine bacteriological examination *Staphylococcus nepalensis* growth was detected at a titer of less than 100 CFU/mL. *S. nepalensis* is a coagulase-negative staphylococcus that was first discovered in Nepal in goats with pneumonia and is

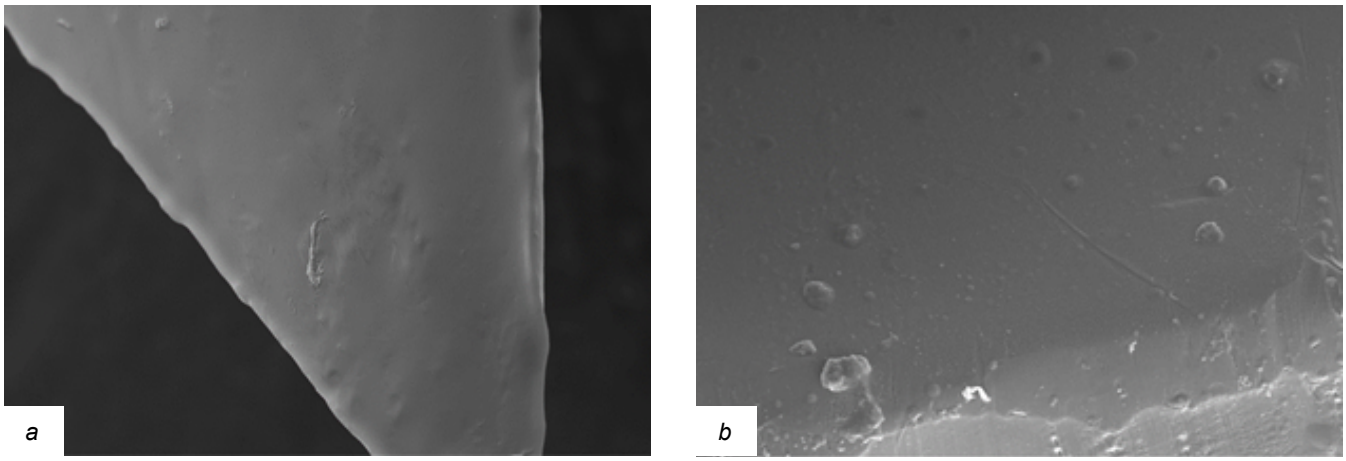

Fig. 9. SEM image of the surface of removed fragments of the catheter from the bladder of animals.
a — absence of *P. aeruginosa* biofilm on the surface of the removed fragment of the catheter, × 5000; *b* — absence of *P. aeruginosa* biofilm on the surface of the removed fragment of the catheter, × 10,000.

