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Original article

# Research on the formation of *Pseudomonas aeruginosa* biofilms as a factor in the development of antibiotic resistance in fatal pneumonias

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**Abstract.** *Background and aim:* Antibiotic resistance of community-acquired pneumonia pathogens and the emergence of multiresistant bacteria is a problem of global importance. Determining the ability to form biofilms will provide an opportunity to develop new ways to overcome bacterial resistance to antimicrobial drugs. Thus, this study aimed to determine *Pseudomonas aeruginosa* isolates antibiotic resistance and to study the architecture of its biofilms. *Methods:* 22 fatal cases of secondary pneumonia due to gunshot trauma for the period from 2022 to 2023 during the war in Ukraine were studied. Isolation and identification of pure culture were provided using Micro-la-test kits. The sensitivity to antimicrobial drugs was studied using «SENSILAtest G-I, G-II» and Kirby-Bauer Disk Diffusion Susceptibility Test. To form biofilms bacteria was grown on the surface of coverslips in the 35 mm Petri dishes for 24 h. Biofilm formation was assessed by laser scanning confocal microscopy and scanning electron microscopy. *Results:* Examination of biological samples revealed 22 strains of microorganisms. Gram-negative microorganisms predominated among pathogens (63.6%) and *Pseudomonas aeruginosa* isolates were 50%. Determining the sensitivity established that to antimicrobial drugs took place in 71.4% of cases (5 strains), and poly-resistantance was found in 28.6% of cases (2 strains). *Conclusions*: the study of structural and functional features of biofilm formation showed that biofilms were formed according to classical stages. Dense biofilms determined the phenotypic variability of *Pseudomonas aeruginosa* with the development of antibiotic resistance to antimicrobial drugs, which is a barrier to the use of antimicrobial therapy. (www.actabiomedica.it)

**Key words:** pneumonia, antibiotic resistance, isolates of *Pseudomonas aeruginosa*, biofilms

## Introduction

Antibiotic resistance (antimicrobial resistance) and the emergence of multidrug-resistant bacterial strains are problems of global importance that pose serious threats to humanity. Today the situation in Ukraine, as in the world as a whole, is disappointing. The main reason is considered to be the irrational use of antibacterial therapy, because it leads to the selection of chemoresistant pathogen strains (1).

According to the World Health Organization, antibiotic resistance is one of the world's 10 main

threats to human health. It is predicted that by 2050 infections, caused by antibiotic-resistant microorganisms, may cause about 10 million annual deaths. The range of bacteria that become resistant to any known antimicrobial is steadily increasing (2).

Pneumonia is an acute infectious disease of mainly bacterial aetiology, which is characterized by focal lesions of the lungs and the obligatory presence of intra-alveolar exudation. A distinction is made between community-acquired pneumonia (CAP), i.e. outside a medical institution and in-hospital (nosocomial) pneumonia. CAP is the prominent cause of mortality and morbidity with an important clinical impact across the globe (3, 4).

Today pneumonia is mostly caused by gram-negative bacteria, in particular *Pseudomonas aeruginosa.* The main problem of treating such pneumonia is related to drug resistance, which is due to the ability of bacteria to form biofilms as a factor in the phenotypic variability of the pathogen (5, 6).

Bacteria in the biofilm state develop specific communications called quorum sensing (QS) (7, 8). Thus, the QS of *P. aeruginosa* regulates the production of virulence factors, such as extracellular exopolysaccharides, proteases and phenazines, which determine the processes of adhesion, colonization and dissemination of the pathogen (9-12). Thus, *P. aeruginosa,* using QS, develops resistance to various groups of antibiotics, including aminoglycosides, quinolones, carbapenems and β-lactams (13-17). The spread of antibiotic-resistant bacteria in hospitals and community-acquired infections is one of the main problems of antibiotic therapy (18, 19).

