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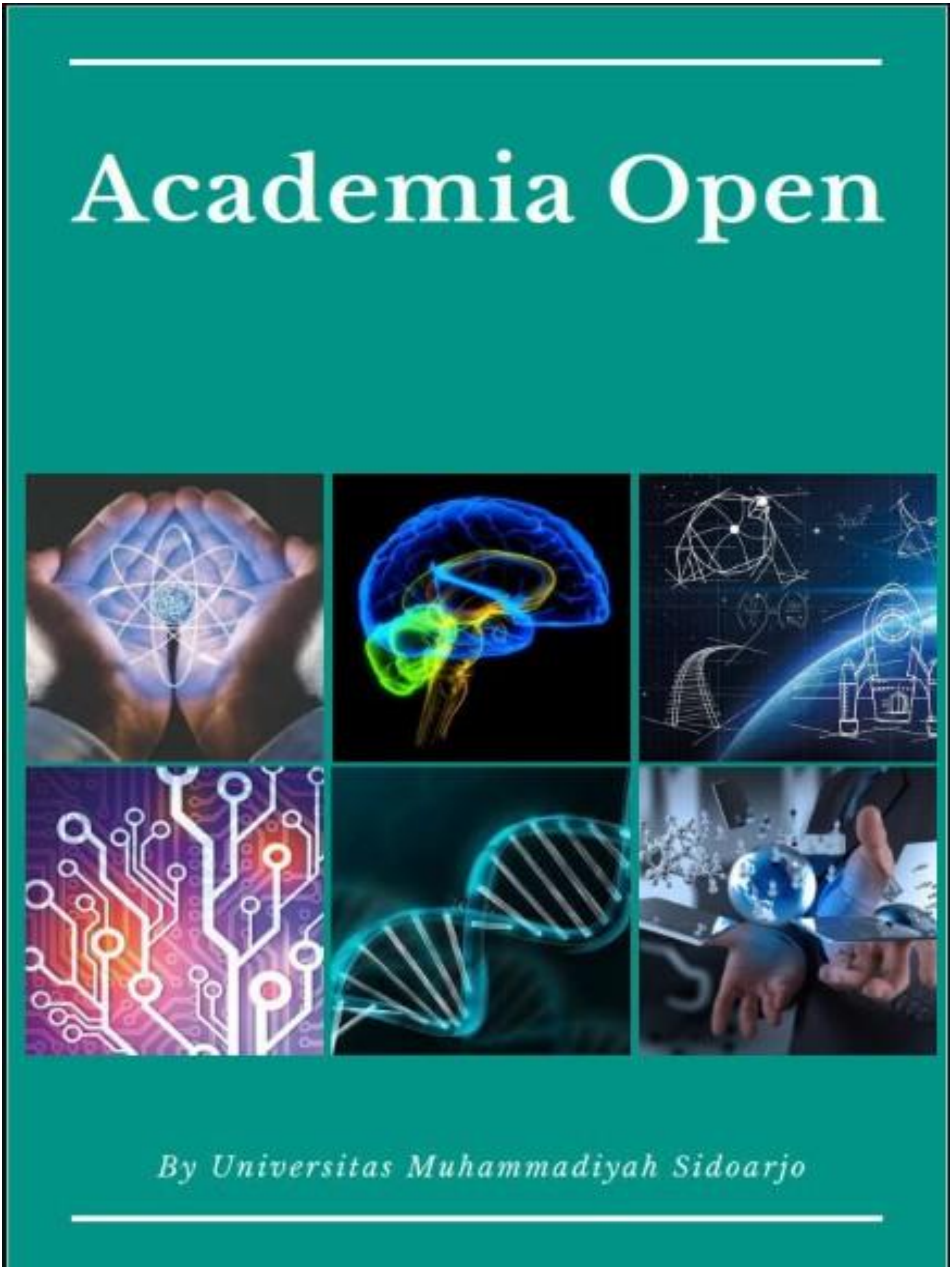

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Antimicrobial Photodynamic Therapy for the Treatment of Antibiotic-Resistant Surgical Infections

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Abstract

General Background: The global rise of antibiotic resistance has become a critical challenge in modern medicine, contributing to increased mortality, prolonged hospitalization, and economic burden. **Specific Background:** Antibiotic-resistant pathogens such as methicillin-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter* spp., and *Pseudomonas aeruginosa* are frequently associated with nosocomial infections in postoperative patients. **Knowledge Gap:** The declining efficacy of conventional antibacterial drugs necessitates the exploration of alternative antimicrobial strategies. **Aims:** This study aimed to improve strategies for controlling antibiotic-resistant microorganisms through the application of antimicrobial photodynamic therapy using methylene blue. **Results:** In vitro findings demonstrated that methylene blue and laser irradiation (632 nm) applied independently showed no antimicrobial activity, whereas their combined application produced a pronounced bactericidal effect. The highest inhibition zones were observed for *Acinetobacter* spp. (up to 40 ± 1.6 mm), followed by *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and MRSA. **Novelty:** The study highlights the strong antimicrobial potential of methylene blue–mediated photodynamic therapy against a broad spectrum of resistant microorganisms. **Implications:** This approach represents a promising alternative method for the treatment and prevention of infectious complications in surgical practice, particularly in the context of increasing antimicrobial resistance.