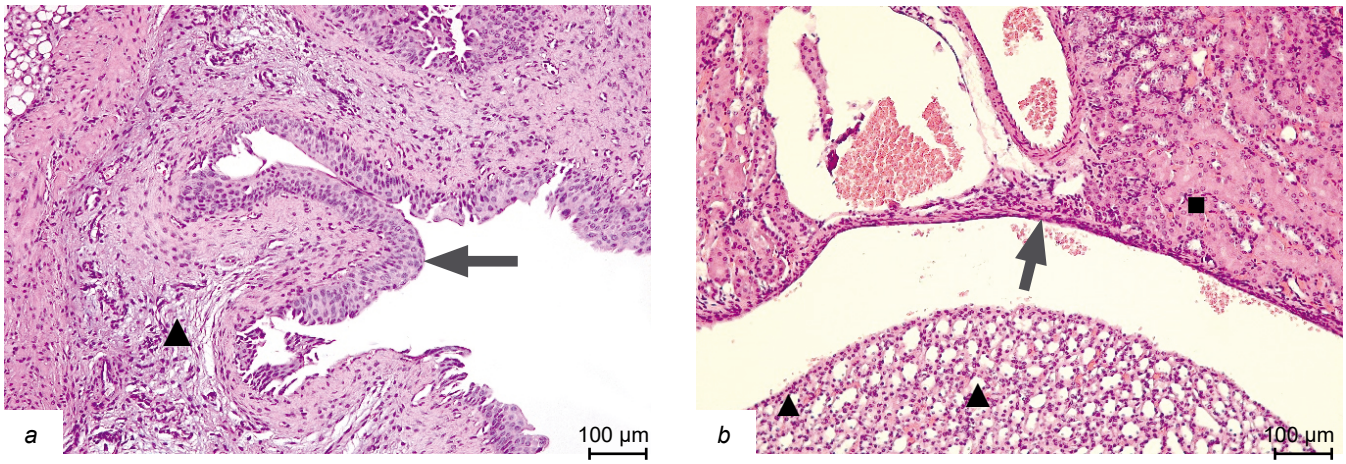

Fig. 10. Urothelium without signs of inflammation and dystrophy (arrow) on a micro-preparation of the bladder (*a*) and kidney (*b*). Electron microscopy, stained with hematoxylin and eosin, ×100.
a — the triangle indicates the lamina propria of the mucosa with slight edema and slight hyperemia of the vessels; *b* — the arrow indicates urothelium without signs of inflammation and dystrophy, the triangle indicates a papilla. The square marks the tubules of a kidney with a typical structure.

considered part of the normal flora of mammals, including mice, including the gastrointestinal tract; it can also cause infections [17]. The isolation of this microorganism could be associated with contamination of the administered catheter or endogenous colonization of this locus. At the same time, on the 2nd and 5th days of the experiment, one of these mice had a negative result in a bacteriological examination of urine, while the other mouse had *S. nepalensis* growth in urine at a titer of 10^1 and 10^3 CFU/mL respectively, which is also confirmed by SEM data: a biofilm of *S. nepalensis* was identified on the catheter extracted from the bladder of this mouse (**Fig. 11**). On the remaining catheters from other animals in this group, no biofilm forms of microorganisms were found during electron microscopic analysis.

In the morphological study, no microscopic signs of inflammation were found in the examined material in 5 out of 6 animals in the negative control group. In contrast, in the mouse that excreted *S. nepalensis* in its urine, the morphological picture in the tissues corresponded to subacute active cystitis and subacute active pyelitis.

The results of bacteriological, morphological, and electron microscopic studies performed during the experiment among all groups of animals are presented in the **Table**.

It is important to note that safety issues, including toxicological assessment and analysis of local irritant effects, were not included in the scope of the current phase of work and require separate study in further research.

Discussion

Currently, there is active discussion of alternative replacements for antimicrobials for the effective prevention and treatment of infectious diseases. The search

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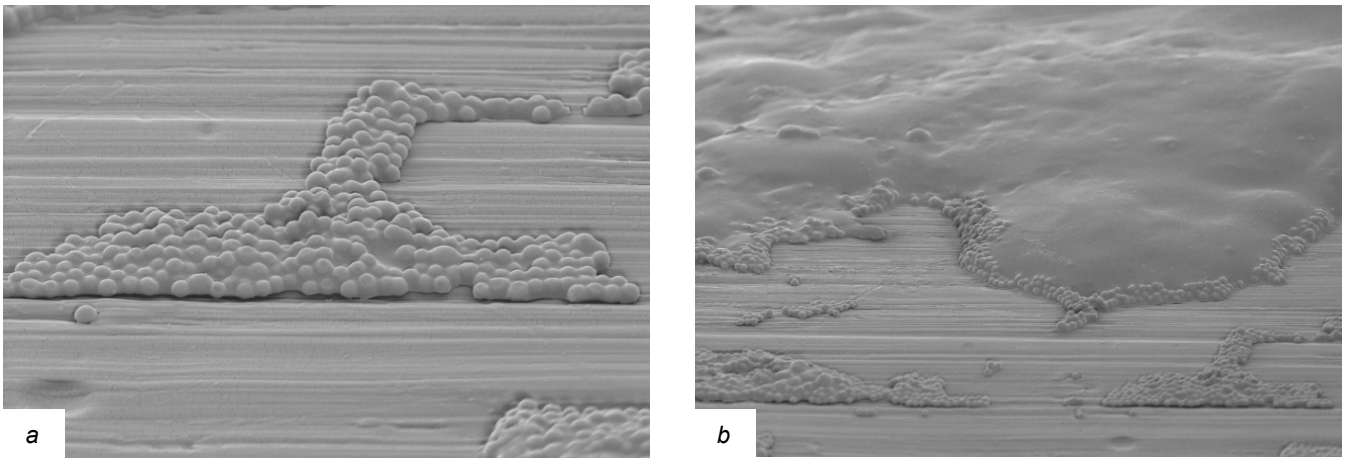