One of the leading mechanisms of the development of bacterial pneumonia is infection with antibiotic-resistant strains of the macroorganism with suppression of the immune status as a result of gunshot and shrapnel injuries. At the same time, the persistence of bacteria in the patient's body in the form of dense biofilms, which is the main factor in the pathogenicity and virulence of *P. aeruginosa* is important (20, 21). In fact, this leads to the inactivation of natural anti-infective resistance factors, such as the systems of complement, lysozyme, platelet cationic protein, etc. (23). The ability of microorganisms to survive antibiotic therapy is determined by their indifference to physico-chemical factors and acquisition of resistance to host defense mechanisms (24, 25).

Cellular mechanisms of non-specific protection play an important role mainly in the respiratory part of the lungs. The main cells here are leukocytes, neutrophils, eosinophils and macrophages. During massive bacterial aggression, these cells release such chemokines as Il-8, TNF-α, Il-1, components of the complement system, as well as G-CSF, which is important in the development of pneumonia (26-28). Humoral protective factors are provided by immunoglobulins A and G, lymphoid cells, macrophages of lymphoid tissue and lymph nodes of the lungs and bronchi (29). Ig A provides agglutination of bacteria and neutralizes their toxins. Ig G in the lower respiratory tract agglutinates and opsonizes bacteria, and activates the complement system, due to which the chemotaxis process of neutrophils and macrophages is accelerated. But when these defense mechanisms are violated, the emergence of an infectious process becomes possible. Thus, when mucus secretion is impaired, the process of adhesion of bacteria to epithelial cells is facilitated. This leads to damage to the latter and increased adhesion of the pathogen, followed by the formation of dense biofilms, which is the main factor in the pathogenicity and virulence of bacteria. This is the pattern of a vicious circle of colonization formation (30-36).

Therefore, bacteria can adapt to environmental changes, including exposure to low and high doses of antibiotics and use such resistance mechanisms as reduced membrane permeability, the presence of drug efflux pumps and their enzymatic inactivation and in addition the spread of antibiotic resistance genes due to the mobility of genetic factors (37, 38). The problem of developing of antibiotic resistance of *P. aeruginosa*, as a factor in types of pneumonia that lead to death, requires more thorough and in-depth research, namely, detailing the stages of biofilm formation (39-41).

**The aim of this study** is to determine the mechanism underlying the resistance of *P. aeruginosa* isolates to antibiotic drugs and study the architecture of the biofilm formed by multi-resistant isolates as a factor in the protection of bacteria against antibiotics.

## Material and Methods

*Study design and subjects*

The research was carried out as part of a scientific research project «Experimental substantiation of antimicrobial agent complex application based on determination of peculiarities of microbiological properties of pathogens of purulent-inflammatory diseases» (№ 0120U102569, 2020-2024) of the Department of Microbiology, Virology and Immunology named after Prof. D.P. Grynyov. In this study biological cadaveric material of military personnel with gunshot injuries and community-acquired pneumonia was collected from corpses stored in the hospital's morgue. Their medical records were studied retrospectively. At the time of hospitalization, all patients were examined according to the protocol for the treatment of pneumonia: chest x-ray, clinical sputum analysis, sputum analysis for Mycobacterium tuberculosis, sputum culture to determine sensitivity to antibiotics, standard clinical and biochemical analysis of blood and urine, C-reactive protein, D - dimer, procalcitonin, bacterial culture of blood for sterility, bronchoscopy if indicated. An informed written consent was obtained from the patients` relatives prior to the study.

*Microbiology testing*

For microbiological research, the kits that enable the identification of clinical strains were used. The tests are placed in the wells of a segmented microtitration plate.

The sensitivity of clinical strains of microorganisms to antimicrobial drugs was studied using Kirby-Bauer Disk Diffusion Susceptibility Test (a standardized protocol to test whether particular bacteria are susceptible to a known concentration of certain antibiotics) and kits "MIC G-I, G-II" (Czech Republic). MIC G-I, G-II tests are designed to test the antimicrobial susceptibility of bacteria from the Enterobacteriaceae family based on the determination of the lowest concentration of certain antibiotics, which inhibits bacterial growth. Both kits are recommended to be used together to test susceptibility to antibiotics used in the treatment of serious infections, especially in hospitalized patients.

