

## Effectiveness of antimicrobial drugs against planktonic and biofilm forms of bacteria isolated from children with urinary tract infections

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### Key words:

biofilms, urinary tract infections, microbial drug resistance.

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**The aim** of our work is to determine the susceptibility of clinical isolates of bacteria in biofilm to the most common antimicrobial drugs used in clinical practice.

**Materials and methods.** By means of serial dilutions of antimicrobial drugs, we analyzed the effectiveness of amoxicillin clavulanate, ceftriaxone, cefixime, gentamicin, furazidin and silver nanoparticles on planktonic and biofilm forms of 60 strains of bacteria isolated from children with lower urinary tract infections, acute and chronic pyelonephritis.

**Results.** Through the use of serial dilutions method it was found that 77 % of planktonic strains were susceptible to amoxicillin clavulanate, 70 % – to ceftriaxone, 47 % – to cefixime, 78 % – to gentamicin and 92 % – to furazidin. The percentage of the strains susceptible to amoxicillin clavulanate after biofilm formation was 63 %, to ceftriaxone – 17 %, to cefixime – 13 %, to gentamicin – 37 %, to furazidin – 80 %.

**Conclusions.** It was established that biofilm bacteria gain resistance to all the investigated drugs, but the change of susceptibility manifests itself in varying degrees. The number of resistant strains of bacteria depends on the form of the urinary tract infection, in acute and chronic pyelonephritis the number of resistant strains is much greater than in infection of the lower urinary tract. Planktonic and biofilm forms of bacteria were the most effectively influenced in vitro by furazidin, amoxicillin clavulanate and gentamicin. Cephalosporins (ceftriaxone, cefixime) were less effective against both forms of bacteria.

### Ключові слова:

біоплівка, інфекція сечовивідних шляхів, резистентність мікроорганізмів.

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## Ефективність антимікробних препаратів проти планктонних і біоплівкових форм бактерій, що виділені від дітей з інфекцією сечових шляхів

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**Мета роботи** – визначення чутливості клінічних ізолятів бактерій у складі біоплівки до найпоширеніших антимікробних препаратів, що використовуються у клінічній практиці.

**Матеріали та методи.** За допомогою серійних розведень антимікробних препаратів проаналізували ефективність амокцициліну клавуланату, цефтріаксону, цефіксиму, гентаміцину, фуразидину та наночастинок срібла на планктонних і біоплівкових формах 60 штамів бактерій, що виділені від дітей з інфекціями нижніх сечових шляхів, гострим пієлонефритом і рецидивом хронічного пієлонефриту.

**Результати.** За допомогою методу серійних розведень встановили, що 77 % планктонних штамів чутливі до амокцициліну клавуланату, 70 % – цефтріаксону, 47 % – цефіксиму, 78 % – гентаміцину, 92 % – фуразидину. Частка штамів, що чутливі до амокцициліну клавуланату після утворення біоплівки протягом 24 годин у 96-лунковому планшеті, дорівнювала 63 %, до цефтріаксону – 17 %, цефіксиму – 13 %, гентаміцину – 37 %, фуразидину – 80 %.

**Висновки.** Бактерії, що містяться в біоплівці, посилюють стійкість до всіх досліджених препаратів, але зміна чутливості проявляється по-різному. Кількість резистентних штамів бактерій залежить від форми інфекції сечовивідних шляхів, при гострому та хронічному пієлонефриті кількість резистентних штамів більша, ніж при інфекції нижніх сечових шляхів. На планктонні та біоплівкові форми бактерій найефективніше впливали in vitro фуразидин, амокцицилін клавуланат і гентаміцин. Цефалоспорини (цефтріаксон, цефіксим) були менш ефективними щодо обох форм бактерій.

### Ключевые слова:

биопленка, инфекция мочевыводящих путей, лекарственная устойчивость микроорганизмов.

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## Эффективность антимикробных препаратов против планктонных и биопленочных форм бактерий, выделенных от детей с инфекцией мочевых путей

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**Цель работы** – определение чувствительности клинических изолятов бактерий в составе биопленки к наиболее распространенным антимикробным препаратам, используемым в клинической практике.

**Материалы и методы.** С помощью серийных разведений антимикробных препаратов проанализировали эффективность амокцициллина клавуланата, цефтриаксона, цефиксима, гентамицина, фуразидина и наночастиц серебра на планктонных и биопленочных формах 60 штаммов бактерий, выделенных от детей с инфекциями нижних мочевых путей, острым и хроническим пиелонефритом.

**Результаты.** С помощью метода серийных разведений установлено, что 77 % планктонных штаммов чувствительны к амокцициллину клавуланату, 70 % – цефтриаксону, 47 % – цефиксиму, 78 % – гентамицину, 92 % – фуразидину. Доля штаммов, чувствительных к амокцициллину клавуланату после образования биопленки, составляла 63 %, цефтриаксону – 17 %, цефиксиму – 13 %, гентамицину – 37 %, фуразидину – 80 %.

