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A multidisciplinary approach to the treatment of long-term-to-heal wounds: targeting the disruption of biofilms

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Abstract. ***Background.*** *The persistent problem of long-term non-healing wounds, particularly in patients with diabetic foot syndrome, is increasingly associated with the formation of polymicrobial biofilms, which significantly reduce the effectiveness of standard antimicrobial therapy. The present study purposed to investigate the molecular mechanisms of antibiotic resistance among key pathogens involved in wound infection and to assess the efficacy of photodynamic therapy (PDT) compared to conventional antimicrobial agents against biofilms in vitro.* ***Materials and methods.*** *A total of 66 clinical isolates were obtained from 41 patients with diabetic foot syndrome undergoing treatment at the V.T. Zaitsev Institute of General and Urgent Surgery of the NAMS of Ukraine. The cohort (mean age 61.0 ± 6.3 years) consisted of 58.5 % men and 41.5 % women. The microbial spectrum included Staphylococcus aureus (30 isolates) and various Gram-negative pathogens (36 isolates). Genetic markers of resistance were identified by multiplex polymerase chain reaction, and susceptibility phenotypes were determined using standard disk diffusion and minimum inhibitory concentration (MIC) assays. Biofilms were modelled on 96-well plates and 35-mm dishes. The efficacy of PDT (6% 5-aminolevulinic acid + red light 635 nm) was assessed in comparison with standard antibiotics.* ***Results.*** *Results demonstrated a high prevalence of resistance genes: CTX-M-2 (26.6 % in S.aureus, 16.7 % in Gram-negatives), VIM (5.6 %), NDM (2.8 %), VanA and VanB (6.7 % in S.aureus), Erm (16.6 % in S.aureus), Tet (23.3 % in S.aureus), QnrB (13.3 % in S.aureus). Phenotypic resistance was highest for metronidazole (52.3 %) and cephalosporins (22.7 %), with strong correlations between genetic markers and phenotypic results. MIC testing revealed that conventional antibiotics, while effective against planktonic cells, failed to eradicate mature biofilms. PDT exhibited significant antibiofilm activity against both planktonic and biofilm forms of S.aureus, E.coli, K.pneumoniae, P.aeruginosa, A.baumannii, and C.albicans. Combined biofilms (C.albicans + S.aureus; S.aureus + K.pneumoniae) showed limited response to combined antibiotic/antifungal therapy, whereas PDT induced notable structural disruption. Microscopy confirmed biofilm damage and planktonic dispersion post-PDT, likely mediated by singlet oxygen generation. These findings underscore the need for integrating PDT into the multidisciplinary management of chronic wound infections, especially where antibiotic resistance and biofilms are prominent.* ***Conclusions.*** *PDT represents a promising adjunctive modality for enhancing wound healing outcomes by effectively targeting biofilms. Future clinical studies are needed to validate these in vitro results and to optimize treatment protocols.*

Keywords: *diabetes mellitus; diabetic foot syndrome; long-term non-healing wounds; biofilms; photodynamic therapy; antimicrobial resistance*

Introduction

According to the US Centers for Disease Control and Prevention (CDC), more than 70 % of human infectious diseases are accompanied or mediated by the formation of biofilms [1]. Despite the fact that infectious diseases remain the leading cause of death worldwide, it is obvious that the use of antibiotics is the basis of etiotropic therapy for most diseases. Microorganisms living as biofilms exhibit characteristic features such as collective cooperation, source capture, and increased survival after antimicrobial treatment.

The appearance of antibiotics in the arsenal of medical therapy has allowed for a sharp reduction in mortality and a significant increase in people's life expectancy over the past 70 years. However, the growth of antibiotic resistance

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in recent years has drawn the attention of researchers to the phenomenon of biofilms as one of its leading causes [2, 3]. Five main types of resistance to antibiotics have been described for individual (planktonic) microbial cells: antibiotic inactivation, target modification, active removal of the antibiotic from the microbial cell (efflux), violation of the permeability of the outer membrane of the microbial cell, and the formation of a metabolic shunt [1]. Scientific information accumulated at this time allows us to see that almost all types of known planktonic resistance are present from microbes in biofilms. The effectiveness of penetration of antibiotics is largely related to their ability to overcome the surface membrane and intercellular matrix of biofilms. The composition of the latter contains a significant number of different lipids, qualitatively like membrane lipids [2]. However, in biofilms there are also special forms of resistance inherent only to them, which can be associated with at least three or even more types of mechanisms [3, 4].

Many human diseases and colonization of medical devices implanted in patients are associated with microorganisms growing in biofilms, and these microorganisms are highly resistant to antimicrobial treatments. Biofilm formation initiates the disease process through various mechanisms, such as detachment of individual bacterial cells or clusters of cell aggregates, production of endotoxins, increased evasion of host immune system surveillance, and establishment of a protective barrier, etc. Biofilms can be good, bad, or neutral: biofilms that are part of the natural environment are neutral, while biofilms that grow on open wounds after infection are harmful. Enhanced survival and evasion of the host immune system makes biofilms responsible for persistent chronic infections [5]. The complexity of biofilm activity and behavior requires interdisciplinary research to develop an effective solution against the destruction that can be caused by this structure [6, 7]. A biofilm is an ideal niche for the exchange of genetic information between bacteria. Antibiotic-resistant bacteria are able not only to secrete protective enzymes or proteins that can protect neighboring antibiotic-sensitive bacteria in the biofilm [8], but also to transfer genes responsible for antibiotic resistance to other, even unrelated, bacteria [9].