Isolation and identification of a pure culture of pneumonia pathogens were carried out using Micro-la-test kits (Czech Republic). The sensitivity of clinical strains of microorganisms to antimicrobial drugs was studied using a microtest system with semiquantitative registration of results "SENSILA test G-I, G-II" and Kirby-Bauer Disk Diffusion Susceptibility Test. Biofilms were grown in Petri dishes on glass coverslips.

*Scanning electron microscopy*

Samples were imaged with a field emission scanning electron microscope Vega 3 LMH (Tescan).

*Laser scanning confocal microscopy (LSCM)*

Intravital cell viability was assessed by LSCM after fluorescence staining nucleoid DNA with DAPI/PI-based «*Bacstain* Bacterial Viability Detection Kit» (Dojindo, Cat. No. BS08). DAPI dye (λEx – 405 nm, λEm – 461 nm) is a minor groove binder specific to the AT sequence of DNA and permeates into bacteria to stain nucleic acids regardless of membrane damage. PI (λEx – 493 nm, λEm = 636 nm) is a parallel intercalator into the DNA double helix that stains nucleic acid which passes only through damaged bacterial membranes. The samples were stained for 15 minutes in the dark. Bacteria-associated and extracellular polysaccharide matrix was label-free visualized by green autofluorescence (λEx – 488 nm, λEm – 532 nm). Live-cell imaging was conducted using an Olympus FV10i-LIV LSCM equipped with a 60/1.2 NA water immersion lens. Confocal images were acquired with a scanning mode format of 1024×1024 pixels. The pinhole aperture was 1 Airy unit. Z-reconstruction of serial single optical sections was performed with a scanning mode of 1024×1024 pixels with an electronic zoom at 2.0 and a Z stack of 0.2 μm/slice. The shown confocal images are representative images of ten fields of view in different regions of the coverslip. Post-rendering of the obtained images of optical sections was performed using Olympus excellence software (Olympus licensed).

The imaging was carried out in triplicates with three independent repetitions.

*Statistical data*

Data were compared using the Mann-Whitney test. If more than two variables were compared, the Dunn`s and Kruskal-Wallis tests were used. The difference between variables was considered statistically significant if *p* values did not exceed 0,005.

This study was approved by the meeting of the Department of Microbiology, Virology and Immunology named after D.P.Grynyov, Kharkiv National Medical University (Protocol No.2, 01/30/2024).

## Results

The biological material of 22 military (21 men), aged between 30-50 years was studied. The patients were hospitalized with a diagnosis of gunshot wound, bilateral community-acquired pneumonia, pulmonary and cardiac failure of the 3-4th degree. 27% of patients did not have the classic symptoms of pneumonia (fever, dyspnoea, cough), which led to a delay in diagnosis at the prehospital level. In two patients, the disease progressed from local infection (lung wound) to systemic infection with a spectrum of complications associated with the development of sepsis (SCAP). That required admission to the intensive care unit with prolonged artificial ventilation of the lungs for more than 7 days. These patients had a history of chronic obstructive pulmonary disease. All patients diagnosed with CAP received combined antibiotic therapy, mucolytics and detoxification therapy. It was a combination of 3-4th generation quinolones with 3-4th generation of cephalosporin. Upon transition to stage 4th of pneumonia, meropenem group of drugs was prescribed. SpO2 < 85-88% was an indication for oxygen therapy.

At the time of admission, 73% of patients had profuse sputum, but only 27% of them underwent bacterial examination of sputum in the prehospital phase (outpatient type of facility).

The bacteriological analysis of biological material in the case of CAP showed that gram-negative microorganisms (63.6%), more often *P. aeruginosa,* predominate among the detected pathogens.