Fig. 11. SEM image of the surface of the removed catheter fragment.
a — bacterial biofilm *S. nepalensis*, × 8250; *b* — overview image of a bacterial biofilm of *S. nepalensis*, covered with a surface film, with *S. nepalensis* cells bordering it, × 2890.

for alternative ways to combat opportunistic microorganisms includes the need to research and develop new antimicrobial factors. These circumstances explain the increased interest in therapeutic BP [18].

According to many experts, the best option is BP drugs, which can be used both in combination with antimicrobials and as an independent therapeutic agent [19, 20]. The development and industrial production of new BP drug forms is actively progressing. However, to date, the use of BP for the destruction of bacterial biofilms has been an insufficiently researched area.

The fact that the use of BP for biofilm destruction is a promising direction is evidenced by the increased interest in this issue among specialists of various profiles [21]. In recent years, close attention to this problem has been confirmed by a significant increase in the number of scientific publications [22, 23]. At the same time, the number of publications on this topic that use electron microscopy to study the process of destroying bacterial biofilms with BP using the SEM method is limited.

In a recent *in vitro* study, F. Zurabov et al. performed electron microscopic analysis of *Klebsiella pneumoniae* biofilms using SEM, adding a phage cocktail of three types of sensitive BP with depolymerase activity to the microbial community [24]. According to the authors, the use of this BP cocktail proved to be quite effective and led to the inhibition of biofilms, but complete lysis of bacterial cells was not detected in this experiment. The results demonstrated the ability of the studied phage cocktail, sensitive to *K. pneumoniae* cells, to effectively destroy the exopolysaccharide matrix of biofilms, which performs a protective function in microbial communities.

According to R.R. Pallavali et al., the action of lytic phages sensitive to biofilm forms of multidrug-resistant strains of *P. aeruginosa*, *E. coli*, *K. pneumoniae* and *Staphylococcus aureus* isolated from the wounds of burn patients led to a decrease in biomass and lysis of bacterial cells [25]. The biomass of biofilms of *P. aeruginosa*, *S. aureus*, *K. pneumoniae* and *E. coli* decreased by more than 50–80% depending on the duration of phage exposure. SEM analysis revealed morphophysiological changes in the structure of biofilms resulting from phage-mediated lysis.

Another promising direction in phage therapy for combating biofilm forms of bacteria is the use of BP enzymes — phage endolysins and depolymerases.

According to data obtained by W. Chen et al. as a result of electron microscopic studies using SEM, the use of a hybrid protein based on Klebsiella BP endolysins against ESKAPE pathogens led to changes in the morphology of microorganisms in the form of roughness on the surface of bacteria, small holes in the cell wall, and cell deformation, up to their complete lysis [26].

An electron microscopic study by the same authors, devoted to the analysis of the effect of phage endolysins on mature biofilms of *Acinetobacter baumannii* and *Enterococcus faecalis*, showed that exposure to these enzymes for 24 hours led to almost complete destruction of the biofilms.

A study by M. Shahed-Al-Mahmud et al. demonstrated the ability of the depolymerase of the recombinant spike protein BP *A. baumannii* (φAB6) to exhibit enzymatic activity for 4 hours and destroy formed biofilms by loosening the protein-exopolysaccharide matrix [27].

In addition to studies describing the destructive effect of BP on biofilm forms of bacteria, a number of authors have devoted their research to analyzing the use of BP for the prevention of biofilm formation.

