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In vitro evaluation of antimicrobial and anti-biofilm properties of antiseptics against multidrug resistant clinical *Escherichia coli* strains, isolated from combat wounds

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The necessity for the investigation of novel approaches and strategies for the treatment of multidrug-resistant *E. coli* related infections becomes more and more essential.

Purpose – to investigate and compare the level of antimicrobial and anti-biofilm activity of antiseptic preparations against MDR clinical isolates of *E. coli*.

Materials and methods. In vitro effectiveness of modern antiseptics; octenidine 0.1% (OCT), polyhexanide 0.1% (PHMB), chlorhexidine 0.5% (CHG), miramistin 0.01% (MRM), decamethoxine 0.1% 0.02% (DCM), povidone-iodine 10% (PVP-I), was determined against forty-six polyresistant clinical strains of *E. coli*. MIC, MBC were found by standard methods, the value of which was interpreted as a bacteriostatic and bactericidal index of antiseptic activity (BS IAA and BC IAA). The effect of antiseptics on the immature biofilm was modelled using the Christensen test.

Results. MIC and MBC values were the lowest in DCM and OCT. The highest values of the antiseptic activity index (IAA>4) were determined for the antiseptics PHMB 0.1%, OCT 0.1% and DCM 0.1%. It was found that the feasibility of using MRM at a concentration of 0.01% is questionable as the BS IAA is above the threshold value, while the BC IAA is not. The effectiveness of PVP-I 1% against MDR *E. coli* was found insufficient. Sub-bacteriostatic concentrations of DCM, CHG, and PHMB reliably inhibited the formation of *E. coli* biofilms within 24 hours. MRM and PVP-I in sub-bacteriostatic concentrations stimulated biofilm formation.

Conclusions. Based on the analysis of all conducted studies, 0.1% and 0.02% DCM, 0.05% CHG, 0.1% OCT, 0.1% PHMB, 10% and 2% PVP-I are the most active against MDR clinical isolates of *E. coli*.

The research was carried out in accordance with the principles of the Helsinki Declaration. The study protocol was approved by the Local Ethics Committee of the participating institution. The informed consent of the patient was obtained for conducting the studies.

No conflict of interests was declared by the authors.

Keywords: *Escherichia coli*, antiseptics, biofilms, infection, combat wounds, susceptibility to antiseptics, anti-biofilm-forming activity.

Оцінка in vitro антимікробних та антибіоплівкових властивостей антисептиків проти клінічних штамів *Escherichia coli* з множинною лікарською стійкістю, виділених із бойових ран

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Необхідність розроблення нових підходів і стратегій лікування інфекцій, пов'язаних із полірезистентною *E. coli*, стає все більш суттєвою.

Мета – дослідити та порівняти рівень антимікробної та антибіоплівкової активності антисептичних препаратів щодо клінічних ізолятів *E. coli* з множинною лікарською стійкістю (МЛС).

Матеріали та методи. *In vitro* ефективність сучасних антисептиків – октенідину 0,1% (ОСТ), полігексаніду 0,1% (PHMB), хлоргексидину 0,5% (CHG), мірамістину 0,01% (MRM), декаметоксину 0,1% і 0,02% (DCM), повідон-йоду 10% (PVP-I) – тестували проти 46 клінічних МЛС штамів *E. coli*. МІК, МБЦК визначали стандартними методами, значення яких інтерпретували у вигляді бактеріостатичного і бактерицидного індексу активності антисептика (БС ІАА та БЦ ІАА). Вплив антисептиків на незрілу біоплівку моделювали за допомогою тесту Крістенсена.

Результати. Значення МІК і МБЦК були найнижчими для DCM і ОСТ. Найвищі значення індексу антисептичної активності (ІАА>4) визначено для антисептиків PHMB 0,1%, ОСТ 0,1% та DCM 0,1%. Виявлено, що доцільність використання MRM у концентрації 0,01% є сумнівною, оскільки БС ІАА перевищує порогове значення, а БЦ ІАА – ні. Ефективність PVP-I 1% проти МЛС *E. coli* визнано недостатньою. Суббактеріостатичні концентрації DCM, CHG і PHMB надійно пригнічували утворення біоплівок *E. coli* протягом 24 год. MRM і PVP-I у суббактеріостатичних концентраціях стимулювали утворення біоплівки.

Висновки. Виходячи з аналізу всіх проведених досліджень, 0,1% і 0,02% DCM, 0,05% CHG, 0,1% ОСТ, 0,1% PHMB, 10% і 2% PVP-I є найактивнішими проти клінічних штамів *E. coli* з МЛС.

Дослідження виконано відповідно до принципів Гельсінської декларації. Протокол дослідження ухвалено Локальним етичним комітетом зазначеної в роботі установи. На проведення досліджень отримано інформовану згоду пацієнтів.

Автори заявляють про відсутність конфлікту інтересів.

Ключові слова: *E. coli*, антисептики, біоплівки, інфекція, бойові поранення, чутливість до антисептиків, антибіоплівкоутворююча активність.

Introduction

The ecology of *Escherichia coli* (*E. coli*) is typical of Enterobacteria: its reservoir is the distal intestine of humans and animals. *E. coli* is a symbol of normal microflora, and before the approval of the idea that obligate anaerobes are dominant both quantitatively and in their significance; it firmly occupied a leading position here. The genetic plasticity of *E. coli* allows for greater variability and adaptation to different growth conditions and niches. Geno- and phenotypic diversity manifests itself in a wide range of lifestyles and virulence, ranging from non-virulent to highly pathogenic forms. Improved competitiveness, as a result of the high adaptability of *E. coli*, is manifested not only by intestinal fitness, but also by extra intestinal virulence. Progressive resistance to antimicrobial drugs is a significant manifestation of adaptive abilities. New hybrid strains are emerging that are equipped with antibiotic resistance and virulence determinants [8,22,28,49].

E. coli is currently one of the most threatening pathogens in healthcare-associated infections (HCAs) and therefore causes considerable medical, economic problems in both acute care and long-term care facilities. Among the HCAs, the infections of soft tissue and skin are the most common, and these are deep and superficial incisional surgical site infections, vascular access infections, infected pressure ulcers, and of course infected burns and injuries, which are the focus of this study [49,50].