During the study of biological samples of patients, 22 strains of microorganisms were detected (Figure 1). Non-fermenting gram-negative aerobic rod-shaped microorganisms *P. aeruginosa* were cultured in 31.8% of cases (in the amount of $1x10^{8-9}$ CFU, Figure 2). Gram-negative facultatively anaerobic bacteria *Klebsiella pneumoniae* was isolated in 18.2% of cases (in the amount of $1x10^{6-8}$ CFU). *Escherichia coli* was detected in 9.1% of cases (in the amount of $1x10^{4-8}$ CFU) and gram-negative, immobile facultatively anaerobic capnophilic pathogenic bacterium *Haemophilus influenzae* was found in 4.5% of cases (in the amount of $1x10^{8}$ CFU). Gram-positive bacteria were represented by:

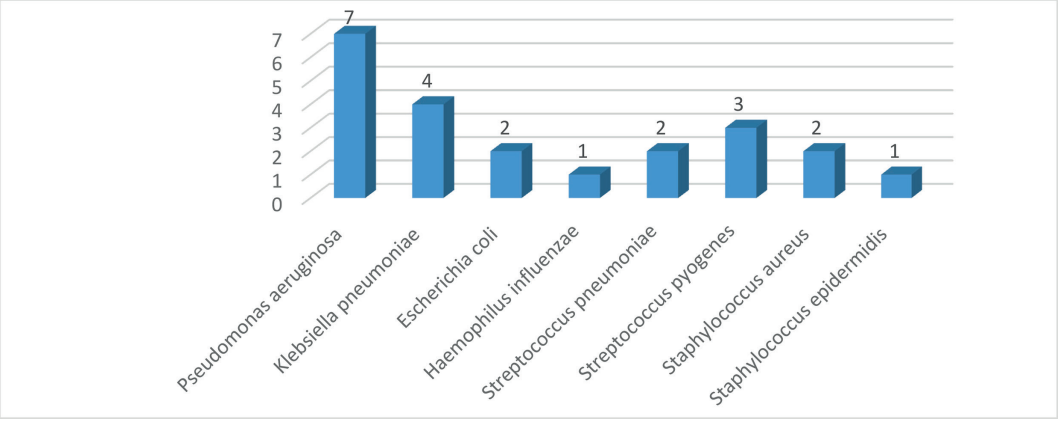


**Figure 1.** Composition of etiological factors (absolute number of strains) of community-acquired bacterial pneumonia from patients' biological samples.

**Figure 2.** Isolates of *P. aeruginosa.*

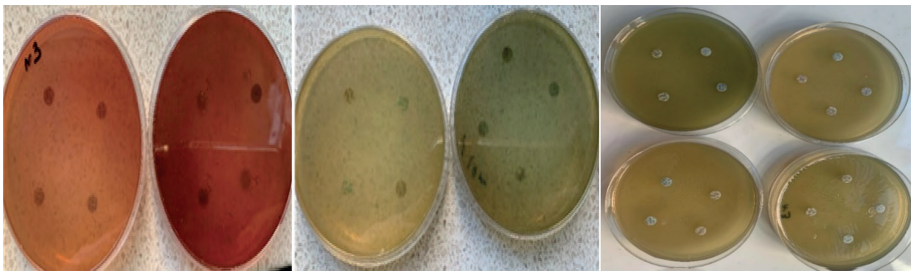

**Figure 3.** Determining the sensitivity of *P. aeruginosa* isolates to antimicrobial drugs by the Kirby-Bauer Disk Diffusion Susceptibility Test method.

*Streptococcus pyogenes* in 13.7% of cases (in the amount of $1x10^{6-9}$ CFU), *Staphylococcus aureus* (in the amount of $1x10^{5-8}$ CFU) and *Streptococcus pneumoniae* (in the amount of $1x10^{6-8}$ CFU) in 9.1% each, *Staphylococcus epidermidis* in 4.5% of cases (in the amount of $1x10^{6}$ CFU).