An example is the study by W. Fu et al., in which electron microscopic analysis of samples showed that pretreatment with a mixture of BP *P. aeruginosa* M4 *in vitro* for 2 hours at 37°C prior to bacterial inoculation

Results of bacteriological, morphological and electron microscopic studies performed during the *in vivo* experiment

Mouse serial number	Result of bacteriological urine culture before the experiment, CFU/mL			Presence of formed biofilm	Severity of inflammation in tissue			
	Before experiment	2nd day of experiment	5th day of experiment		Microscope slide of kidney		Microscope slide of bladder	
					presence	severity	presence	severity
1. Experimental group								
1.1	No growth of facultative anaerobic microflora (FAM) detected	*P. aeruginosa*, 10^1	*P. aeruginosa*, 10^1	Yes	Yes	+	Yes	+
1.2	No growth of FAM detected	*P. aeruginosa*, 10^2	No growth of FAM detected	No	No	–	No	–
1.3	No growth of FAM detected	*P. aeruginosa*, 10^2	No growth of FAM detected	No	Yes	+	Yes	+
1.4	No urine collected	*P. aeruginosa*, 10^1	No growth of FAM detected	No	No	–	No	–
1.5	No growth of FAM detected	*P. aeruginosa*, 10^3	*P. aeruginosa*, 10^4	No	Yes	+++	No	–
1.6	No growth of FAM detected	*P. aeruginosa*, 10^2	No growth of FAM detected	No	No	–	No	–
2. Positive control								
2.1	No growth of FAM detected	No urine collected	No growth of FAM detected	No	No	–	No	–
2.2	No growth of FAM detected	*P. aeruginosa*, 10^1	*P. aeruginosa*, 10^3	Yes	Yes	++	Yes	++
2.3	*A. viridans*, < 100	*P. aeruginosa*, 10^2	*P. aeruginosa*, 10^3	Yes	Yes	++	Yes	++
2.4	No growth of FAM detected	*P. aeruginosa*, 10^2	*P. aeruginosa*, 10^3	Yes	Yes	+	Yes	+
2.5	No urine collected	*P. aeruginosa*, 10^2	*P. aeruginosa*, 10^3	Yes	Yes	++	Yes	++
2.6	No growth of FAM detected	*P. aeruginosa*, 10^1	*P. aeruginosa*, 10^2	Yes	Yes	++	Yes	++
3. Negative control								
3.1	*S. nepalensis*, < 100	No growth of FAM detected	No growth of FAM detected	No	No	–	No	–
3.2	*S. nepalensis*, < 100	*S. nepalensis*, 10^1	*S. nepalensis*, 10^3	Yes	Yes	+	Yes	+
3.3	No growth of FAM detected	No growth of FAM detected	No growth of FAM detected	No	No	–	No	–
3.4	No urine collected	No growth of FAM detected	No growth of FAM detected	No	No	–	No	–
3.5	No growth of FAM detected	No growth of FAM detected	No growth of FAM detected	No	No	–	No	–
3.6	No growth of FAM detected	No growth of FAM detected	No growth of FAM detected	No	No	–	No	–

prevented biofilm formation [28]. The data obtained were confirmed by the absence of both microcolonies and single bacterial cells on the surface of the Foley UC.

Another study using the SEM method, conducted by J.J. Curtin et al., confirms the possibility of preventing the formation of *S. epidermidis* biofilms on Foley catheters by pretreating them with BP for 24 hours *in vitro* [29].

Thus, a number of studies on the use of BP to combat biofilm forms of microorganisms that cause HAIs have noted morphophysiological changes in the structure of biofilms in the form of destructive changes in the intercellular exopolysaccharide matrix and changes in the morphology of bacterial cells that are part of biofilms, which manifested itself in changes in the surface of the cell wall and various deformations of cells, up to their complete lysis.

The results of our study demonstrated the destructive effect of BP against biofilms, which manifested itself at both the cellular and population levels of microbial communities of microorganisms.