Highlights:
- Combined Light and Photosensitizer Application Produces Strong Bactericidal Activity
- Highest Microbial Inhibition Observed in Acinetobacter Species Cultures
- Broad-Spectrum Action Against Gram-Positive and Gram-Negative Resistant Pathogens

Keywords: Photodynamic Therapy, Antibiotic Resistance, Methylene Blue, Nosocomial Infections,

Surgical Infections.

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Introduction

In the 21st century, antibiotic resistance has become a global medical problem with serious social and economic consequences. According to international expert estimates, antimicrobial resistance currently causes more than 700,000 deaths annually worldwide, and by 2050 this number may reach 10 million deaths per year [4, 5, 13, 14, 15].

A particularly important role in the development of hospital infections is played by microorganisms belonging to the ESKAPE group. These include *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* [1]. These pathogens demonstrate high resistance to most antibacterial drugs and are responsible for the majority of nosocomial infections [2, 13, 14].

Another problem associated with antimicrobial resistance is the decrease in the number of newly developed antibiotics. Over the past 20 years, the number of antibacterial drugs approved by the FDA for clinical use has decreased by approximately 75% [4].

In surgical practice, purulent-inflammatory diseases of the abdominal organs and their complications continue to represent some of the most serious pathological conditions in abdominal surgery. The mortality rate associated with these disorders remains high, ranging from 19% to 70% [6, 16].

Patients undergoing abdominal surgery often require prolonged mechanical ventilation. Under these conditions, tracheobronchitis and pneumonia frequently develop due to colonization of the bronchial tree by opportunistic microorganisms. The development of such complications may also be associated with bacterial translocation from the gastrointestinal tract [8, 9, 12].

Given the increasing prevalence of antibiotic-resistant microorganisms, the search for alternative antimicrobial methods is becoming particularly important. Among modern physical methods of infection control, photodynamic therapy is considered one of the most promising approaches [17, 19, 20].

Photodynamic therapy involves the use of a photosensitizer activated by light of a specific wavelength. One of the most accessible photosensitizers is methylene blue. Under exposure to red-spectrum light, methylene blue transfers energy to molecular oxygen, forming singlet oxygen. This highly reactive oxygen species damages proteins, lipids, and nucleic acids, leading to the destruction of microbial cells [17, 19, 20].

For photodynamic therapy procedures, a diode laser operating within the red wavelength spectrum (about 630–670 nm) can be utilized as the irradiation source [18].

The objective of this study was to improve the effectiveness of combating antibiotic-resistant bacteria using photodynamic therapy [3, 7, 10, 11].

Materials and Methods

The microbiological investigations were undertaken in the laboratory facilities of the Department of Microbiology at Tashkent State Medical University.

The study included bacteriological analysis of bronchial lavage samples obtained from patients with endobronchitis that developed after prolonged mechanical ventilation following surgery for acute abdominal diseases.

A total of 49 operated patients were included in the study.

A total of 147 clinical samples were examined.

The following microorganisms were identified: Escherichia coli – 18.2%; Staphylococcus aureus – 10.7%; Candida spp. – 10.7%; Pseudomonas aeruginosa – 7.4%; Acinetobacter spp. – 6.2%; Klebsiella pneumoniae – 6.0%; MRSA – 3.3%.

The antimicrobial activity of photodynamic therapy was studied using methylene blue at concentrations: 0.01%; 0.05%; 0.1%.

Microbial suspensions were standardized to a concentration of approximately 10^8cells/ml. Antimicrobial activity was assessed using the agar diffusion technique. Circular wells with a diameter of 7 mm were formed in the agar medium, after which 0.01 ml of methylene blue solution was introduced into each well. The inoculated plates were then incubated at 37 °C for a period of 24–48 hours.

The antimicrobial effect was determined by measuring the diameter of microbial growth inhibition zones and interpreted according to the following criteria:

- ≤10 mm – resistant;
- 11–14 mm – moderately resistant;

• ≥15 mm – sensitive.