Understanding the fact that microorganisms in the composition of biofilms acquire new properties, which imply a significant decrease in sensitivity to standard methods of etiotropic therapy, which includes antibiotics, forces to investigate new methods of exposure. A multiple increase in the dosage of antibacterial agents is ineffective, because achieving effective concentrations in *in vivo* conditions is unattainable for obvious reasons. Therefore, a few approaches affecting the components of biofilms are considered to reduce their resistance/integrity level using a combination of antibacterial drugs [10] and antiseptics, organic acids and metal salts [11], microbial metabolites and bacteriophages [12], enzymes of various origin [13], mucolytics [14]. Methods that affect the matrix components, signal molecules, adhesion factors, micro- and nanotechnologies [11] are recognized as promising, and the variety of signal molecules as possible objects of influence justifies the use of combinations of hydrolytic enzymes with proteo-, lipo- and amylolytic activity and the possibility impact on nucleic acid-containing components of biofilms.

Despite the large arsenal of means for local application, the problem of treating long-term non-healing wounds is far from being resolved. In recent years, physical and chemical methods of wound treatment, used both separately and in combination, began to be used to solve this problem. A promising direction in the treatment of purulent and long-term non-healing wounds is the use of phototherapy (PT) and photodynamic therapy (PDT).

Currently, a new technology — PDT — is being intensively developed all over the world. This technique can be used in many different areas of medicine, including purulent surgery. PDT has undeniable advantages over traditional antibacterial therapy (ABT). The effectiveness of PDT does not depend on the spectrum of sensitivity of pathogenic microorganisms to antibiotics [3, 14]. Pathogenic microorganisms, in contrast to the effect of antibiotics on them, do not develop resistance to PDT. Photodynamic damage is local in nature, and the bactericidal effect is limited by the laser irradiation zone of photosensitized tissue, which makes it possible to avoid the side effect observed with the use of antibiotics and antiseptics for the treatment of surgical infection with local PDT. When using the PDT method, different effectiveness of the treatment was revealed depending on the bacterial flora sown from the wound contents. The need to study the effect of PDT on the course of the wound process caused by various groups of bacterial flora is obvious to all researchers in this field.

The aim of the study was to clarify the mechanisms of the formation of antibiotic resistance in the main causative agents of wound infection, as well as to compare the effectiveness of photodynamic therapy and antibiotics in relation to biofilms *in vitro*.

Materials and methods

Clinical isolates of pathogens were obtained from the wounds of 41 patients with ischemic and mixed forms of diabetic foot syndrome (DFS), who were treated in the department of acute vascular diseases Zaitsev Institute of General and Urgent Surgery of the NAMS of Ukraine in 2022–2025. All of them suffered from type 2 diabetes and had chronic ischemia of the lower limb with localization of ulcerative-necrotic lesions of soft tissues in the foot. The average age of patients in the study was 61.0 ± 6.3 years. Gender data: 24 (58.5 %) — men, 17 (41.5 %) — women. The duration of the disease is ≥ 5 and ≤ 15 years, the duration of the wounds is from 3 months to 1 year. All patients received signed informed consent, and the Ethics Committee approved the study. The material for the study was wound contents.

To take the material, a standard sterile paper endodontic pin was used, which was placed at the edge of the wound for 30 seconds to absorb the liquid part, and then transferred to an Eppendorf-type test tube with 0.5 ml of Amies or Stewart semi-liquid transport medium. Transportation was carried out in special thermal containers at a temperature of no higher than –4 °C for 12 hours (as a rule, 3–4 hours).

The total number of strains isolated from wounds in patients with diabetes with chronic limb wounds and subjected to molecular biological research was 66, 30 strains were *S.aureus* and the other 36 were Gram-negative pathogens.

Reference strains *Staphylococcus aureus* ATCC 25923 — 4, *Candida albicans* NCTC 885-653 and *Candida krusei* ATCC 24408, *E.coli* ATCC2592, *K.pneumoniae* ATCC70060, *Pseudomonas aeruginosa* ATCC27853, *Acinetobacter baumannii* ATCC19606 were used in control experiments.

For quantitative 4-sector sowing, the Melnikov-Tsarev method was used on 5% blood hemin agar (5 μg/ml hemin, 0.1 μg/ml menadione). Subsequent counting of colonies after standard cultivation (for 7 days at 37 °C) was performed with a binocular research stereomicroscope (Nikon, Japan). Microbial number (results of quantitative research) was expressed through colony-forming units (CFU/ml), or their decimal logarithm. A complex of morphological, cultural, biochemical, and chemotaxonomic features was used to identify selected cultures. Determination of the species affiliation of pure cultures of Gram-negative bacteria based on the assessment of biochemical properties was also carried out using diagnostic kits from ARI (France) and Roche (Germany).

To control the phenotype of sensitivity to antibacterial drugs, the traditional Kirby-Bauer disk diffusion method was used, and to determine the minimum inhibitory concentration (MIC) of the studied drugs the cassette micromethod was used.

To determine the sensitive phenotype the standard protocol of the disco-diffusion method was carried out. Sowing of the culture under study was carried out with "lawn" at a suspension concentration according to the McFarland turbidity standard of 108 CFU/ml. Standard agar was used to determine the sensitivity of HiMedia Labs (India). The results were recorded after incubation in a thermostat at 37 °C for 24 hours. An automatic colony counter Scan 500 (Interscience) with computer support for data registration was used for quick recording of results and documentation of the obtained data.

When using disk-diffusion and cassette micromethods, a research stereomicroscope (Nikon) was also used to record data.