Currently, the volume of scientific research devoted to modeling CAUTI in laboratory animals with subsequent evaluation of BP efficacy remains insufficient, which necessitates further in-depth study of this problem [30].

F. Mehmood Khan et al. in a review devoted to the use of phage therapy in *in vivo* animal models, presented various routes of administration of BP to infected

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mice [31]. However, among the listed options for BP administration, the transurethral route was absent.

A.N. Singh et al. evaluated the effectiveness of a phage cocktail with different routes of administration and dosages for combating simulated UTI in mice caused by colistin-resistant *K. pneumoniae* [32]. The phage cocktail was administered to mice transurethrally, rectally, subcutaneously, and orally after the onset of UTI. The authors found that higher concentrations of phages and transurethral, oral, and rectal routes of administration were optimal. Transurethral administration required several doses, while oral and rectal administration required approximately 6–9 repeated administrations.

In contrast to these studies, M.S. Conover et al. modeled not only UTI but also CAUTI in mice. In the experimental model of CAUTI, a silicone-coated RenaSIL tube was inserted into the bladder, followed by instillation of a bacterial suspension of *E. faecalis*. Within 1 day, CAUTI developed, characterized by active progression of the infection associated with the formation of *E. faecalis* biofilms on the surface of the invasive device. In turn, when using the UTI model, 1 day after the onset of infection, the authors noted a decrease in the concentration of the pathogen in the urine with its subsequent eradication. This approach allowed for a more accurate simulation of the clinical situation in which the presence of UC in the bladder contributes to the development of a persistent bacterial infection associated with biofilm forms of microorganisms.

The results of the presented studies by different authors indicate that, despite the growing interest in phage therapy, there are insufficient studies devoted to modeling CAUTI and evaluating the effectiveness of BP. The data obtained emphasize the need for further research aimed at optimizing phage therapy for CAUTI, in particular, the study of transurethral administration of BP. This will allow the development of more effective strategies to combat persistent biofilm-associated infections, which is especially relevant in the context of growing antibiotic resistance.

Recent studies confirm the promise of using phages in the fight against infections associated with biofilm forms of microorganisms, including CAUTI. Experimental data demonstrate their ability to destroy the exopolysaccharide matrix of biofilms, cause lysis of bacterial cells, and prevent their formation. However, despite *in vitro* successes, questions about the optimal methods of administration (including transurethral), dosage, and efficacy of phages *in vivo* require further study.

The development of combined strategies that combine phage therapy with other antimicrobial approaches is particularly relevant. In-depth research in this area may be the key to overcoming antibiotic resistance and creating new methods for treating chronic and recurrent infections, including those associated with biofilm forms of microorganisms associated with medical devices.

Conclusion

Using SEM, it was established that the modeled biofilms of *P. aeruginosa* and *E. coli* formed on Foley's UC *in vitro* were subjected to destruction by lytic BP with destruction of the exopolysaccharide matrix and pronounced cell lysis.

Electron microscopic analysis of control samples of Foley UC using SEM showed that without BP treatment, microbial communities in the form of biofilms formed on them after 24 hours. At the same time, physiologically active and dividing cells, united into a single structure, were detected on the surface of the UC. After 48 hours, abundant clusters of bacterial cells were observed on the UC without BP treatment, forming a single biofilm structure together with fragments of the intercellular matrix.

Our data from the study evaluating the effectiveness of BP for the destruction of *P. aeruginosa* biofilms *in vivo* in an experimental model of CAUTI in mice indicate that BP can be successfully used to combat CAUTI associated with biofilm forms of microorganisms and lead to the destruction of the exopolysaccharide matrix of biofilms with lysis of the cells that make up the biofilm.

The effectiveness of BP in destroying microbial biofilms formed on the surface of UC in experimental *in vitro* and *in vivo* models has been clearly demonstrated.

When patients develop CAUTI associated with biofilm forms of microorganisms and removal of the invasive device is not possible, the use of BP by flushing through the UC may be considered.

The results of experimental studies have demonstrated that BP can be an effective means of combating HAIs caused by microorganisms that form biofilms.

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