The duration of hospitalization of patients with HCAI increases threefold, the risk of death – from 4 to 15 times. The most severe forms of HCAI are caused by hospital strains with multidrug resistance to antimicro-

bials. Infections caused by multidrug resistant (MDR) pathogens often negate not only the results of treatment in general and intensive care units, but also the results of expensive high-tech and life-saving interventions [23].

The hospital-acquired *Enterobacteriaceae* can cause serious wound infections. This is due to the elimination of gram-positive microflora from the skin in a hospital and their active colonization by gram-negative microflora. At any localization of the primary focus, generalization of infection caused by representatives of the *Enterobacteriaceae* family is possible, with the development of purulent-septic disease [40].

Enterobacteria as *Proteus mirabilis*, *Escherichia coli*, *Klebsiella pneumoniae subsp. pneumoniae* are the priority pathogens of purulent-inflammatory diseases of the skin and soft tissues. Some researchers have noted an increase in the frequency of isolation of *E. coli* from samples in recent years [39]. Clinical strains of *E. coli* are among frequently detected MDR isolates, as increasing representatives of carbapenem-resistant *Enterobacteriaceae*, which produce extended-spectrum beta-lactamase and has become a global health threat [43]. According to the World Health Organization (WHO) report, they are classified as critical priority pathogens, their emergence and spread limits therapeutic options [42].

Over the past two decades, the negative impact of biofilm-forming microorganisms on chronic wounds has been increasingly recognized, and the ability of biofilm formation as a virulence factor is a fundamental reason that chronic wounds do not heal in a timely manner [38]. Such wounds are a significant problem both in military medical centres and in public healthcare facilities, as they are difficult to treat. The biofilm

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creates ideal conditions for the exchange of resistance genes, so chronic patients may be at high risk of carrying, acquiring and spreading antibiotic-resistant microorganisms [39].

Nowadays Ukraine suffers from hostilities affecting both soldiers and civilians. Combat injuries are often associated with numerous life-threatening complications, and wound infections are the main consequences of these war injuries. The microbial profile of combat wound infections is diverse, with antibiotic-resistant Gram-negative bacteria becoming the predominant component in the later stages of management. The danger of the emergence and spread of MDR pathogens of war-related wound infections lies in the fact that this problem can have consequences not only locally, but also on a global scale [29,35,36,44,47].

E. coli resistance to antibiotics is increasing every year, with more and more infections being caused by MDR strains. Researchers around the world understand that they are dealing with a genetically enhanced, multifaceted and versatile microbe. And with each passing year, the need to promote alternative strategies for the treatment of these infections is becoming more and more evident [8].

There is always a need to improve modern concepts of effective treatment and prevention of infectious complications of wounds in view of the spread of antibiotic resistance and taking into account the biofilm status of the pathogen. Application of antiseptics to combat colonization and infection directly at the portal of entry to prevent the generalization of the infectious process is an extremely important stage in the prevention and treatment of infectious complications. A comprehensive understanding of the susceptibility of MDR *Escherichia* in various forms of existence to modern topical antimicrobials will determine the rational use of these agents to maintain their activity in the future.

The **purpose** of the work – to investigate and compare the level of antimicrobial and anti-biofilm activity of antiseptic preparations against MDR clinical isolates of *E. coli* obtained from patients with infected combat burns and shrapnel wounds of different localization.

Materials and methods of the research

This article is a continuation of a study, part of which was published earlier [11], and was devoted to the antimicrobial properties of antiseptics against MDR clinical strains of Gram-negative bacteria, isolated from combat wounds. Our study is a fragment of scientific project «Research of the biological properties of pathogens of HCAs and the development of means of combating them».

Bacterial strains analysed

Forty-six MDR *E. coli* clinical strains (68% of the total number), which were used to determine the effectiveness of antiseptic drugs, were received from patients with infected combat burns and shrapnel wounds of different localization, which were received during military operations on the territory of Ukraine.

The reference strains from the American Type Culture Collection (ATCC) *E. coli* ATCC 25922 (β -lactamase negative), *E. coli* ATCC 35218 (β -lactamase producing strain) were used as a control.

The identification of isolates was carried out by standard microbiological methods. The tinctorial, morphological, cultural and biochemical properties of the isolates were taken into account.

The biochemical profile was studied using «ENTEROtest 24 N» («Lachema, Czech Republic»), OFtest («Lachema, Czech Republic») OXItest («Lachema, Czech Republic»).

The disk diffusion method was used to determine the antimicrobial susceptibility of isolates based on CLSI and EUCAST (Clinical Laboratory Standards Institute and European Committee for Antimicrobial Susceptibility Testing) standards.

The resistance profile of the isolates was determined according to the definition criteria recommended by ECDC (European Centre for Disease Prevention and Control), and CDC (Centres for Disease Control and Prevention). *E. coli* strains were characterized as MDR if acquired resistance to at least one antimicrobial agent from three or more groups of drugs was determined.

Phenotypic resistance of *E. coli* clinical isolates to antimicrobial agents belonging to such antimicrobial categories was revealed: aminoglycosides, carbapenems, antipseudomonal penicillins with β -lactamase inhibitors, non-extended spectrum cephalosporins; the 1st and the 2nd generation cephalosporins, extended-spectrum cephalosporins of the 3rd and the 4th generations, fluoroquinolones, monobactams, penicillins, penicillins with β -lactamase inhibitors, phenicols, polymyxins.

Tested Substances

The activity of the following antiseptic substances from the group of quaternary ammonium compounds and halogen-containing compounds (namely, pharmaceutical products available in Ukraine) was determined against reference and clinical *E. coli* strains:

1. OCT – octenidine dihydrochloride 0.1% (Octenisept® farblos/incolore, Schulke & Mayr GmbH, Germany);
2. PHMB (polyhexamethylene biguanide) – polyhexanide solution 0.1% (Prontosan®, B Braun Medical, Germany);

3. CHG – chlorhexidine digluconate 0.5% (Chlorhexidine-Viola® Viola, FF, JSC, Ukraine);

4. MRM – miramistin 0.01% (Miramistin®, Darnitsa PrAT, Ukraine);

5. DCM – decamethoxine 0.1% (was prepared from the substance powder of Decamethoxine®, Yuria-Pharm, Ukraine);

6. DCM – decamethoxine 0.02% (Decasan®, Yuria-Pharm, Ukraine);

7. PVP-I – povidone-iodine 10% (Betadine®, EGIS Pharmaceuticals PLC, Hungary).

PVP-I was used in the study at an initial concentration of 10% and recommended working dilutions of 1:5 (2%) and 1:10 (1%).