**Выводы.** Бактерии, содержащиеся в биопленке, усиливают устойчивость ко всем исследованным препаратам, но изменение чувствительности проявляется в разной степени. Число резистентных штаммов бактерий зависит от формы инфекции мочевыводящих путей, при остром и хроническом пиелонефрите количество резистентных штаммов больше, чем при инфекции нижних мочевых путей. На планктонные и биопленочные формы бактерий наиболее эффективно влияли *in vitro* фуразидин, амоксициллин клавуланат и гентамицин. Цефалоспорины (цефтриаксон, цефиксим) были менее эффективными в отношении обеих форм бактерий.

Resistance to antimicrobial agents is acknowledged a worldwide threat to human health: in the United States alone, every year at least 2 million cases of serious bacterial infections are registered that are resistant to treatment with one or more antibiotics, out of which 23 000 are fatal [2]. EARS-Net 2016 data show that antimicrobial resistance remains a serious threat to public health in Europe [6].

At the same time The National Institute of Health in USA estimates: 80 % of all microbial infectious diseases are associated with biofilms – groups of microbial cell adherent to themselves and/or surface and enclosed in self-secreted slime like matrix. Several of scientific reports indicate importance of biofilm in urinary tract infections (UTI) – development of chronic infectious and demonstrate increased tolerance to disinfectants and antibiotics [3,10].

The investigations of the majority of European countries [4,5,9] indicate that more than half of antibiotics are prescribed at the level of primary healthcare. Children are the largest part of the population aided at this level. Moreover, up to 50 % of UTI in young children are missed in primary care [1] and not prescribed antibiotics at first presentation [7].

According to statistic reports of the Ministry of Healthcare of Ukraine, prevalence of renal and urinary tract diseases in children over the past 5 years in Ukraine significantly increased, namely: from 40 to 56 per 1000 child population. Given the fact that children are more vulnerable to acute and chronic complications, including kidney scarring and renal failure, they require immediate treatment using appropriate antibiotics. According to the current Ukrainian national treatment protocols, only the minimum inhibitory concentration and minimum bactericidal concentration of antibiotics is indicated to planktonic forms of bacteria, and the fact that resistance of biofilm forms of bacteria is dramatically increases [8] is ignored.

## The aim

The aim of our work is to determine the susceptibility of clinical isolates of biofilm bacteria to the most common antimicrobial drugs used in clinical practice.

## Materials and methods

The study was held at the Department of Pediatrics No. 4 and the Department of Microbiology, Virology and Immunology of Bogomolets National Medical University. In the course of the study, the analysis was conducted of amoxicillin clavulanate, ceftriaxone, cefixime, gentamicin, furazidin and silver nanoparticles effectiveness against 60 strains of bacteria isolated from 85 patients who received medical treatment in Children's Clinical Hospitals No. 6 and No. 7 in the city of Kyiv within the period from September 2016 until January 2017. The patients were divided into three groups.

*Group No. 1:* 46 patients formed the "Lower Urinary Tract Group". The criteria for inclusion in the group No. 1 were: complaints of painful urination, dysuria, urgency urinary, lower abdominal pains and leukocyturia at relatively good performance status of the patient. 23 subsequently analyzed strains of bacteria were isolated from the 1 group patients.

*Group No. 2:* 19 patients were included in the "Acute Pyelonephritis Group". The criteria for inclusion in the group No. 2 were complaints of kidney pain, increased body temperature up to low-grade fever or higher, abdominal pain, headache, dizziness, nausea vomiting and acute pyelonephritis characteristic changes in physical, instrumental and laboratory examination. 19 strains of bacteria were isolated from the 2 group patients.

*Group No. 3:* 20 patients were included in the "Chronic Pyelonephritis Recurrence Group". The criteria for inclusion in the group No. 3 were clinical signs of acute pyelonephritis and past medical history of pyelonephritis recurrence episodes. 18 strains of bacteria were isolated from the 3 group patients.

A bacteriologic study with an extended antibioticogram of the urine samples of all patients was conducted at the Laboratory of Microbiology, Virology and Mycology of the State Institution "Institute of Urology of National Academy of Medical Sciences of Ukraine".

### *Determination of biofilm and planktonic bacteria forms susceptibility to antibiotics*

Susceptibility of planktonic forms of bacteria to antibiotics was detected by the disc test with the use of Mueller–Hinton agar. We also determined the minimal inhibitory concentration by the method of standard serial dilutions in the broth using 96-well plates for antibiotics ceftriaxone, cefixime, amoxicillin clavulanate, gentamicin, as well as for furazidin and silver nanoparticles.

The assessment of the susceptibility results was carried out in accordance with CLSI protocols.