Determining the sensitivity of *P. aeruginosa* isolates to antimicrobial drugs showed that all isolates were resistant to: Amikacin (AMK), Ampicillin (AMP), Ampicillin/sulbactam (AMS), Cefalexin (CEX), Cefuroxime (CXM), Cefotaxime (CTX), Ciprofloxacin (CIP), Gentamicin (GEN), Trimetoprim/sulfamethoxazole (T/S), Aztreonam (AZT), Ceftazidime (CAZ), Ceftazidime/clavulanate (CZC), Cefepime (CEP), Meropenem (MER), Netilmicin (NET), Piperacilin/tazobactam (PIT), Tigecyclin (TGC) (Figure 3). For example, the aminoglycoside antibiotic (AMK) and quinolone (CIP) demonstrated reduced diffusion through *P. aeruginosa* biofilm models, similar to the effects of β-lactam antibiotics.

Studying the structural and functional features of biofilm formation by multi-resistant isolates of *P. aeruginosa* using scanning microscopy established that the adhesion of individual bacterial cells occurs with the subsequent formation of conglomerates surrounded by a matrix with the subsequent formation of a biofilm (Figure 4). The process of irreversible fixation lasts from 12 to 14 hours for poly-resistant strains of *P. aeruginosa* and from 14 to 18 hours for pan-resistant isolates. Generally, the process of primary biofilm formation lasts from 16 to 20 hours for poly-resistant strains of *P. aeruginosa* and from 18 to 24 hours for pan-resistant isolates from the beginning of adhesion.

Bacterial cells arranged in the form of dense elongated sticks were visible under the film. It was found that daily biofilms of *P. aeruginosa* isolates have a dense structure in the form of a gel.

With the help of luminescence microscopy, it were revealed dense areas of biofilm, in which the accumulation of cells with high luminescence was indicated (Figure 5).

As a result of the quantitative analysis of the conducted study, it was established that poly-resistant isolates of *P. aeruginosa* formed biofilms, the density of which was 2.48±0.14 optical density (OD) and in pan-resistant isolates it was 4.16±0.18 OD.

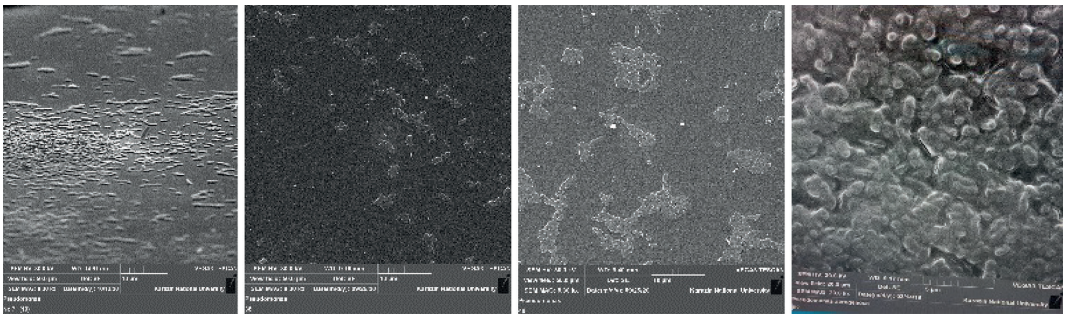

**Figure 4.** P. aeruginosa biofilm formation: I stage - adhesion of isolates; II stage - fixation; III stage - co-aggregation; IV stage - clustering.

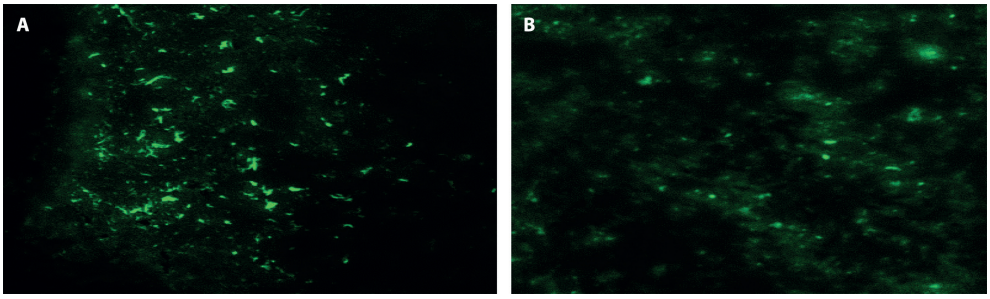


**Figure 5.** Ability of *P. aeruginosa* to form biofilms. A – biofilm of poly-resistant strains of *P. aeruginosa*; B – biofilm of pan-resistant strains of *P. aeruginosa*.