Susceptibility assays on planktonic cells

MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) determination.

In the study the antimicrobial activity of antiseptics was evaluated by determining of their minimum inhibitory (bacteriostatic) (MIC) and bactericidal (MBC) concentrations against control and clinical strains of *E. coli*. The MIC of the antimicrobials was defined by the Standard macro method of double serial dilutions (the guidelines of Ukraine No.167 April 5, 2007; Standards for Antimicrobial Susceptibility Testing, in accordance to the Clinical and Laboratory Standards Institute guidelines (CLSI, USA)) [10,34]. Cultivating of the microorganisms was performed on Mueller–Hinton broth (HiMedia Laboratories, India). Consecutive two-fold dilutions of the studied antiseptics were prepared, starting with working concentrations.

For inoculation, a suspension of microorganisms was prepared at a concentration of 5×10^6 CFU/ml. When recording the results, the MIC value was determined as the minimum concentration of antiseptic substance, which prevented the visible growth of bacteria after incubation for 48 hours at 37°C. For determining MBC, 0.1 ml were taken from tubes in which no growth was observed and then inoculated onto the surface of Petri dishes with TSA (Laboratorios Conda S.A, Spain)), after which they were incubated for 48 hours at 37°C. MBC was taken as the lowest concentration of the antiseptic, at which colony growth does not occur under the given conditions. Three replicates were performed for each isolate of *E. coli* and antimicrobial component [11].

BS IAA (bacteriostatic index of antiseptic activity) and BC IAA (bactericidal index of antiseptic activity) determination. The antimicrobial efficacy of antiseptics was compared using the IAA (index of antiseptic activity) indicator.

The bacteriostatic and bactericidal action was differentiated; therefore, the BS IAA and the BC IAA were calculated accordingly.

The BS IAA indicator is the ratio of the working concentration of a certain antiseptic to its MIC in relation to a given microorganism. Accordingly, the BC IAA indicator is the ratio of the working concentration to the MBC of the antiseptic. IAA allows evaluating and comparing the effectiveness of antiseptics against microorganisms, regardless of their working concentrations. The interpretation of the results is based on the fact that under natural conditions the activity of antiseptics is reduced by four times, so the antiseptic was considered active if the IAA was greater than four (IAA>4) [3,11,26].

Susceptibility assays on biofilm formation

Quantitative crystal violet assay. A quantitative crystal violet assay, or the microtiter-plate Christensen test, was applied for determining of the biofilm-forming ability of *E. coli* isolates.

The ability of antiseptics to inhibit immature biofilm was determined by culturing microorganisms with the simultaneous presence of sub-bacteriostatic concentrations of antiseptics for 24 hours. Subsequently, a spectrophotometric assessment of biofilm formation by *E. coli* isolates was carried out in optical density units (ODU assessment).

Each strain of the 46 isolates was exposed to a specific sub-MIC concentration of antiseptics for the certain strain.

The culture of each strain was inoculated into a test tube with tryptic soy broth (TSB, EMD Millipore, USA) and 1% glucose, and incubated for 24 hours at 37°C. Then the resulting culture was diluted 1:50 with fresh tryptic soy broth with 1% glucose 2 times more concentrated). Then, 100 µl of the prepared suspension and 100 µl of an antiseptic solution at a concentration of $2 \times 1/3$ MIC were added to a sterile 96-well flat-bottom microtiter plate (USA Scientific, Inc), achieving final concentration of antiseptic in the well equal to 1/3 MIC.

After incubation at 37°C for 24 h, non-adherent bacteria were removed by washing thrice with phosphate buffer saline, pH 7.2 (Sigma, USA; cat. no. P-3813). Slime and adherent cells were fixed with absolute methanol and stained with 220 µl of crystal violet 0.1% w/v (Merck, Germany) for 15 min at room temperature. The wells were then rinsed thrice with PBS to remove unbound CV dye and dried at 37°C for 30 min. Then 220 µl of ethanol (95%) was added to each well.

Spectrophotometer STAT FAX®4300 (Netherlands) was used for all spectrophotometric measurements (at a wave-

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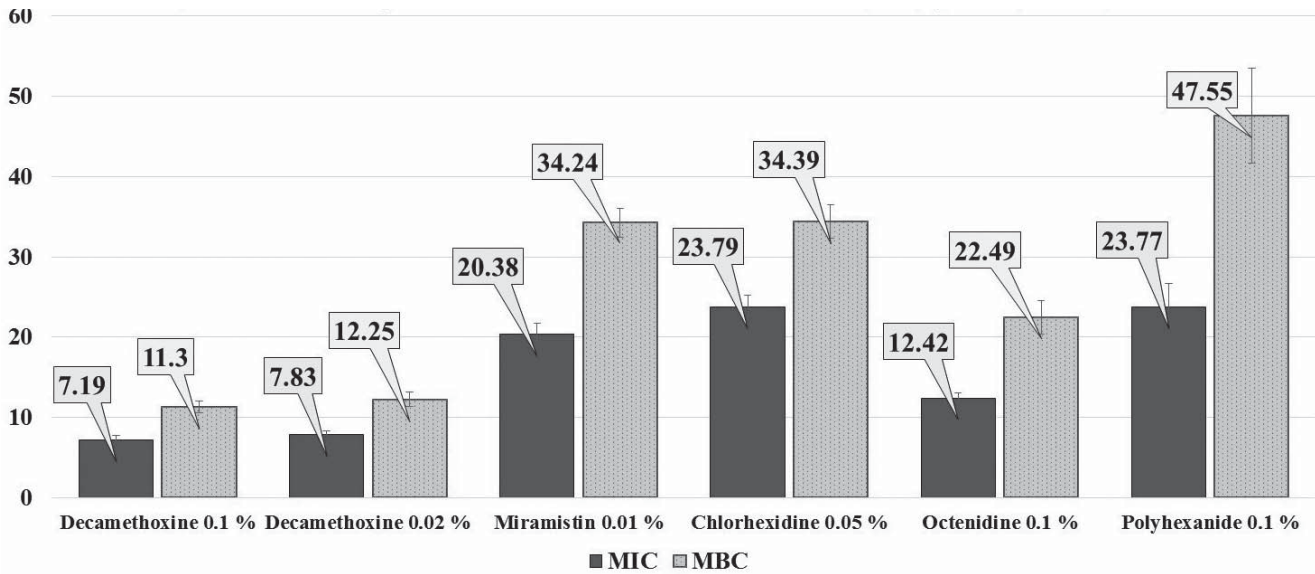


Fig. 1. Characteristics of the antiseptics' activity in relation to *E.coli* clinical strains, received from patients with infected combat wounds, in µg/ml (M±m)

length of 620 nm). The study was carried out in triplicate for each strain of *Escherichia coli*.