Results and Discussion

The study material comprised the results of bacteriological cultures obtained from bronchial lavage fluid of 49 operated patients who underwent prolonged mechanical ventilation in the intensive care unit of City Clinical Hospital No. 4 named after I. Ergashev. A total of 147 clinical samples were examined, of which 63 (43.0%) yielded various types of microorganisms. The most frequently isolated strains were *Escherichia coli* (18.2%), *Staphylococcus aureus* (10.7%), *Acinetobacter spp*. (6.2%), *Klebsiella pneumoniae* (6.0%), *Candida spp*. (10.7%), *Pseudomonas aeruginosa* (7.4%), and methicillin-resistant *Staphylococcus aureus* (MRSA) (3.3%).

In the first set of plates inoculated with the above microbial cultures, disks impregnated with MB at concentrations of 0.01%, 0.05%, and 0.1% were applied (in vitro experiment). The plates were then covered and incubated at 37 °C for 24 hours (control group 1). In the second set (control group 2), only laser irradiation was applied to the bacteria without MB, followed by incubation at 37 °C for 24 hours. In the third set of plates, under the same conditions, bacteria treated with MB were subjected to photodynamic exposure (main study group).

In both control groups, the microbiological results were negative, indicating that methylene blue at all tested concentrations when used alone, as well as laser irradiation alone, did not exhibit antibacterial activity against the tested microorganisms in any experimental series.

The results of photodynamic exposure on bacteria, including representatives of nosocomial infections and methicillin-resistant *Staphylococcus aureus*, are presented below.

Table 1. shows the results of experimental studies on *Acinetobacter* spp.

Photosensitizer concentration (MB), %	Irradiation exposure (s)			
	Initial	90	120	180
	Zone of microbial growth inhibition, mm (M ± m)			
0.01	0	17 ± 0.67	33 ± 1.2	38 ± 1.4
0.05	0	17 ± 0.65	34 ± 1.3	40 ± 1.6
0.10	0	17 ± 0.65	34 ± 1.4	40 ± 1.5
Control 1 (without photosensitizer)	0	0	0	0
Control 2 (without irradiation)	0	0	0	0

MB — methylene blue; M ± m — mean ± standard error.

As shown in Table 1, Acinetobacter spp. demonstrated high sensitivity to photodynamic therapy (PDT) at all irradiation exposure modes. At the same time, methylene blue (MB) and laser irradiation applied separately did not exhibit bactericidal activity against *Acinetobacter* spp.

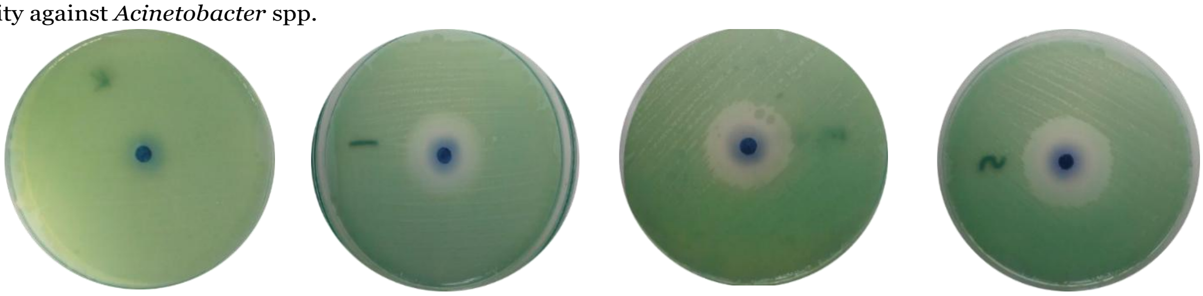

Figure 1. Antibacterial activity of a 0.05% methylene blue solution against *Acinetobacter* spp.

As shown in Figure 1, *Acinetobacter spp*. proved to be highly sensitive to the photodynamic effect of methylene blue at all tested concentrations. The zone of microbial growth inhibition reached an average of up to 40 ± 1.4 mm. In contrast, laser irradiation and methylene blue used separately did not demonstrate bactericidal activity.

Klebsiella pneumoniae, as a cause of purulent endobronchitis during prolonged mechanical ventilation after abdominal surgery, was observed in 6% of patients. Our previous studies have shown that these microorganisms exhibit very high resistance to antibiotics, with more than 90% of strains producing β-lactamases.

Table 2 presents the results of bench (in vitro) testing of photodynamic exposure against *Klebsiella pneumoniae* cultures.