Along with the antimicrobial drugs most often used in the treatment of wounds, the effect on biofilms was assessed by PDT using 5-aminolevulinic acid (5-ALA) as a photosensitizer (PS): 6% 5-ALA phosphate gel was applied to plastic cups with biofilms, and then 2-time dark reaction was treated with pulsed LED lamps with a wavelength (λ) of 635 nm (which corresponded to red light) Treviolux and SmartComfort lamps (Germany). All LED devices had a peak radiation intensity of 80 mW/cm^2, which was measured using a USB 2000 spectrometer (Ocean Optics, Florida, USA). The study period is zero day.

Biofilms were modeled as follows. The bacterial culture was sown on agar slants and incubated in a thermostat for 24 hours at a temperature of 37 °C. Washing from the agar culture was carried out by adding 1 ml of saline solution and adjusted to the McFarland density standard. The formation of biofilms on the bottom of plastic plates with 96 wells was carried out as follows: 150 μl of nutrient medium (nutrient broth for *S.aureus* and Gram-negative pathogens, Sabouraud medium for *C.albicans*) and 10 μl of culture were added to each well except for the last hole, which constituted benchmarks. Tablets were incubated in a thermostat at 37 °C for 24 hours. The next day, the contents of the wells were collected and washed three times with saline solution. 150 μl of distilled water and 15 μl of 1% alcohol solution of gentian violet were added to the wells and incubated at room temperature for 45 minutes. The dye was removed, and the wells were washed three times with distilled water. Next, 250 μl of ethanol was added to the wells and incubated at room temperature for 45 minutes. After that, the optical density of biofilms was measured at a wavelength of 545 nm on a SF-46 photometer.

Also, the formation of biofilms was carried out in plastic cups with a diameter of 35 mm, on the bottom of which a sterile cover glass was placed. 18th-hour culture of strains diluted 1 : 100 was introduced in 4 ml so that the suspension was evenly distributed on the bottom of the cup.

To determine control parameters, a sterile nutrient was placed into several cups. The cups were placed in a thermostat at 37 °C for 24 hours. The next day, the contents of the cups were carefully selected so as not to destroy the formed biofilm, 4 ml of distilled water and 400 μl of 1% alcohol solution of gentian violet were added and left at room temperature for 45 minutes. After that, the solution was carefully removed, and the cups were washed three times with distilled water. The cups were dried, the former biofilms were studied under a microscope and photographed.

Genetic markers of bacteria and fungi were determined using multiplex polymerase chain reaction (PCR). Accelerated sample preparation was used for DNA isolation. Gel electrophoresis in 1.6% agarose gel with ethidium bromide staining was used to detect cloned DNA samples. An ultraviolet transilluminator TSR-25 M (Vilber Lourmat, France) with a wavelength of 312 nm was used to view and photograph the gels. In the positive control sample, 5 luminous bands of pink color were determined, measuring 1000, 745, 512, 360, 197 bp, in the negative control sample these bands were absent. In the studied samples, with a positive result of PCR, from one to five luminous bands were determined, located according to the size of the genome at the same level as the bands in the positive control.

DNA detection was performed by electrophoresis. Separation of amplification products was carried out by horizontal electrophoresis (in 2% agarose gel). A TSR-25 M transilluminator (Vilber Lourmat, France) with a radiation wavelength of 312 nm was used for viewing and photographing gels. In the studied samples, the genes of the chromosomal regions responsible for the formation of resistance to antibacterial drugs were identified: Mes (to cephalosporins), VanA (to glycopeptides), VanB (to vancomycin), OXA-48, NDM and VIM (to carbapenems), plasmid genes were identified Qnr A and B. To document the obtained results, the gels were photographed in transmitted ultraviolet light with a digital video camera connected to a computer (Gel Imager software that allows video image analysis).

Optical density was evaluated on a Dynex DynaRead microplate reader. The amount of the formed biofilm (expressed in the form of optical density (OD) at 570 nm.

Light microscopy was carried out on a research stereomicroscope (for studying colonies of microorganisms grown on nutrient media) and an Invitrogen EVOS digital color fluorescence microscope using an immersion lens. This microscopic technique made it possible to take photographs of preparations of the research material (smears of pure cultures, colonies, and biofilms) on a digital camera included in the microscope kit.

Research was carried out in the biotechnological laboratory of the Ukrainian Association of Biobanks (Austria, Graz).

All received digital data were processed using the Epi Info computer program (version 8.0) using the variational statistics method, as well as the Excel program from the Microsoft Office 2007 and 2010 service package. Numerical data were presented as arithmetic mean and standard error (M ± m). The null hypothesis for comparison groups was tested using non-parametric Wilcoxon-Mann-Whitney (U) and Kolmogorov-Smirnov tests. Differences between groups were considered statistically significant at $p < 0.05$.

Results

We managed to identify genetic markers of resistance to beta-lactam antibiotics (CTX-M and MecA to cephalosporins), including carbapenems (VIM and NDM, but not OXA-48), as well as to glycopeptides (VanA and VanB), macrolides (Erm), tetracyclines (Tet) and plasmids Qnr (A, B) — to fluoroquinolones (Table 1).

The gene CTX-M-2, responsible for resistance to cephalosporins-1, was detected most often in representatives of causative agents of wound infections. It was detected in 8 out of 30 strains of *S.aureus* (26.6 %) and in 6 out of 36 strains of Gram-negative pathogens (16.7 %). The differences were statistically significant ($p = 0.026$).

The second gene controlling resistance to cephalosporins, Mec-1, was detected less often: in 3 out of 30 strains of *S.aureus* (frequency 10 %), and in 2 out of 36 strains of Gram-negative bacteria (frequency 5.5 %). The differences were statistically significant ($p = 0.031$).