The cut-off optical density (OD_c) was indicative of biofilm formation and was defined as the sum of the arithmetic mean of negative controls and a triple value of its standard deviation (OD_c = $\bar{x} + 3\sigma$). Non-inoculated culture medium served as a negative control.

If the optical density was <0.120, then the biofilm-forming ability was assessed as low, if =0.121–0.239, then – as medium, if it was >0.240, then it was assessed as high.

The positive control with which the results were compared was the average value of optical density for each strain without the addition of an antiseptic (instead of it, saline was added to the suspension) [9,11,13].

Statistical analysis

To evaluate the degree of reliability of the obtained data, a variational statistical method of analysis was used, including the calculation of the arithmetic mean (M), the mean arithmetic error (m), the mean error (t), and the reliability of the difference (p). Microsoft Office Excel (version 16.0.5056.1000, 2016) and the Statistica software package (version 12.5.192.7, StatSoft Inc.) were used for statistical processing.

Differences were considered statistically significant at p≤0.05. The relationship between biofilm formation by *E. coli* isolates in the presence of antiseptics and the susceptibility of these isolates to a particular antiseptic was established using the Pearson correlation coefficient (r) [11].

Results of the research

For our study, we were interested in isolates with a resistance profile corresponding to the MDR category.

As a result of the analysis of phenotypic resistance, 46 isolates were characterized as MDR, which accounted for 68% of the total number of isolates obtained. Thus the resistance rates of selected MDR *E. coli* isolates were 23.9% to Gentamicin, 28.3% to Tobramycin, 15.2% to Amikacin, 26.1% to Piperacillin-tazobactam, 21.7% to Imipenem, 39.1% to Meropenem, 78.3% to Cefuroxime, 43.5% to Ceftriaxone, 52.2% to Ceftazidime, 41.3% to Cefepime, 73.9% to Ciprofloxacin, 45.7% to Aztreonam, 84.8% to Ampicillin, 65.2% to Ampicillin-sulbactam, 19.6% to Chloramphenicol. And all these isolates were susceptible to colistin.

Antimicrobial activity on planktonic *Escherichia coli* cells

This *in vitro* study was designed to determine the activity of modern antiseptics against MDR clinical isolates of *E. coli* in planktonic and biofilm form. Thus, at the first stage of the study, under the action of antiseptics on planktonic forms of clinical isolates of the wound pathogen *E. coli*, which is classified by WHO as a critical priority pathogen due to its antibiotic resistance profile (*E. coli* resistant to carbapenems), high efficiency of the main antiseptics from the surfactant group was revealed.

Quantitative indicators of the bacteriostatic and bactericidal action of the studied antiseptics in the form of a MIC and a MBC respectively are illustrated by Fig. 1. Coefficients of reliability of the difference between the MIC of the studied antiseptics are shown in Table 1. Coefficients of reliability of the difference between the MBC can be found in Table 2.

The highest antimicrobial activity against clinical strains of *E. coli* among the studied antiseptics from the group of quaternary ammonium compounds (QAC) was found in decamethoxine (0.1% and 0.02%) and octeni-

Table 1

Coefficients of reliability of the difference between MIC of the antiseptics against *E.coli* clinical strains (p1)

MIC of antiseptics to compare	MIC of Decamethoxine 0.1%	MIC of Decamethoxine 0.02%	MIC of Chlorhexidine 0,05%	MIC of Octenidine 0.1%	MIC of Miramistin 0.01%	MIC of Polyhexanide 0.1%
MIC of Decamethoxine 0.1%	1.0000	>0.10	<0.001	<0.001	<0.001	<0.001
MIC of Decamethoxine 0.02%	>0.10	1.0000	<0.001	<0.001	<0.001	<0.001
MIC of Chlorhexidine 0,05%	<0.001	<0.001	1.0000	<0.001	<0.1	>0.10
MIC of Octenidine 0.1 %	<0.001	<0.001	<0.001	1.0000	<0.001	<0.001
MIC of Miramistin 0.01 %	<0.001	<0.001	<0.1	<0.001	1.0000	>0.10
MIC of Polyhexanide 0.1 %	<0.001	<0.001	>0.10	<0.001	>0.10	1.0000

Note: *MIC – minimum inhibitory concentration of antiseptics.

dine (0.1%). Their minimum inhibitory and minimum bactericidal concentrations were the lowest. The average values of the minimum inhibitory concentrations of decamethoxine 0.1%, decamethoxine 0.02% (decasan) and octenidine (0.1%) were $7.19 \pm 0.53 \mu\text{g/ml}$; $7.83 \pm 0.49 \mu\text{g/ml}$ and $12.42 \pm 0.67 \mu\text{g/ml}$. Bactericidal properties were determined in the presence of concentrations that were $11.30 \pm 0.76 \mu\text{g/ml}$ (decamethoxine 0.1%); $12.25 \pm 0.88 \mu\text{g/ml}$ (decamethoxine 0.02%) and $22.49 \pm 2.08 \mu\text{g/ml}$ (octenidine 0.1%) (Fig. 1).

Miramistin, chlorhexidine and polyhexanide have demonstrated a sufficiently high BS and BC activity against clinical strains of *E. coli*. Effective inhibition of the growth of *Escherichia* was received with the use of miramistin at a concentration of $20.38 \pm 1.31 \mu\text{g/ml}$; chlorhexidine – at a concentration of $23.79 \pm 1.39 \mu\text{g/ml}$ and polyhexanide – at a concentration of $23.77 \pm 2.96 \mu\text{g/ml}$. As for the bactericidal action of these antiseptics, the highest value of the minimum bactericidal concentration was determined for polyhexanide ($47.55 \pm 5.91 \mu\text{g/ml}$), which is twice its bacteriostatic concentration. The MBC values for miramistin and chlorhexidine were $34.24 \pm 1.8 \mu\text{g/ml}$ and $34.39 \pm 2.12 \mu\text{g/ml}$, respectively (Fig. 1).