Table 2. Results of photodynamic exposure on *Klebsiella pneumoniae* cultures (n = 10)

Photosensitizer concentration (MB), %	Irradiation exposure (s)			
	Initial	90	120	180
	Zone of microbial growth inhibition, mm (M ± m)			

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0.01	0	14 ± 0.52	16 ± 0.59	23 ± 0.85
0.05	0	15 ± 0.57	17 ± 0.64	23 ± 0.82
0.10	0	16 ± 0.61*	19 ± 0.72**^	25 ± 0.91
Control 1 (without photosensitizer)	0	0	0	0
Control 2 (without irradiation)	0	0	0	0

Note: *Significant compared with the photosensitizer concentration of 0.01% (*P < 0.05; *P < 0.01).*
^Significant compared with the photosensitizer concentration of 0.05% (^P < 0.05).

Legend: Differences compared with the corresponding zones of microbial growth inhibition at a photosensitizer (PS) concentration of 0.01% are statistically significant at P ≤ 0.05.

The data presented in Table 2 demonstrate that zones of microbial growth inhibition of *Klebsiella pneumoniae* under photodynamic exposure were observed at all tested irradiation modes (99, 120, and 180 s). At the same time, these zones showed a tendency to increase with rising photosensitizer concentration (within the range of 0.01% to 0.05%) (P ≤ 0.05) and were practically independent of the applied irradiation exposure times.

Thus, the inhibition zones reached their maximum already at an exposure time of 99 s for all tested modes and ranged from 15 to 23 mm. The results of bench (*in vitro*) experiments are presented in Figure 2.

In the study of photodynamic inactivation of *Klebsiella pneumoniae* strains, it was established that laser irradiation and methylene blue (MB) used separately did not suppress bacterial growth. Therefore, a complete absence of antimicrobial activity against the studied *Klebsiella pneumoniae* strains was observed when these factors were applied individually, whereas their combined application was effective.

Figure 2. Antibacterial activity of a 0.05% methylene blue solution against *Klebsiella pneumoniae*.

Thus, photodynamic therapy (PDT) is characterized by pronounced antimicrobial activity against *Klebsiella pneumoniae*.

As is well known, *Pseudomonas aeruginosa* occupies a leading position in the overall structure of nosocomial infections and represents an extremely resilient microorganism with broad adaptive capabilities and the ability to rapidly develop resistance to antibiotics [2]. Table 3 presents the results of photodynamic therapy using methylene blue at various concentrations against *Pseudomonas aeruginosa* cultures.

Table 3. Results of photodynamic exposure on *Pseudomonas aeruginosa* cultures (n = 10)

Photosensitizer concentration (MB), %	Irradiation exposure (s)			
	Initial	90	120	180
	Zone of microbial growth inhibition, mm (M ± m)			
0.01	0	24±0,82	26±0,97	30±1,1
0.05	0	25±0,88	27±1,0	34±1,3*
0.10	0	25±0,92	27±0,99	36±1,4**
Control 1 (without photosensitizer)	0	0	0	0
Control 2 (without irradiation)	0	0	0	0

Note: *Significant compared with the photosensitizer concentration of 0.01% (*P < 0.05; *P < 0.01).*
^Significant compared with the photosensitizer concentration of 0.05% (^P < 0.05).

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Figure 3. Antibacterial activity of a 0.05% methylene blue solution against *Pseudomonas aeruginosa*.

As shown in Table 3 and Figure 3, photodynamic therapy (PDT) against the resistant bacterium Pseudomonas aeruginosa demonstrates a pronounced bactericidal effect. The zone of growth inhibition reached up to 25 ± 1.2 mm at an exposure time of 99 s and increased to 34 ± 1.4 mm at an exposure time of 180 s.

Table 4 presents the results of bench (*in vitro*) testing against methicillin-resistant *Staphylococcus aureus* (MRSA). Considering the significant role of MRSA in thoracoabdominal surgery in general and in purulent endobronchitis in particular, bench experiments were conducted to assess the resistance of MRSA to photodynamic exposure. The results of these bench tests are summarized in Table 4.

Table 4. Results of photodynamic exposure on MRSA cultures (n = 10)

Photosensitizer concentration (MB), %	Irradiation exposure (s)			
	Initial	90	120	180
	Zone of microbial growth inhibition, mm (M ± m)			
0.01	0	14±0,53	14±0,51	16±0,58
0.05	0	18±0,65***	19±0,73***	21±0,81***
0.10	0	18±0,68***	19±0,71***	21±0,79***
Control 1 (without photosensitizer)	0	0	0	0
Control 2 (without irradiation)	0	0	0	0

Note: *Significant compared with the photosensitizer concentration of 0.01% (*P < 0.05; *P < 0.01).*
^Significant compared with the photosensitizer concentration of 0.05% (^P < 0.05).