Susceptibility of biofilm forms to antibiotics was determined as follows: after 24-hour incubation, 200 µl of nutrient broth, bacterial culture (0.5 according to McFarland) was diluted in the proportion of 1:100 in 96-well plate. The wells were washed three times with isotonic solution. 200 µl of nutritive broth with 1% of glucose and antibiotic in double dilutions were added to the biofilm formed on the bottom. After 12 hours of incubation at 37 °C bacterial growth in the wells of the plate was assessed through optical density of the broth determination with microtiter plate reader (Humareader) at a wavelength of 630 nm.

Analysis of the results was performed using the SPSS program version 12. The results were considered statistically significant at  $P < 0.05$ .

## Results

Out of the isolated bacteria *E. coli* prevailed – 53 %, *S. epidermidis* – 20 %, *E. faecalis* – 12 %, 15 % – others.

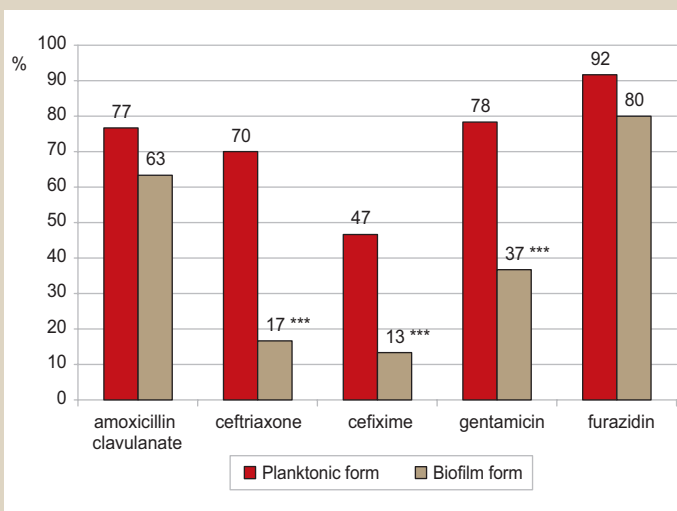


Fig. 1. Amount of bacteria in planktonic and biofilm forms susceptible to antimicrobial drugs (determined by the method of serial dilutions).

\*\*\*: P < 0.001 (difference between planktonic and biofilm forms of bacteria).

Table 1. Resistance to antimicrobial drugs among planktonic forms of clinical isolates from children with urinary tract infection

Antibiotic	Number of resistant strains (disc test), %	Number of resistant strains (dilutions method), %
Amoxicillin clavulanate	19.0	23.0
Ceftriaxone	11.0	30.0*
Cefixime	35.0	43.0
Gentamicin	22.0	22.0
Furazidin	8.0	8.0

\*: P < 0.05.

Antibiotic susceptibility of planktonic forms of 60 clinical isolates was determined through the method of serial dilutions and the disc test.

Through the use of serial dilutions method it was revealed that 77 % of planktonic strains were susceptible to amoxicillin clavulanate, 70 % – to ceftriaxone, 47 % – to cefixime, 78 % – to gentamicin and 92 % – to furazidin.

Only ceftriaxone demonstrated statistically significant difference in the results received by the method of serial dilutions and the disc test, other antibacterial agents demonstrated similar activity (Table 1).

After biofilm formation, the number of strains resistant to antimicrobial drugs statistically significantly increased (except furazidin). The number of the strains susceptible after biofilm formation to amoxicillin clavulanate equaled 63 %, to ceftriaxone – 17 %, to cefixime – 13 %, to gentamicin – 37 %, to furazidin – 80 %.

Change in susceptibility to antibiotics depending on the microorganism form is shown in the Fig. 1.

The investigation of silver nanoparticles activity concerning the investigated strains, conducted at the Institute of Life and Environmental Sciences of Ukraine, showed that they possess antibacterial activity against planktonic forms at the level of 100 mg/l (90 % of strains), for 10 % of the strains inhibitory concentration was at the level of 10 mg/l. Bacteria in biofilm became more resistant to silver nanoparticles, namely: 62 % of the strains retained the ability to multiply at a concentration of 100 mg/l, for 38 % of biofilm bacteria the minimal inhibitory concentration ranged from 10 mg/l to 50 mg/l (Fig. 2).