Thus, clinical strains of *E. coli* were most susceptible to octenidine and decamethoxine. The minimum bacteriostatic concentrations of octenidine were significantly (Table 1) lower than those of miramistin by 1.64 times ($p < 0.001$), chlorhexidine – by 1.92 times ($p < 0.001$), polyhexanide – by 1.91 times ($p < 0.001$). The values of the minimum bactericidal concentrations of octenidine were significantly (Table 2) lower than those of miramistin by 1.52 times ($p < 0.001$), chlorhexidine – by 1.53 times ($p < 0.001$), polyhexanide – by 2.11 times ($p < 0.001$).

The bacteriostatic effect of decamethoxine against clinical strains of *E. coli* significantly (Table 1) exceeded

that of miramistin by 2.71 times ($p < 0.001$), chlorhexidine – by 3.17 times ($p < 0.001$), octenidine – by 1.65 times ($p < 0.001$), polyhexanide – by 3.17 times ($p < 0.001$). The bactericidal activity of decamethoxine was significantly (Table 2) higher than that of miramistin by 2.91 times ($p < 0.001$), chlorhexidine – by 2.92 times ($p < 0.001$), octenidine – by 1.91 times ($p < 0.001$), polyhexanide – by 4.04 times ($p < 0.001$).

To summarize the above, it can be emphasized that among the quaternary ammonium antiseptics studied, clinical *E. coli* isolates were most susceptible to decamethoxine and octenidine, and least susceptible to polyhexanide.

MICs of povidone iodine against *E. coli* averaged $2989.13 \pm 147.84 \mu\text{g/ml}$, and bactericidal concentrations were $3695.65 \pm 186.16 \mu\text{g/ml}$.

We compared the activity of drugs that belong to the same chemical group of antiseptics (quaternary ammonium compounds). Povidone iodine belongs to the halide-containing compounds. Its active substance is present in the initial solution in much higher concentrations and cannot be compared with the concentrations of quaternary ammonium compounds. In this case, it is possible to evaluate the activity of an antiseptic and compare it with other drugs using the antiseptic activity index.

The indicators BS IAA and BC IAA were calculated. Interpretation of the results of calculating the differentiated IAA allows not only comparing antiseptics from different chemical groups with different initial concentrations, but also assessing the appropriateness of using the drug against a given microorganism.

For 2% and 1% concentrations of povidone iodine, the antiseptic activity indices were additionally calculated, since dilutions of povidone-iodine 1:5 and 1:10 are recommended for use by the instructions (Fig. 2).

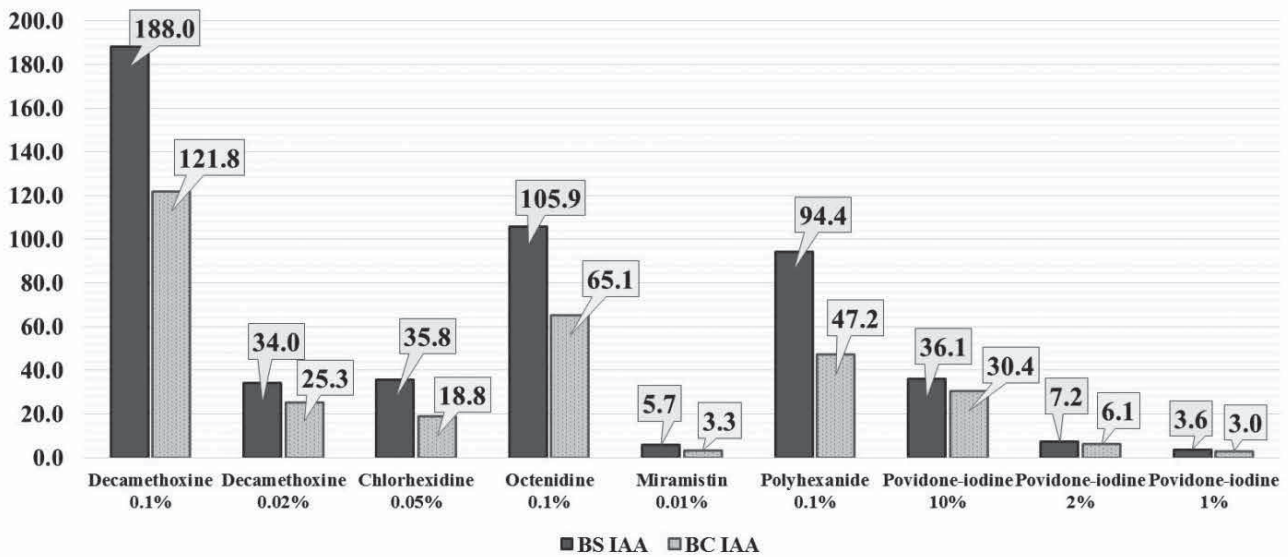


Fig. 2. The average data of bacteriostatic and bactericidal IAA against clinical strains of *E. coli*

The values of BS IAA and BC IAA for povidone-iodine 10% were 36.1 and 30.4, for povidone iodine 2% – 7.2 and 6.1, for povidone iodine 1% – 3.6 and 3.0 respectively. For octenidine 0.1%, BS IAA and BC IAA values of 105.9 and 65.1 were determined. The BS IAA and BC IAA values for chlorhexidine 0.05% were 35.8 and 18.8 respectively. For polyhexanide 0.1%, the values of BS IAA and BC IAA were 94.4 and 47.2. There were determined values of BS IAA (5.7) and BC IAA (3.3) for miramistin 0.01%. The BS IAA of decamethoxine 0.1% was 188.0, and the BC IAA for decamethoxine 0.1% was 121.8. The bacteriostatic IAA and bactericidal IAA for decamethoxine 0.02% were 34.0 and 25.3 respectively (Fig. 2).

Influence of antiseptics in vitro on the immature biofilm of *E. coli* isolates.

All studied strains were biofilm-forming. It was established that MDR clinical strains of *E. coli* have average

properties of biofilm formation. The average meaning of the absorption degree of dye by biofilms in the control wells was 0.212 ± 0.004 optical density units (ODU).