The LSCM analysis (Figure 6, Figure 7) depicts live bacteria with intact membranes in blue (DAPI blue-fluorescent nucleic acid stain) whereas, non-viable bacteria with damaged membranes incorporate propidium iodide (red-fluorescent nucleic acid stain) and are stained red. For analysis single slices (thickness 0.2 μm) of a confocal stack were provided. In 24 h-old biofilm indicated high viability (>95%) and uninhibited biofilm formation without membrane damage.

LSCM visualized *P. aeruginosa* cells forming a 24 h-old layer on the surface of the coverslip. Single slices (thickness 0.2 μm) of a confocal stack were provided. Composite image of green, blue and red emission bands: green fluorescence (polysaccharide matrix autofluorescence) was excited with 488 nm; blue fluorescence (nucleoid DNA+DAPI) was excited with 405 nm and red fluorescence (damaged cells nucleoid DNA+PI) was excited with 493 nm. Scale bar = 2 μm was used.

LSCM provided a three-dimensional reconstruction of a 24 hours-old biofilm layer of *P. aeruginosa* cells, created from a confocal stack of Olympus cellSence software (Figure 7). Composite image of green, blue and red emission bands: green fluorescence (polysaccharide matrix autofluorescence excited with 488 nm); blue fluorescence (nucleoid DNA+DAPI excited with 405 nm) and red fluorescence (fluorescence of the damaged nucleoid DNA+PI excited with 493 nm).

The primary functions of cell-associated and cell-free produced polysaccharide matrix are to hold the bacterial community together, fix bacterial cells to solid surfaces, and maintain proper hydration and nutrients availability. However, polysaccharide matrix plays a key role in the formation of biofilm and also in biofilm resistance to antimicrobial agents and protects bacteria from stresses like desiccation and oxidizing agents (42). The LSCM imaging demonstrated that biofilm-forming bacteria embedded in polymeric extracellular matrices that consist of thick polysaccharides layer.

## Discussion

In the management of CAP patients, assessment of severity is fundamental not only to assign the

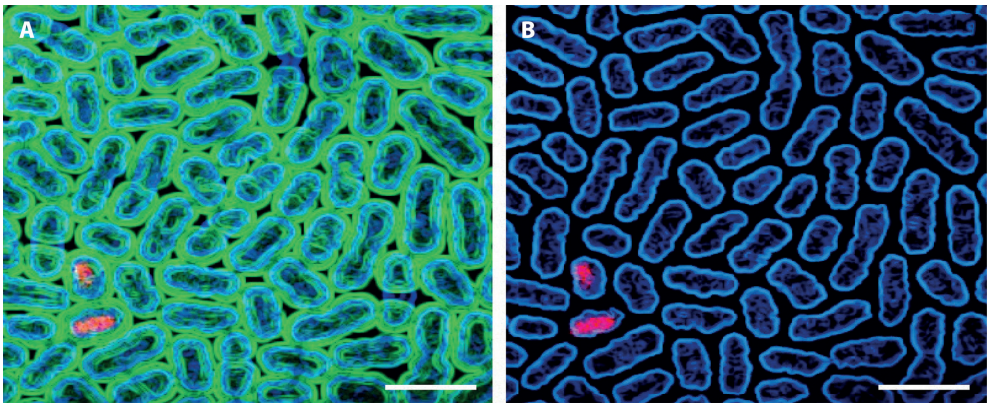


**Figure 6.** LSCM visualization of *P. aeruginosa* cells forming a 24 hours-old layer on the surface of the coverslip. A – multicolor imaging of bacterial polysaccharide matrix and nucleoids; B – bacterial nucleoid imaging.