It is known that a higher level of pathogen resistance is associated with carbapenem resistance genes, which can be divided into several groups. The VIM gene was detected in 1 strain of *Pseudomonas aeruginosa* and 1 strain of *Acinetobacter baumannii* (frequency 5.6 %). The second gene encoding type 2 carbapenem resistance, NDM, was identified in 1 strain of *K.pneumoniae* and in no case in *Acinetobacter baumannii*. The third gene of this group (OXA-48) was not identified in any case.

Resistance to glycopeptide antibiotics (vancomycin, teicoplanin), coded by genes VanA and VanB, was revealed in two strains of *S.aureus* (6.7 %).

The Erm gene, encoding resistance to macrolides, was detected in 5 strains of *S.aureus* (16.6 %) and in 4 strains of Gram-negative pathogens (11.1 %). The differences are statistically significant ($p = 0.05$).

The Tet gene, encoding resistance to tetracyclines, was detected in 7 strains of Gram-positive pathogens out of 30 (23.3 %) and in 4 (11.1 %) of Gram-negative pathogens. The differences are statistically significant ($p = 0.025$).

Plasmid resistance to QnrB fluoroquinolones was revealed in 4 strains of *S.aureus* (13.3 %) and in 2 strains of *Klebsiella pneumoniae* (5.5 % of the total number of Gram-negative pathogens). Differences ($p = 0.025$).

Among the analyzed cultures were strains with multiple resistance to groups of antibacterial drugs: *Klebsiella pneumoniae* — 1 strain out of 5 (14.3 %), *S.aureus* — 4 out of 5 (13.3 %), *Pseudomonas aeruginosa* — 2 (28.6 %) strain and *Acinetobacter baumannii* — 1 (11.1 %) strain — to three groups of drugs.

We compared the results of the phenotypic determination of resistance to antibacterial drugs by the disk-diffusion method and the detection of the corresponding resistance genes using PCR, which allowed us to trace the relationship between the frequency of occurrence of individual genetic markers of resistance and the results of the standard method of determining the sensitivity of microorganisms that form biofilms on wounds.

The following discs were used for this purpose: metronidazole, lincomycin, methicillin (ampicillin), amoxicillin + sodium clavulanate, spiramycin (erythromycin), vancomycin, tetracycline (doxycycline), imipenem, cipro- and moxifloxacin (Table 2).

Table 1. Frequency of specific genetic markers of resistance to antimicrobial drugs in causative agents of wound infection, n (%)

Type of pathogen	Genetic markers								
	CTX-M-2	MecA	VIM	NDM	VanA	VanB	Erm	Tet	Qnr (A, B)
S.aureus	8 (26.6)	3 (10)	–	–	2 (6.7)	2 (6.7)	5 (13.3)	7 (23.3)	4 (13.3)
Pseudomonas aeruginosa	2 (10)	–	1 (5)		–	–	1 (5)	2 (10)	
K.pneumoniae	2 (28.6)	1 (14.3)	–	1 (14.3)	–	–	2 (28.6)	1 (14.3)	2 (28.6)
Acinetobacter baumannii	2 (22.2)	1 (11.1)	1 (11.1)	–	–	–	1 (11.1)	1 (11.1)	

Table 2. The frequency of isolation of resistant and sensitive strains of wound infection pathogens (absolute number of strains, %)

Disks with drugs	Frequency of resistant strains R	Frequency of sensitive strains S	Ratio R/S
Metronidazole	23 (52.3)	6 (13.5)	3.9*
Ceftriaxone	10 (22.7)	12 (27.3)	0.8
Methicillin	5 (11.4)	27 (61.4)	0.2*
Amoxicillin + sodium clavulanate (amoxiclav)	6 (13.6)	40 (90.9)	0.03*
Vancomycin	10 (22.7)	28 (63.6)	0.4*
Doxycycline	2 (4.6)	37 (81.8)	0.06*
Imipenem	2 (2.3)	41 (95.5)	0.02*
Ciprofloxacin	6 (13.6)	28 (63.6)	0.2*
Moxifloxacin	2 (4.6)	30 (68.2)	0.07*
Spiramycin	6 (13.6)	24 (54.5)	0.3*

Note: * — differences between groups were considered statistically significant at p < 0.05.

It is obvious that the most frequently used (over the past 20–30 years) in surgical practice (metronidazole and cephalosporins) showed the highest number of resistant strains — 52.3 and 22.7 %, respectively.

The number of methicillin-resistant strains was 11.4 %, which correlated with the detection of CTX-M-2 resistance genes in 16.7 % of strains (r = 0.789; p = 0.032). When using beta-lactamase-protected drugs (amoxiclav), the rate of resistant strains decreased to 2.3 %, which correlated with the detection of the Mec-1 resistance gene in 5.5 % of strains (r = 0.648; p = 0.022).

Resistance to carbapenems was revealed in only 2.3 % of strains, and the VIM gene in 2.8 %, that is, a high direct correlation dependence was shown (r = 0.799; p = 0.01).

Resistance to macrolides was detected in 13.6 % of strains, and the Erm gene in 11.1 % of strains, that is, a high direct correlation dependence was also shown (r = 0.764; p = 0.01).

Resistance to tetracyclines was detected in only 4.6 % of strains, and the Tet gene in 11.1 % of strains, that is, no statistically significant correlation dependence was detected (r = 0.234; p = 0.052).