Determination of anti-biofilm forming activity showed that sub-bacteriostatic concentrations of decamethoxine (an average of $2.40 \pm 0.18 \mu\text{g/ml}$), chlorhexidine (an average of $2.61 \pm 0.16 \mu\text{g/ml}$), and polyhexanide (an average of $7.92 \pm 0.99 \mu\text{g/ml}$) reliably (coefficient of reliability $p < 0.001$ for decamethoxine and chlorhexidine, $p < 0.01$ for polyhexanide) inhibited the formation of *E. coli* biofilms within 24 hours. Under the decamethoxine influence, the average value of the optical density of *E. coli* biofilms decreased 1.08 times, compared with the control and amounted to 0.198 ± 0.001 ODU, under chlorhexidine – by 1.05 times and was 0.201 ± 0.001 ODU, and in the presence of polyhexanide y 1.06 times and was 0.200 ± 0.001 ODU.

Table 2

Coefficients of reliability of the difference between the MBC of the studied antiseptics against *E. coli* clinical isolates (p2)

MIC of antiseptics to compare	MBC of Decamethoxine 0.1%	MBC of Decamethoxine 0.02%	MBC of Chlorhexidine 0.1%	MBC of Octenidine 0.1%	MBC of Miramistin 0.01%	MBC of Polyhexanide 0.1%
MBC of Decamethoxine 0.1%	1.0000	>0.10	<0.001	<0.001	<0.001	<0.001
MBC of Decamethoxine 0.02%	>0.10	1.0000	<0.001	<0.001	<0.001	<0.001
MBC of Chlorhexidine 0.1%	<0.001	<0.001	1.0000	<0.001	>0.10	< 0.05
MBC of Octenidine 0.1%	<0.001	<0.001	<0.001	1.0000	<0.001	<0.001
MBC of Miramistin 0.01%	<0.001	<0.001	>0.10	<0.001	1.0000	>0.10
MBC of Polyhexanide 0.1%	<0.001	<0.001	< 0.05	<0.001	< 0.05	1.0000

Note: *MBC – minimum bactericidal concentration.

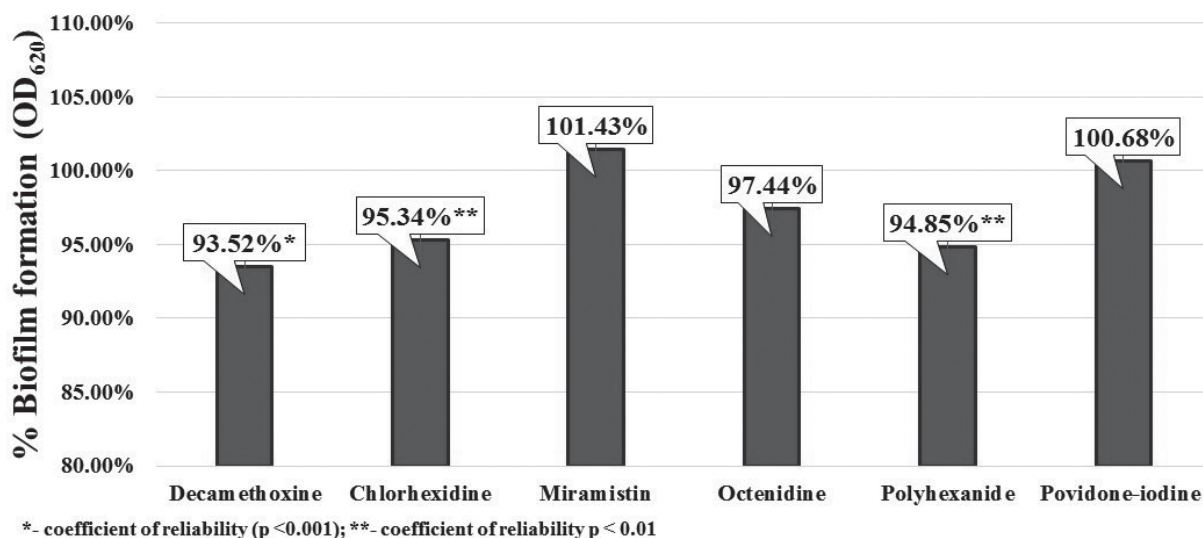


Fig. 3. Percentage indicator of biofilm-forming ability of *E. coli* (n=46) in the presence of studied antiseptics compared to the intact control

1/3 MIC of octenidine ($4.14 \pm 0.22 \mu\text{g/ml}$) showed a lower anti-biofilm-forming effect, but the difference was not statistically significant. The results of this study are clearly demonstrated in the ability of *E. coli* isolates to form biofilms in the presence of the studied antiseptics compared to the intact control (Figure 3).

Evaluating the anti-biofilm forming effect of the studied antiseptics, decamethoxine, chlorhexidine and polyhexanide had exhibited the strongest effect on immature biofilms. The inhibitory effect was 93.52%, 95.34% and 94.85% in comparison with the control (100%). For octenidine this value was 97.44% (Figure 3).

In contrast, sub-MIC of miramistin ($6.79 \pm 0.44 \mu\text{g/ml}$) and povidone iodine ($996.38 \pm 49.28 \mu\text{g/ml}$) slightly stimulated biofilm formation. The stimulating effect compared to the control was 101.43% and 101.68%, respectively.

Decamethoxine and polyhexanide in sub-MICs demonstrated the strongest effect on immature biofilm and significantly inhibited the formation of biofilm by *E. coli* by 6.48% ($p < 0.001$) and 5.15% ($p < 0.01$), respectively, compared to the control. Chlorhexidine and octenidine showed a less intensive effect. Chlorhexidine inhibited biofilm formation by 4.66% ($p < 0.01$) and octenidine – by 2.56% ($p > 0.10$) comparably to untreated control. Miramistin and povidone iodine in sub-bacteriostatic concentrations stimulated the protective reaction of microorganisms in the form of increased biofilm formation by 1.43% and 0.68%, respectively.

A negative correlation between the biofilm-forming abilities of the isolates in the presence of sub-MIC octenidine and decamethoxine and their sensitivity to them was proven. Pearson correlation coefficients for decamethoxine and octenidine were $r = -0.67$ and -0.53 ,

respectively. Thus, for these antiseptics, the inhibition of biofilm-forming properties depends precisely on the antiseptic concentration, and not on the sensitivity of *E. coli* to them.

Biofilm-forming properties of strains of *E. coli* correlated well with their sensitivity to chlorhexidine ($r = 0.49$). The properties of *E. coli* to form biofilms are poorly correlated with their sensitivity to polyhexanide ($r = 0.11$).