As shown in Table 4, methylene blue (MB) at concentrations of 0.05% and 0.1% used in photodynamic therapy (PDT) exhibited bactericidal activity at all laser irradiation exposure times, with the sensitivity index reaching up to 21 ± 1.4 mm (Figure 4).

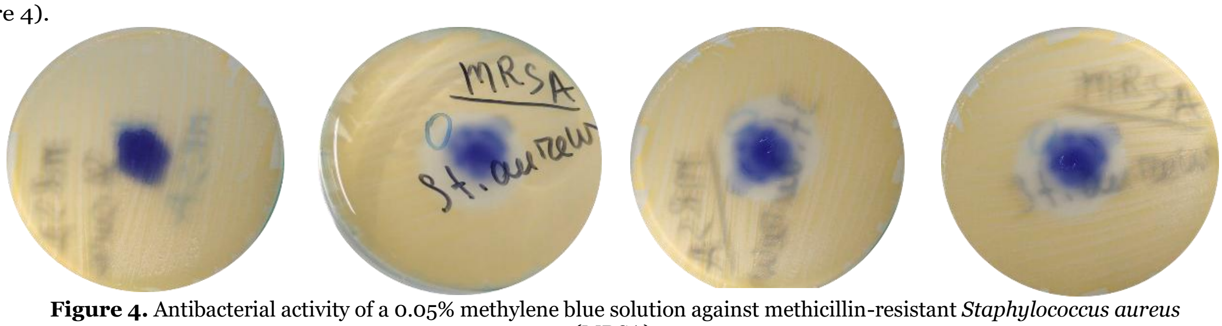

Figure 4. Antibacterial activity of a 0.05% methylene blue solution against methicillin-resistant *Staphylococcus aureus* (MRSA).

Thus, photodynamic therapy using methylene blue demonstrates pronounced antimicrobial activity against a broad spectrum of pathogenic microorganisms, including both Gram-positive and Gram-negative bacteria. As demonstrated by our *in vitro* experiments, methylene blue and laser irradiation at a wavelength of 632 nm, when applied separately, did not exhibit antibacterial activity against either Gram-negative or Gram-positive bacteria, including MRSA. Therefore, this method of infection control may find wide application as an antimicrobial approach for the treatment and prevention of various purulent-inflammatory processes.

The *in vitro* results obtained in the present study determined the direction of subsequent investigations, namely, the evaluation of the effectiveness of PDT using methylene blue *in vivo* in an animal model of purulent endobronchitis.

Table 5 presents the results of the antimicrobial activity of PDT against resistant microflora. As shown in Table 5, PDT with methylene blue exerted antimicrobial effects against all tested microorganisms; however, the magnitude of this effect varied among different pathogens.

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Table 5. Antimicrobial activity of photodynamic therapy at a methylene blue concentration of 0.05%

№№	Test culture	Number of strains	Zone of growth inhibition (M ± m), mm	
			90s	180s
1.	*Acinetobacter* spp.	10	17±0,65	40±1,6
2	*Klebsiella pneumoniae*	10	15±0,57	23±0,82
3	*Pseudomonas aeruginosa*	10	25±0,88	34±1,3
4	Methicillin-resistant *Staphylococcus aureus* (MRSA)	10	18±0,65	21±0,81

Note: The table presents the results of the bactericidal effect of laser irradiation at exposure times of 90 and 180 s, with an energy density of 25–35 J/cm².

As shown in Table 5, at a methylene blue (MB) concentration of 0.05% and a laser irradiation exposure time of 180 s, high antimicrobial activity was observed against most tested cultures (with zones of microbial growth inhibition reaching up to 40 ± 1.6 mm) compared with an exposure of 99 s. It should be noted that the highest activity was recorded against *Acinetobacter spp.*

Thus, photodynamic therapy using methylene blue demonstrates pronounced antimicrobial activity against a broad spectrum of antibiotic-resistant microorganisms.

Conclusion

1. Methylene blue solutions at concentrations of 0.01%, 0.05%, and 0.1%, when applied independently, did not demonstrate antibacterial activity against antibiotic-resistant microorganisms.

2. Laser irradiation at a wavelength of 632.8 nm used as a single modality also showed no significant antimicrobial effect.

3. However, the combined application of methylene blue as a photosensitizer together with laser irradiation resulted in a pronounced bactericidal effect against antibiotic-resistant bacterial strains.

4. Methylene blue–based photodynamic therapy may serve as a promising adjunctive method for the prevention and treatment of purulent-inflammatory complications.

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