Resistance to glycopeptides was detected in 13.6 % of strains, and VanA and VanB genes in 3.3 % of strains, that is, no correlation was also detected (r = 0.242; p = 0.056).

Resistance to fluoroquinolones of different generations (ciprofloxacin and moxifloxacin) was revealed in 13.6 and 4.6 % of strains, respectively, and QnrB plasmids in 5.5 % of strains, while a direct high correlation was established for moxifloxacin (r = 0.785; p = 0.01).

The next stage of the study was a comparison of the effect of antimicrobial agents on biofilms of isolates and reference strains of causative agents of wound infection by the IPC method.

To determine the MIC of biofilms of isolates and reference strains and pathogens, the following antimicrobial drugs were taken: broad-spectrum beta-lactam antibiotic

Table 3. Indicators of optical density of MIC of antimicrobial drugs on biofilms of isolates and reference strains, c.u.

Antimicrobial agents	Average optical density of isolates λ = 570 nm/molecular mass (× 10^{-6})	Average optical density of reference strains λ = 570 nm/molecular mass (× 10^{-6})
S.aureus		
Imipenem	0.1484 ± 0.0600/0.10	0.1437 ± 0.0500/0.10
Vancomycin	0.1348 ± 0.0300/0.10	0.1229 ± 0.1000/0.10
Ceftriaxone	0.1635 ± 0.0500/0.10	0.1587 ± 0.0700/0.10
Azithromycin (sumamed)	0.1556 ± 0.0900/0.10	0.1486 ± 0.1400/0.10
Levofloxacin	0.1617 ± 0.1400/0.10	0.1557 ± 0.0900/0.10
PDT	0.2006 ± 0.0800/0.01	0.2004 ± 0.1100/0.01
E.coli		
Imipenem	0.1486 ± 0.0800/0.10	0.1333 ± 0.0500/0.10
Vancomycin	–	–
Ceftriaxone	0.1615 ± 0.0500/0.10	0.1593 ± 0.0500/0.10
Azithromycin (sumamed)	–	–
Levofloxacin	0.1603 ± 0.1200/0.10	0.1548 ± 0.0700/0.10
PDT	0.1950 ± 0.0500/0.01	0.1941 ± 0.0600/0.01
K.pneumoniae		
Imipenem	0.1478 ± 0.0500/0.10	0.1425 ± 0.0700/0.10
Vancomycin	–	–
Ceftriaxone	0.1633 ± 0.0300/0.10	0.1513 ± 0.0100/0.10
Azithromycin (sumamed)	–	–
Levofloxacin	0.1603 ± 0.1400/0.10	0.1543 ± 0.0700/0.10
PDT	0.2003 ± 0.0400/0.05	0.2001 ± 0.0500/0.05
Pseudomonas aeruginosa		
Imipenem	0.1496 ± 0.0800/0.10	0.1427 ± 0.0700/0.10
Vancomycin	–	–
Ceftriaxone	0.1695 ± 0.7000/0.10	0.1578 ± 0.0300/0.10
Azithromycin (sumamed)	–	–
Levofloxacin	0.1629 ± 0.1600/0.10	0.1533 ± 0.0300/0.10
PDT	0.1970 ± 0.0700/0.04	0.1965 ± 0.0400/0.05
Acinetobacter baumannii		
Imipenem	0.1484 ± 0.0600/0.10	0.1437 ± 0.0500/0.10
Vancomycin	–	–
Ceftriaxone	–	–
Azithromycin (sumamed)	–	–
Levofloxacin	0.1517 ± 0.1400/0.10	0.15217 ± 0.09000/0.10
PDT	0.1950 ± 0.0300/0.03	0.1953 ± 0.0800/0.02

Table 4. Antimicrobial drugs used to determine the effect on planktonic cells and biofilms of causative agents of wound infection

Antimicrobial agents	Concentration (μg/ml)
Imipenem	5
Vancomycin	10
Ceftriaxone	16
Azithromycin (sumamed)	16
Levofloxacin	16

Table 5. Antimicrobial agents used to determine the effect on planktonic cells and biofilms of C.albicans

Antimicrobial agents	Concentration (μg/ml)
Terbinafine	64
Ketoconazole	32
Fluconazole	32
Amphotericin B	10

from the carbapenem group — imipenem, glycopeptide antibiotic — vancomycin, 3rd generation cephalosporins — ceftriaxone, semi-synthetic macrolide — azithromycin, fluoroquinolone 3rd generation levofloxacin.

Biofilms of isolates and reference strains of wound infection pathogens were grown in 96-well plastic tablets, MIC was determined by the method of serial dilutions. The optical density of biofilms and reference cultures treated with FS and irradiated with red light and treated with probiotics was also evaluated. It should be noted that after processing the tested samples with probiotics, the effect was obtained after 72 hours.

The results are given in Table 3.

Analyzing the results of the study, it was established that the effectiveness of antimicrobial drugs on biofilms of isolates and reference strains of *S.aureus* is absent. For vancomycin, the MIC was determined to be 10 μg/ml, when using ceftriaxone, the MPC was 16 μg/ml, for sumamed, the MIC was 10 μg/ml, for fluoroquinolones — 16 μg/ml. PDT was effective in relation to biofilms and reference strains of *S.aureus* (optical density 0.2006 ± 0.0800 and 0.2004 ± 0.1100, respectively).

A similar trend was observed in relation to Gram-negative strains of causative agents of wound infection. Thus, the reference strains of *E.coli* in biofilms were resistant to the studied antimicrobial agents, while the MIC for cephalosporins was determined as 1.0 μg/ml, and for carbapenems — 0.1 μg/ml. High efficiency in relation to biofilms was shown by PDT (0.1950 ± 0.0500 and 0.1941 ± 0.0600 units of OSH for the studied and reference strains, respectively).