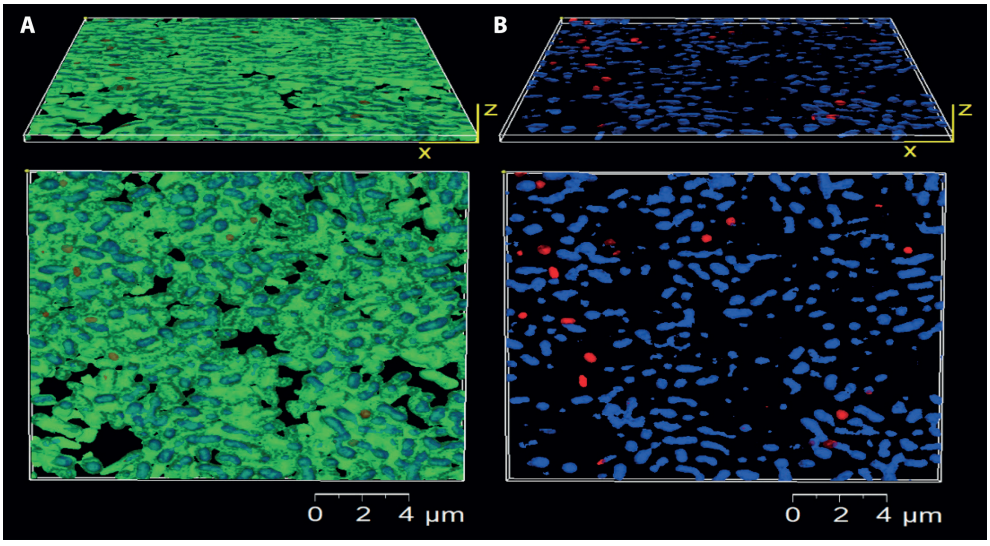


**Figure 7.** LSCM three-dimensional reconstruction of a 24 hours-old biofilm layer of *P. aeruginosa* cells. A – multicolor imaging of bacterial polysaccharide matrix and nucleoids; B –imaging of nucleoids only.

appropriate site of care but also to select empiric antibiotic therapy.

In the current study, the laboratory-microbiological aspects of *P. aeruginosa* biofilms formation as a factor in the development of antibiotic resistance were analyzed. These results of our research correlate with other data in the idea of *P. aeruginosa* biofilms formation as a factor in the development of antibiotic resistance (6, 15, 33).

The Center for Disease Control estimates that 65% of all human infections are caused by bacteria with biofilm phenotype and the National Institutes of Health estimates that this number is closer to 80% (43). Our study indicated the prevalence of *P. aeruginosa* (63.6%) among gram-negative microorganisms isolated from the biological material in patients with CAP. *P. aeruginosa* is characterized by high prevalence and fatality rates (22), equipped with a suite of virulence determinants and a complex regulatory network of intracellular and intercellular signals that allow the bacteria to adapt to antibacterial drugs and evade host defences (32). Multidrug- and totally- drug-resistant strains of *P. aeruginosa* are increasing threats that contribute to high mortality in these patients (22, 27).

The presence of bacterial biofilms complicates the treatment of infectious diseases, in particular pneumonia (17, 26). Biofilm-associated bacteria are involved in a wide range of infections, such as respiratory tract infections, chronically infected wounds, and medical device-associated infections (42). They also promote antibiotic resistance by blocking the access of antibiotics and host immune cells, leading to drug resistance in bacteria (34). The inability of antibiotics to kill biofilm bacteria has been classically attributed to a decreased ability of these agents to penetrate biofilms due to the mechanical and chemical properties of the extracellular matrix. A dense polysaccharide matrix of biofilm was visualized in our study using LSCM.

In this study, we found that all *P. aeruginosa* isolates were resistant to many groups of antimicrobials prescribed to patients. Furthermore, increased tolerance of *P. aeruginosa* biofilms to amikacin and ciprofloxacin was observed. Detection of β-lactam antibiotics effectiveness showed that the effectiveness against actively reproducing bacteria was inversely proportional to the activity of biofilm bacteria, confirmed by other data (22). It was shown that *P. aeruginosa* has high levels of intrinsic resistance to most antibiotics due to limited outer membrane permeability (27).