Discussion

Multidrug-resistant *Enterobacteriaceae* as causative agents of infectious processes of various localization have become a significant challenge for infection control [48]. WHO reported that drug-resistant microorganisms have been found all over the world and account about 50% of infections caused by *E. coli*, *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, which were resistant to majority of antibiotics [37].

Antiseptic of wounds is promising and gives a chance to control the infectious process in the wound due to the use of highly effective antimicrobial agents, compatible with the wound surface, capable of counteracting the spread multidrug-resistant pathogens (MDRPs) [25].

Taking into account global concerns about resistance to antibiotic and their limited therapeutic options, the use of antiseptics as local medication to prevent manifestation of bacterial resistance is very important [8].

The most effective antiseptics include surfactants, in particular QACs, which effect bacteria due to the amphiphilic nature of their molecule and their destructive effect on the membranes of prokaryotes. Antiseptics have

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a wider spectrum of activity compared to antibiotics. While the mechanism of action of antibiotics is to affect certain intracellular specific targets, antiseptics have multiple targets for their effects both on the surface and inside bacterial cells. In addition, antiseptics can be used in higher concentrations when applied directly to the skin, mucous membranes and wounds, thus destroying bacteria, despite the presence of antibiotic resistance [4,27].

The pandemic of global coronavirus disease (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in a significant increase of uncontrolled use of disinfectants, including QAC. Persistence of QAC sub-inhibitory concentrations on various surfaces in combination with their widespread use of rapidly increased the rate of selection of resistant bacteria, which in turn can result in a decrease of the effectiveness of modern antiseptics and disinfectants [7,19,32].

Regular monitoring of the susceptibility of MDR *Escherichia* in various forms of existence to topical agents is necessary for their reasonable rational use today and maintenance of their efficacy in the future.

Antimicrobial activity on planktonic E. coli cells MIC and MBC analysis

At the first stage of the study, we tested the effectiveness of the impact of antiseptics on planktonic forms of MDR *Escherichia*.

Maillard et al., after analyzing previous studies of the sensitivity of bacteria to modern widely used antiseptics, reported a decrease in sensitivity to all biocides, which is clearly related to the increasing prevalence of resistance determinants [33]. Nevertheless, our study revealed sufficiently high in vitro effectiveness of the tested antiseptics, which are widely used. Based on the MIC and MBC values, it can be concluded that the activity of decamethoxine and octenidine is higher. The MICs for chlorhexidine, miramistin, and polyhexanide were equivalent.

The results of the current study do not deny those from other countries. R. López-Rojas et al., showed polyhexanide bactericidal activity against all high-risk clones of MDR nosocomial pathogens (*E. coli*) at significantly lower concentrations and time of activity than those commercially used [31].

R. Alvarez-Marín et al. emphasize that OCT can be extremely useful in the eradication of emerging highly resistant Gram-negative pathogens associated with nosocomial infections, including MDR *E. coli* [1].

Ruben Barreto et al. recently reported that compared with CHG, PHMB and OCT, PVP-I had a wider antimicrobial spectrum against Gram-negative bacteria, but at

the same time, it was pointed out that CHG was found to be effective against MDR strains *E. coli* [2,6].

However, there are increasing reports of microbial resistance to CHX among clinically relevant Gram-negative bacterial species [16,45,46]. There is evidence of decrease of *E. coli* susceptibility to chlorhexidine [41].

Unfortunately, bacterial resistance is often associated with antiseptic overuse / contamination and its widespread addition to different personal care and cleaning products [16].

Chlorhexidine digluconate is a widely used healthcare cationic antiseptic, an essential drug recognized by the WHO and listed among the 300 most prescribed drugs in the United States as of 2020 [16,21]. The bis-biguanide chlorhexidine (CHG) has recently attracted the attention of researchers because its use has been associated with the emergence of stable resistance to the antibiotic of last resort, colistin [16].

The expression of efflux pumps such as the *qacA/B* gene is a well-documented mechanism resulting in elevated CHG MIC [20,33]. Matthew E. Wand et al. have shown that isolates with an MDR phenotype have a 4-fold increase in resistance to chlorhexidine. The MIC and MBC values for octenidine were low, and octenidine had a stronger effect on these mutants (with *SmvA* efflux pump). But, at the same time, it is emphasized that *SmvA* is the main efflux pump for cationic biocides in several bacterial species, and that increased efflux through *SmvA* can lead to increased chlorhexidine and octenidine tolerance.

The results of our research illustrate the same situation. The MIC and MBC values for octenidine were 1.92 and 1.53 times lower compared to chlorhexidine, respectively [46].

BS IAA and BC IAA analysis

There are currently no approved guidelines to define antiseptic resistance breakpoints from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical Laboratory Standards Institute (CLSI).

Therefore, the IAA indicator is so important for the comparative analysis of antiseptics and for assessing the appropriateness of using certain concentrations of the active substance in the working solution of the drug. Differentiation of bacteriostatic and bactericidal effect in the analysis of IAA is especially relevant at the present time, since the cidal effect of an antiseptic is more preferable to prevent the selection of resistant strains [25].

Analyzing, differentiating and comparing IAA values for antiseptics based on QAC and halogenated compounds, if their IAA (BS and BC) were >4, they were considered active. When analysing the cidal action of antiseptics

tics, it was found that the ratio of BC to BS IAAs varied in the range from 0.5 to 0.83 for QAC and halogen compound antiseptics. Povidone-iodine showed the greatest bactericidal effect, since the ratio of BC to BS IAAs was 0.83 for different concentrations of povidone-iodine. Among QAS antiseptics, decamethoxine had the highest bactericidal effect, since the ratio of BC to BS IAAs was 0.7. For chlorhexidine and polyhexanide, this ratio is 0.5, for miramistin – 0.58, for octenidine – 0.61.

IAA values were highest (IAA >4) for the antiseptics polyhexanide (0.1%), octenidine (0.1%) and decamethoxine (0.1%), since the working solutions of these drugs contain high concentrations of the active substance. At the same time, 0.05% chlorhexidine, 0.02% decamethoxine and 10% and 2% povidone-iodine demonstrated lower IAA values; but despite this, their BS and BC IAA values exceeded the threshold (4) by 6.3–8.5 times and 4.7–9.0 times for 0.02% decamethoxine and 0.05% chlorhexidine, respectively, for 10% and 2% povidone-iodine – by 7.6–9.0 times and 1.5–1.8 times, respectively, these concentrations are considered effective against MDR *E. coli*. Since the BS IAA of miramistin is above the threshold value (=5.7), while the BC IAA is not (=3.3), the feasibility of using miramistin at a concentration of 0.1% is questionable. This concentration of the drug can create conditions for the selection of resistant strains. The effectiveness of povidone-iodine 1% against MDR *E. coli* was found insufficient (BS IAA=3.6; BC IAA=3.0, both <4).