For *K.pneumoniae*, the MIC to carbapenems was 12 μg/ml, for cephalosporins and fluoroquinolones — 20 μg/ml. PDT showed a high efficiency in relation to biofilms (0.2003 ± 0.0400 and 0.2001 ± 0.0500 units of OSH for the studied and reference strains, respectively).

Regarding non-fermenting Gram-negative (GNB) pathogens of wound infection (*Pseudomonas aeruginosa* and *Acinetobacter baumannii*), biofilms of the pathogens were resistant to the studied antimicrobial agents. FS treatment with subsequent irradiation (PDT) proved to be effective (Table 4).

The next stage of the study was the determination of the MIC of planktonic cells and biofilms of isolates and reference strains of *C.albicans*.

To determine the MIC of planktonic cells and biofilms of isolates and reference strains of *C.albicans*, the following antimicrobial drugs were taken: antifungal drugs terbinafine, ketoconazole, fluconazole, and amphotericin B (Table 5), and the effect of PDT was also studied.

As a result of the study, it was established that the effect of antimicrobial drugs on planktonic cells differed between isolates and reference strains of *C.albicans*.

When determining the effect of the drug terbinafine, the average optical density for *C.albicans* isolates was 0.0747 ± ± 0.0900 units of OSH, and 0.0702 ± 0.0700 for reference strains, which has a high efficiency in relation to *C.albicans*, the MIC was 8 μg/ml for isolates and 2 μg/ml for reference strains.

The average optical density when determining the action of ketoconazole was 0.0844 ± 0.0400 IU for isolates and 0.0812 ± 0.1100 IU for reference strains, such data indicate a high efficiency, but the drug is less effective in comparison with terbinafine, BMD was 4 and 1 μg/ml, respectively. The effect of fluconazole was less effective than that of terbinafine and ketoconazole.

Amphotericin B turned out to be a drug with high efficiency, so the average optical density for *C.albicans* isolates was 0.0714 ± 0.0400 c.u. for reference strains 0.0698 ± 0.1500 c.u., MIC for isolates was 1.2 μg/ml.

A moderately effective effect of PDT was found (the average optical density for the isolates was 0.1034 ± 0.0600 c.u. and 0.0934 ± 0.0300 c.u.).

The MIC of antimicrobial drugs on *C.albicans* biofilms was also determined (Table 6).

As a result of the research, it was established that the antimicrobial drugs, the effect of which was studied on *C.albicans* biofilms, were ineffective in comparison with planktonic cells in the same concentrations.

Moderate efficacy against biofilms formed by *C.albicans* was noted after treatment of biofilms with 5-ALA followed by red light irradiation, as well as after treatment with a combination of probiotics.

Considering that many wound infections are caused by associations of microorganisms, and the effect of chemothe-

Table 6. Indicators of optical density MIC of antimicrobial agents on biofilms of isolates and reference strains of C.albicans, c.u.

Antimicrobial agents	Average optical density of isolates λ = 570 nm/molecular mass (× 10^{-6})	Average optical density of isolates λ = 570 nm/molecular mass (× 10^{-6})
Terbinafine	0.1475 ± 0.0700/0.10	0.1456 ± 0.0400/0.10
Ketoconazole	0.1440 ± 0.1200/0.10	0.1390 ± 0.0800/0.10
Fluconazole	0.1435 ± 0.0400/0.10	0.1421 ± 0.0100/0.10
Amphotericin B	0.1293 ± 0.0900/0.10	0.1290 ± 0.0500/0.10
PDT	0.0933 ± 0.0130/0.06	0.0888 ± 0.0600/0.05

rapeutic drugs is ineffective against these microorganisms, the next stage of our research was to determine the combined effect of antimicrobial drugs on two-component consortia (*C.albicans* + *S.aureus* and *S.aureus* + *K.pneumoniae*) of biofilms of reference strains and investigated pathogens (Table 7).

According to the results of the research, we can conclude that the combination of antimycotics with antibacterial drugs was ineffective in relation to the two-component biofilm of *C.albicans* + *S.aureus*. Similar data were obtained regarding the effect of antimicrobial drugs on the combined biofilms of *S.aureus* + *K.pneumoniae*. Moderate bactericidal activity was determined after treatment of FS consortia followed by pulsed irradiation with red light.

Microscopic examination of combined microbial biofilms showed that after treatment with FS and irradiation with red light, the structure of the biofilm was significantly damaged, single planktonic bacteria were visible in the field of view. The bactericidal effect and destruction of the biofilm occurs probably due to the emission of singlet oxygen (Fig. 1).

Discussion

Traditional methods of treatment of chronic wounds may be ineffective in their healing, which requires the use of a multidisciplinary approach to eliminate a complex of factors that contribute to non-healing of wounds. Comprehensive treatment of non-healing wounds requires the work of a multidisciplinary team consisting of wound care specialists, surgeons, nurses, nutritionists and other health professionals to address the complex factors that contribute to non-healing wounds. Effective debridement of the wound is the most important stage that promotes healing [6, 13]. Traditional methods, such as surgical debridement or enzymatic preparations, are still valuable, but new techniques have also appeared. These include biological therapy, in which sterile larvae are applied to the wound for selective removal of necrotic tissues, autolytic debridement with the use of dressings that promote natural enzymatic breakdown of devitalized body tissues.