Respiratory tract infections are a major cause of morbidity and mortality worldwide. Chief among them are infections affecting the lower respiratory tract (25). The outcome of lower respiratory tract infection is also determined by the extent of immune defence and inflammation (26). Pneumonia is a type of acute lower respiratory infection that is common and severe, especially in cases caused by gram-negative microflora, as it is a direct cause of unhealthy aging, decline and death. Although pneumonia results from a microbial infection, the pathogenesis of this disease is found out by the host response (26). The mechanisms responsible for susceptibility must be better elucidated so they can be interrupted. Improved knowledge in these areas may lead to the development of additional comprehensive diagnostic and treatment methods through an in-depth study of mechanisms aimed at reducing mortality in patients with pneumonia.

It should be noted that in 2 cases a septic condition developed, which led to a fatal outcome: during the histological examination, it was found that the pleura was thickened, stratified in places infiltrated with leukocytes, there was an accumulation of leukocytes with fibrin threads on the surface, its vessels were full of blood. Areas of emphysema with thinning and rupture of interalveolar membranes alternate with atelectasis. In the lumens of the alveoli there is a diffuse purulent exudate, sometimes with fibrin impurities. Individual alveoli are collapsed, there is swollen pink content in the lumens with individual macrophages and a large number of clear erythrocytes. Interalveolar membranes are not defined in places, most of them are infiltrated with lymphocytes, plasma cells and alveolar macrophages. The development of sepsis in these patients, can be explained by the predominance of pan-resistant isolates of gram-negative aerobic rod-shaped microorganisms that are highly resistant to antibiotics. Our quantitative analysis showed that pan-resistant isolates of *P. aeruginosa* form biofilms, the OD of which exceeds the OD of biofilms of poly-resistant isolates. The possibility of developing sepsis in the setting of pneumonia is supported by scientific studies by other authors, which indicate that pneumonia is by far the most common cause of sepsis (44, 45).

Finally, information about antibiotic resistance is constantly changing and expanding, thus the survey problems about the development of antibiotic resistance in community-acquired pneumonia should be reconstructed for future research. A greater understanding of this issue will reveal new ways to improve the results of diagnosis and treatment of pulmonary infections.

## Conclusion

Studying the structural and functional features of biofilms formation by antibiotic-resistant *P. aeruginosa* isolates established that on the surface of conglomerates consisting of bacterial cells, biofilms are formed according to the classical stages. The first stage (adhesion or sedimentation) is the primary attachment of the pathogen to the substrate. The second stage (fixation) is the final irreversible attachment of cells of microorganisms to the surface. The third stage (co-aggregation) is characterized by the formation of microcolonies followed by the synthesis of a polymer matrix

and the formation of a monolayer and microcolonies. The fourth stage is the formation of clusters and a mature biofilm (after the fusion of microcolonies, a three-dimensional structure is formed that can change size and shape). The fifth stage is dispersion (destruction stage) which represents the release of bacteria or the loss of single fragments that spread through the organism and attach to the substrate with the formation of a new biofilm.

Dense biofilms determine the phenotypic variability of *P. aeruginosa* with the development of resistance to antimicrobial drugs, which is an obstacle to the use of antimicrobial therapy.

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**Ethic Committee:** Meeting of the Department of Microbiology, Virology and Immunology named after D.P.Grynyov, Kharkiv National Medical University (Protocol No. 2, 01/30/2024).

**Conflict of Interest:** Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

**Authors Contribution:** MM, AC, OB conceived and designed the study and wrote original manuscript. SM, MM, YM, IM, YK collected and organized the data, collected pictures, provided research materials. RP, AF conceived the idea, analyzed and interpreted data. All the Co-Authors contributed to the interpretation of data and provided critical comments on the manuscript for important intellectual content. All authors have critically reviewed, approved the final draft and are responsible for the content and similarity index of the manuscript.

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