Biofilm Formation Analysis

At the next stage of the study, the task was to test the preventive activity of drugs against immature bacterial biofilms, for which sub-bacteriostatic concentrations of antiseptics were added during the biofilm formation process.

The European Wound Management Association (EWMA) has presented and recommended the TIMERS strategy to counteract biofilm-related wound infections. The abbreviation TIMERS includes such elements as T – tissue debridement, I – infection and inflammation control, M – moisture balance, E – edges, epithelization stimulation, R – Repair of tissue and regeneration and S – Social factors that impact healing [5].

Topical antibiotics are considered inadvisable or ineffective in fighting wound bacterial biofilm. Currently, in clinical routine, antiseptics, surgical debridement, maggot therapy, and antimicrobial dressings are used as countermeasures. Antiseptics, depending on the specific wound/infection, are often used in conjunction with wound debridement (T – tissue debridement) and antimicrobial dressings (pillar I – infection and inflammation control) [27].

Bacterial biofilm is an important potential virulence factor contributing to pathogen invasion and persistence, which has been highlighted in many studies. However, routine clinical microbiology study targets only on planktonic microbial forms, without taking into account the possibility of biofilm formation [14,24].

Thus, assessment of the biofilm production capabilities and study of the influence of antiseptics on its formation is an important step in microbiological research.

An antiseptic based on halogenated compounds povidone-iodine and QAC antiseptics affected the stage of biofilm formation in MDR *E. coli* strains differently. Previous studies have reported that octenidine and chlorhexidine have the highest activity against mature biofilms of clinical MDR *E. coli* [17]. Also, Grzegorz Krasowski et al. reported high in vitro activity of polyhexanide and octenidine against biofilm formed by wound pathogens [27].

Recognizing the importance of microbial biofilms, we studied the effectiveness of antiseptics against bacteria in biofilms, namely their effect on biofilm formation (action on immature biofilm). Our study showed that chlorhexidine, decamethoxine and polyhexanide had high anti-biofilm formation activity against MDR *E. coli* clinical isolates.

In the presence of sub-bacteriostatic concentrations of chlorhexidine the biofilm-forming properties of *E. coli* strains had positive correlation with their susceptibility ($r=0.49$). In the presence of sub-bacteriostatic concentrations of polyhexanide, the biofilm-forming properties of studied by us clinical strains had less, but positive correlation with their susceptibility to this antiseptic ($r=0.11$).

Chlorhexidine and polyhexanide should be considered as the most effective anti-biofilm agents due to the significant suppression of biofilm formation by *E. coli* isolates and a positive correlation with their sensitivity to antiseptics.

Decamethoxine at sub-bacteriostatic concentrations most effectively inhibited biofilm formation, and the correlation was negative ($r=-0.67$), which indicates that the ability to effectively inhibit biofilm formation depends on a certain concentration of the drug and not on the increased susceptibility of a particular strain to an antiseptic. For octenidine, a negative correlation was also shown. Thus, decamethoxine and octenidine are able to prevent biofilm formation by *E. coli* strains in a concentration dependent manner.

Previously, M. Loose, et al reported that antiseptics containing the biocides polyhexanide and octenidine were most effective against *E. coli*. Moreover, the determination of anti-biofilm activity demonstrated that Pron-

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tosan® (polyhexanide), as well as Octenisept® (octenidine), were able to prevent the formation of *E. coli* biofilms in a concentration-dependent manner [30].

The fact that PVP-I is also very effective at eradicating biofilms of Gram-negative bacteria was recently reported by Ruben Barreto et al [6].

On the other hand, sub-bacteriostatic concentrations of povidone-iodine and miramistin have stimulated biofilm formation by clinical isolates. Thus, the use of these antiseptics in concentrations lower than MIC can lead to the stimulation of the protective mechanisms of bacteria as biofilm formation.

Many researchers come to the conclusion that the ability to form biofilms is a key pathogenicity factor, the presence of which contributes to successful and stable colonization of the wound site, regardless of the MDRO phenotype [12].

Our study confirms this pattern in relation to clinical strains of *E. coli* with multidrug resistance.

Diagnosis of infection requires qualified specialists, appropriate equipment and time. In order to limit the spread of antibiotic-resistant strains, it is necessary to identify microorganisms and determine antimicrobial susceptibility patterns. These important aspects are often underestimated [39].

Conclusions

Clinical strains of *E. coli* were most susceptible to octenidine and decamethoxine.

IAA values were the highest (IAA >4) for polyhexanide (0.1%), octenidine (0.1%) and decamethoxine (0.1%), since the working solutions of these drugs contain high concentrations of the active substance.

Conducting a comparative analysis of antiseptics by indicator of their IAA, it was found that the feasibility of using miramistin at a concentration of 0.1% is questionable as the BS IAA is above the threshold value, while the BC IAA is not, which can create selective conditions for the emergence of resistant strains. The effectiveness of povidone-iodine 1% against MDR *E. coli* was found insufficient since BS IAA and BC IAA were below the threshold.

On estimating the anti-biofilm effect of polyhexanide, chlorhexidine and decamethoxine had demonstrated the most pronounced effect of these antiseptics on immature biofilms. Miramistin and povidone-iodine in sub-bacteriostatic concentrations stimulated biofilm formation.

Chlorhexidine and polyhexanide should be considered as the most effective anti-biofilm agents due to the significant suppression of biofilm formation by *E. coli* isolates and a positive correlation with their sensitivity to antiseptics.

Decamethoxine at sub-bacteriostatic concentrations most effectively inhibited biofilm formation, and the correlation was negative, which indicates that the ability to effectively inhibit biofilm formation depends on a certain concentration of the drug and not on the increased susceptibility of a particular strain to an antiseptic.

Based on the analysis of all conducted studies, 0.1% and 0.02% decamethoxine, 0.05% chlorhexidine, 0.1% octenidine, 0.1% polyhexanide, 10% and 2% povidone-iodine are the most active against MDR clinical isolates of *E. coli*.

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