Biological treatment is selective treatment using larvae as living medical devices. Advances in the development of dressings have revolutionized the treatment of wounds that do not heal for a long time. Modern dressings, such as hydrogels, foams, alginates and films, provide a moist environment in the wound, facilitate autolytic debridement and facilitate the healing process. In addition, the use of bioactive dressings containing growth factors, extracellular matrix components, or antimicrobial agents may further improve healing outcomes [14–16]. Negative pressure wound therapy (NPWT) has become a valuable tool in the treatment of

Table 7. Indicators of optical density of IPC of antimicrobial agents on combined biofilms of isolates and reference strains of pathogens of wound infection, c.u.

Antimicrobial agents	Average optical density of isolates λ = 570 nm/molecular mass (× 10^{-6})	Average optical density of reference strains λ = 570 nm/molecular mass (× 10^{-6})
C.albicans + S.aureus		
Imipenem + amphotericin B	0.1456 ± 0.0700/0.10	0.1362 ± 0.0700/0.10
Vancomycin + amphotericin B	0.1464 ± 0.0800/0.10	0.1401 ± 0.0300/0.10
Ceftriaxone + amphotericin B	0.1474 ± 0.0500/0.10	0.1406 ± 0.0500/0.10
Sumamed + amphotericin B	0.14326 ± 0.03000/0.10	0.1386 ± 0.0300/0.10
Levofloxacin + amphotericin B	0.1464 ± 0.0500/0.10	0.1408 ± 0.0100/0.10
PDT	0.1416 ± 0.0500/0.08	0.1418 ± 0.0700/0.07
S.aureus + K.pneumoniae		
Imipenem	0.1906 ± 0.0100/0.96	0.1904 ± 0.0300/0.96
Vancomycin	0.1904 ± 0.0300/0.96	0.1902 ± 0.0500/0.96
Ceftriaxone	0.1908 ± 0.0500/0.96	0.1906 ± 0.0700/0.96
Azithromycin (sumamed)	0.1911 ± 0.0100/0.96	0.1908 ± 0.0100/0.96
Levofloxacin	0.1904 ± 0.0300/0.96	0.19602 ± 0.05000/0.96
PDT	0.1886 ± 0.0300/0.96	0.1882 ± 0.0700/0.96

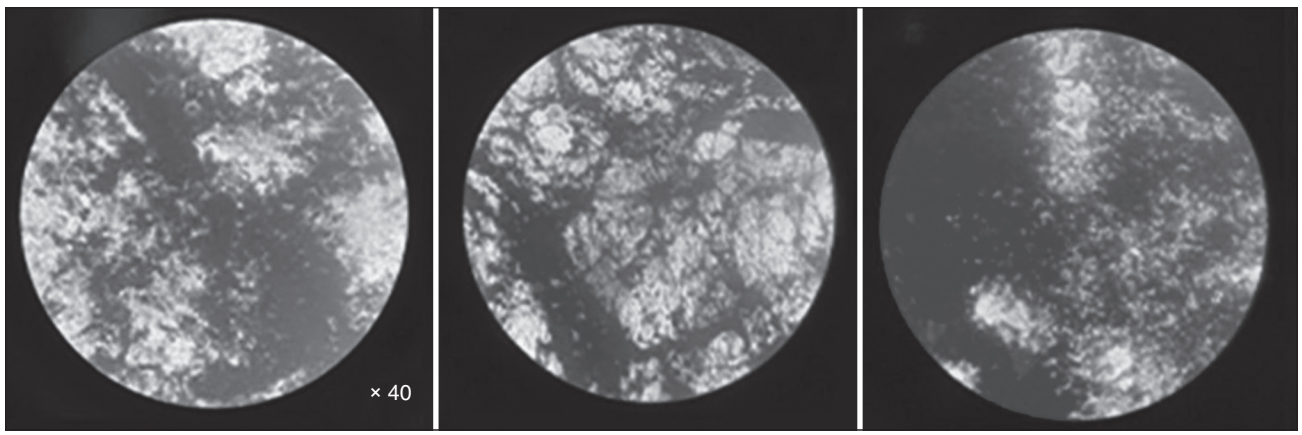

Figure 1. Combined biofilm of A.baumannii + E.coli under the influence of PDT

chronic non-healing wounds. This therapy consists in the application of controlled negative pressure to the wound, which promotes blood flow, reduces edema and stimulates the formation of granulation tissue [15]. To optimize the results of wound healing, NPWT can be combined with modern bandages, local cryotherapy, cell and tissue therapy [17–19]. However, these methods do not have a significant effect on the microbial flora, especially in the composition of biofilms, which requires the systemic appointment of antimicrobial therapy.

According to the results of the first stage of our research, it was established that for most prescribed antibacterial drugs (beta-lactams, carbapenems, macrolides, fluoroquinolones), a reliable relationship was established between the presence of genes encoding resistance and the results of the phenotypic method of determining sensitivity. Cases of its absence (tetracyclines, glycopeptides) can be explained by the presence of additional genetic resistance markers that were not considered in our study, since in addition to the Tet, VanA and VanB genes responsible for resistance to these antibiotics, it may be encoded by other chromosomal genes and plasmids.

The proposed justification will allow to optimize the existing schemes for the use of antibacterial drugs and the use of alternative methods in the complex treatment of wounds, which is currently carried out empirically and therefore does not always achieve the desired effect.

On the basis of the conducted second and third stages of the study, it can be concluded that the concentrations of antimicrobial drugs used in the experiment are effective against planktonic cells causing wound infection and ineffective against biofilms. Thus, in order to achieve an effective effect of drugs on biofilms, it is necessary to increase their concentration by several times, which is possible in vitro, but impossible in clinical practice. PDT showed high efficiency both in relation to planktonic forms and pathogens in biofilms.