## Discussion

Bacteria in biofilm can increase their resistance to antibacterial drugs aided by several mechanisms. One of

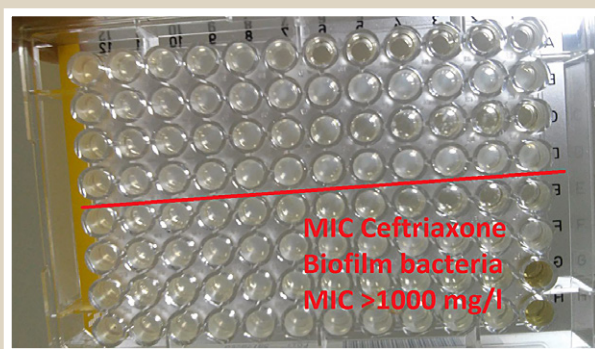
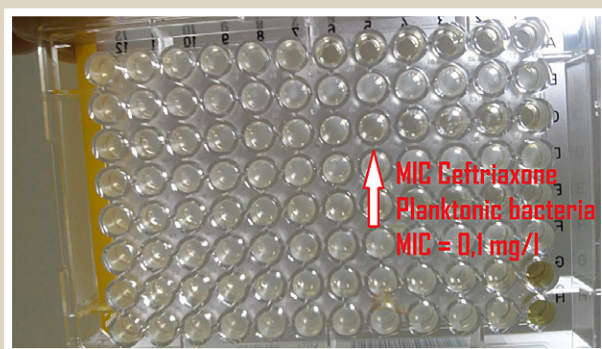


Fig. 2. Change in inhibitive concentration in planktonic and biofilm bacterial forms.

Table 2. Susceptibility to antimicrobial drugs among planktonic and biofilm forms of bacteria divided into clinical groups

Antibiotics	Group 1 (n = 23)		Group 2 (n = 19)		Group 3 (n = 18)	
	Number of susceptible strains, planktonic form, %	Number of susceptible strains, biofilm form, %	Number of susceptible strains, planktonic form, %	Number of susceptible strains, biofilm form, %	Number of susceptible strains, planktonic form, %	Number of susceptible strains, biofilm form, %
Amoxicillin clavulanate	78	74	74	63	78.0	50.0*
Ceftriaxone	83	30*	47	5*	79.0	11.0*
Cefixime	43	13*	32	16	67.0	6.0*
Gentamicin	87	52*	89	21*	72	28*
Furazidin	96	83*	89	84	89	72.0

\*: P < 0.05 (difference between planktonic and biofilm strains in each group).

them prevents molecules of antibiotic from penetrating into biofilm matrix [3]. The probability of this mechanism is indirectly demonstrated in this study, as the increase of the biofilm resulted in the loss of susceptibility to a greater range of antibiotics.

The data on susceptibility to antibiotics, received through the disc method, are almost similar to the data received by the method of serial dilutions of antibiotics and differ only with respect to the indices of susceptibility received by the method of serial dilutions with biofilm bacterial form. The results obtained indicate that the disc method is not able to demonstrate a credible level of biofilm bacterial form susceptibility to antibiotics. Considering the significant difference in susceptibility to antibiotics between the two forms of bacteria, the definition of biofilm-forming bacteria susceptibility by standard methods is more than approximate.

When testing the susceptibility of biofilm-forming bacteria to antimicrobial drugs, the biofilms were formed at the bottom of a 96-well plate (plastic being an adhesive surface). When testing susceptibility of biofilm-forming bacteria, (clinical strains of *P. mirabilis*, *E. coli*, and *S. epidermidis*) biofilms of which were formed on the surface of latex catheters, we found that the minimal inhibitory concentration of ceftriaxone had not changed depending on the surface of biofilm formation. The opposite results were obtained for gentamicin: concentration that had inhibited the biofilm growth on a catheter was 4 times less than the inhibitory concentration for the biofilms formation on plastic. Considering that the process of film formation is extremely sensitive to modeling conditions, the exactitude of susceptibility to antibiotics results may depend not only on bacterial form, but also on a surface a biofilm is formed on.

## Conclusions

Biofilm forms of clinical isolates of bacteria are more resistant against antibiotics and silver nanoparticles than planktonic bacterial forms.

The size of bacterial biofilm affects the range of antimicrobial drugs susceptibility of the bacteria. The greater the biofilm size is, the lesser number of antibacterial drugs it is susceptible to.

The study has shown that the planktonic and biofilm forms of bacteria were the most effectively influenced in vitro by furazidin, amoxicillin clavulanate and gentamicin. Cephalosporins (ceftriaxone, cefixime) were less effective against both forms of bacteria.

Silver nanoparticles demonstrate antibacterial effect on planktonic form of clinical isolate in high concentrations (MIC = 100 mg/l). After biofilm formation the bacteria become more resistant to the nanosilver effect.

**Further research.** To create and probe more accurate model for antibiotic susceptibility testing (flow models etc) in order to obtain the most reliable data as our previous results demonstrate that biofilm mode as well as surface properties could affect the antibiotic susceptibility levels.

To modify treatment of patients with different forms of UTI taking into account the data from biofilm bacteria susceptibility testing and perform comparative analysis to determine whether this approach could contribute to more successful treatment of UTI.

**Conflicts of interest:** authors have no conflict of interest to declare.  
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