The effect of antimicrobial drugs on planktonic cells of reference strains of *C.albicans* is higher than the effect on isolates, which is associated with the loss of their pathogenic properties. The most effective drugs were amphotericin B and terbinafine, less effective were ketoconazole and fluconazole, possibly due to the emergence of resistance to them. The indicators of the effect of PDT were lower compared to the effect of antifungal chemotherapeutic drugs, but had a sufficient effect, which may be related to their high antimicrobial activity. Antifungal drugs were ineffective against *C.albicans* biofilms at concentrations that were effective against planktonic cells. PDT demonstrated moderate efficiency.

Thus, the PDT technique showed sufficiently high activity in relation to the main causative agents of wound infection both in plankton and in the composition of biofilms and consortia.

Conclusions

Genetic resistance determinants such as CTX-M-2 (26.6 %), Tet (23.3 %), and Erm (16.6 %) were prevalent among chronic wound pathogens, especially in *S.aureus* and Gram-negative bacteria.

There was a statistically significant correlation between resistance genes and phenotypic resistance to β-lactams, macrolides, and carbapenems ($r > 0.75$; $p < 0.05$).

Conventional antibiotics were ineffective against biofilms at clinically safe concentrations. MIC values effective for planktonic cells did not significantly reduce biofilm biomass.

PDT significantly reduced biofilm mass in both mono- and multispecies cultures:

— for *S.aureus*: OD reduced to 0.2006 ± 0.0800;

— for *C.albicans*: OD reduced to 0.0933 ± 0.01300;

— for mixed *S.aureus* + *K.pneumoniae*: OD to 0.1886 ± 0.0300.

PDT is a promising adjunctive treatment for chronic, non-healing, biofilm-associated wound infections and may enhance clinical outcomes in multidisciplinary care.

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Мультидисциплінарний підхід до лікування хронічних ран, що погано загоюються: приціл на руйнування біоплівок

Резюме. ***Актуальність.*** Проблема довготривалих незагоєних ран, особливо в пацієнтів із синдромом діабетичної стопи, все частіше пов'язана з утворенням полімікробних біоплівок, що суттєво знижує ефективність стандартної антимікробної терапії. ***Мета:*** вивчити молекулярні механізми антибіотикорезистентності основних збудників інфекцій ран, а також оцінити ефективність фотодинамічної терапії (ФДТ) порівняно зі стандартними антимікробними препаратами проти біоплівок *in vitro*. ***Матеріали та методи.*** Загалом було отримано 66 клінічних ізолятів від 41 пацієнта із синдромом діабетичної стопи, які проходили лікування в Інституті загальної та невідкладної хірургії ім. В.Т. Зайцева НАМН України. У цій когорті (середній вік 61,0 ± 6,3 року) було 58,5 % чоловіків та 41,5 % жінок. У мікробному спектрі переважали *Staphylococcus aureus* (30 ізолятів) та різноманітні грамнегативні патогени (36 ізолятів). Генетичні маркери резистентності виявляли методом мультиплексної полімеразної ланцюгової реакції, а фенотипову чутливість — за допомогою стандартних методів дискової дифузії та визначення мінімальної інгібуючої концентрації (МІК). Моделювання біоплівок здійснювали на планшетах на 96 лунок та чашках діаметром 35 мм. Ефективність ФДТ (6% 5-амінолевулінова кислота + червоне світло 635 нм) порівнювали із такою стандартних антибіотиків. ***Результати.*** Встановлено високу поширеність генів резистентності: CTX-M-2 (26,6 % для *S.aureus*, 16,7 % серед грамнегативних), VIM (5,6 %), NDM (2,8 %), VanA і VanB (6,7 % для *S.aureus*), Erm (16,6 % для *S.aureus*), Tet (23,3 % для *S.aureus*), QnrB (13,3 % для *S.aureus*). Найвищий рівень фенотипової резистентності спостерігався до метронідазолу (52,3 %) та цефалоспоринів (22,7 %), із вираженим кореляційним зв'язком між генетичними маркерами та фенотиповими результатами. Випробування МІК продемонстрували, що традиційні антибіотики ефективні проти планктонних клітин, але не можуть повністю ліквідувати зрілі біоплівки. Натомість ФДТ мала виражену антибіоплівкову активність проти як планктонних, так і біоплівкових форм *S.aureus*, *E.coli*, *K.pneumoniae*, *P.aeruginosa*, *A.baumannii* та *C.albicans*. Комбіновані біоплівки (*C.albicans* + *S.aureus*; *S.aureus* + *K.pneumoniae*) показали обмежену відповідь на комбіновану антибіотико-протигрибкову терапію, тоді як ФДТ викликала помітне структурне руйнування біоплівок. Мікроскопія підтвердила пошкодження біоплівок та дисперсію планктонних клітин після ФДТ, ймовірно, за рахунок утворення синглетного кисню. Отримані результати підкреслюють необхідність інтеграції ФДТ у мультидисциплінарне лікування хронічних інфекцій ран, особливо в умовах поширення антибіотикорезистентності та біоплівок. ***Висновки.*** ФДТ є перспективним допоміжним методом для покращення загоєння ран за рахунок ефективного націлення на біоплівки. Необхідні подальші клінічні дослідження для підтвердження отриманих *in vitro* результатів і оптимізації лікувальних протоколів.

Ключові слова: цукровий діабет; синдром діабетичної стопи; хронічні рани, що погано загоюються; біоплівки; фотодинамічна терапія; антимікробна резистентність