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Research Article

Antibiotic Resistance of Planktonic and Biofilm Forms of *E. coli* to Soluble Nitrofurans

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ABSTRACT

Urinary Tract Infections (UTIs) are a significant cause of morbidity. *Escherichia coli* (*E.coli*) is the most frequent agent (about 80%) of UTIs in humans. Several studies have demonstrated that Biofilm (BF) cells are more resistant to antimicrobial agents than planktonic bacterial cells, which contributes to the persistence of infections. The study aimed to reveal the difference between antibiotic resistance of planktonic and BF form *E.coli* causing UTI. The study was conducted from November 2019 till February 2020 at Vitebsk State Medical University, the Republic of Belarus. The strains were tested for susceptibility to a Furagin soluble (Furaginum soluble = potassium N-(5'-nitro-2'-furilalliliden)-1-aminogidantoin with magnesium carbonate), the novel nitrofuran derivate synthesized and manufactured in Latvia since 1979 (Furamags ®).

The results were based on the minimal concentrations that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates' growth.

38 strains of *E.coli* isolated from patients with uncomplicated UTI had the ability to form a BF. MIC_{50} and MIC_{90} values for planktonic *E.coli* isolates were 4 and 8 μg/ml. MIC_{50} and MIC_{90} values for BF *E.coli* isolates were 32 and 128 μg/ml.

34 strains of *E.coli* isolated from patients with complicated UTI had the ability to form a BF. MIC_{50} and MIC_{90} values for planktonic *E.coli* isolates were 2 and 4 μg/ml. MIC_{50} and MIC_{90} values for BF *E.coli* isolates were 16 and 32 μg/ml.

Furagin soluble has lower MIC as compared with nitrofurantoin and could be used not only for treating cystitis and pyelonephritis but also for treating and preventing catheter-related UTI.

INTRODUCTION

Urinary Tract Infections (UTIs) are a significant cause of morbidity that affects persons of all ages. Approximately 40% of women have had a UTI at some time in their lives. *Escherichia coli* (*E. coli*) is the most frequent agent (about 80%) of UTIs in humans and one of the most common causes of Gram-negative nosocomial infections. Urinary catheters destroy natural barriers and provide a nidus for infection by serving as a substrate for biofilm formation. Several studies have demonstrated that biofilm cells are more resistant to antimicrobial agents than planktonic bacterial cells. The resistance of biofilms to antibiotics contributes to the persistence of infections, such as those associated with implanted devices.

Biofilm formation is an important process for the survival of microbial pathogens in the environment or the mammalian host. Biofilm is a three-dimensional community of bacteria with intricate architecture that lives on surfaces and is encapsulated in a network of hydrated polysaccharides, proteins, and DNA. Persistent bacterial infection and increased antibiotic resistance can often be attributed to biofilm formation on host tissues and implants [1]. The formation of a biofilm begins in stages. In stage one, there is a transient binding of planktonic bacteria to a solid surface with characteristic adhesion. In stage two, there is aggregation and formation of microcolonies surrounded by protective secreted molecules known as Extra Polymeric Substance (EPS) matrix. Finally, there is dispersal that involves shedding from the mature biofilm as planktonic bacteria or as microcolonies. This dispersal stage may promote further colonizing the host with biofilms. This may ultimately benefit the organisms due to limited nutrient availability and waste accumulation [2].

One biofilm-specific property is antibiotic resistance. Resistance to antimicrobial agents is the most important cause of noneffective therapy of biofilm-associated infections, and, importantly, it is multifactorial. The biofilm matrix may be a diffusion barrier to some antibiotics; other factors are the altered microbial physiology and the biofilm environment. Antibiotic activity against biofilm microorganisms cannot be accurately determined using standard CLSI (Clinical and Laboratory Standards Institute) broth microdilution methods for susceptibility testing since these techniques are based on exposing planktonic organisms to the antimicrobial agent. Instead, the biofilm is exposed to the antimicrobial agent, removed from the attached substratum, homogenized, and quantitated as viable cell counts. In the development of a model biofilm system, substratum and hydrodynamics are factors to be considered in addition to culture medium and inoculum. Antibiotic resistance in biofilms is due to multiple mechanisms: intrinsic resistance of the microorganisms involved, decreased antibiotic diffusion through the extracellular matrix, decreased growth of the organism due to nutrient limitation, and activation of the stress response [3].

Importantly, several studies have shown that subminimal inhibitory concentrations of some antibiotics can induce biofilm formation in vitro, a process that may have clinical relevance. The majority of well-documented studies investigating the mechanisms of antibiotic-induced biofilm formation have been performed using the common device associated pathogens, including *S.aureus*, *S.epidermidis*, *E.coli*, and *P.aeruginosa* [4].

The study aimed to reveal the difference between antibiotic resistance of planktonic and biofilm form *E.coli* causing complicated and uncomplicated UTI as defined in the EAU (European Association of Urology) Guideline on Urological Infections [5].

MATERIALS AND METHODS

The study was conducted during the period from November 2019 until February 2020 at Vitebsk State Medical University (Vitebsk, Republic of Belarus). 38 strains of *E.coli* isolated from patients with uncomplicated UTI and 34 hospital strains of *E.coli* isolated from patients with complicated UTI were included in the study. Inclusion criteria were UTI and no prior antibiotic treatment. The exclusion criteria were prior antibiotic treatment. All authors participating in the present study read the Declaration of Helsinki and the study was designed according to its guidelines.

After sample collection by catheterization, the material was immediately transported to the laboratory and further processed within 2 hours at the most. Samples were cultivated on nutrient agar at 37 °C for 24 h. Negative control samples were incubated for another 24 h to ensure no growth. Purification was performed by serial subcultivation on nutrient agar to obtain single colonies in pure culture. The growth of bacteria was considered significant if the number of Colony-Forming Units (CFU)/ml was $\geq 10^3$.

The gold standard method for biofilm detection was carried out as described by Christensen, et al. [6]. In brief, a colony of *E.coli* was isolated from a fresh agar plate and inoculated in 2 ml of Mueller Hinton broth. The broth was incubated overnight at 37 °C. The culture was then diluted to 1:100 with a fresh medium. A sterile individual plate with 96 flat-bottom polystyrene wells was filled

with 200 µL of the diluted culture. The control organisms were also processed similarly. The plate was incubated at 37 °C for 24 hours. After incubation, the contents of each well were removed by gentle tapping. The wells were washed with 200 µL of phosphate buffer saline to remove free-floating bacteria. Biofilms formed by bacteria adherent to the wells were fixed by 2.5% glutaraldehyde solution and stained with 0.25% Crystal Violet (CV). Excess stain was washed gently, and the plate was kept for drying; after that 200 µl of 33% acetic acid was added to the wells for 10 minutes. The biofilm-forming capacity was further confirmed spectrophotometrically at OD620. The cutoff OD for the spectrophotometric determination of biofilm formation was calculated as three standard deviations above the mean OD of the negative control. Classification of bacterial adherence was calculated as per given formulas for all tested bacteria as one of three possible categories: weak (ODc < OD ± 2OD), moderate (2ODc < OD ± 4OD), and strong (4ODc < OD) biofilm formation [7]. Tests for susceptibility to antibiotics were performed by the broth microdilution method with fresh Mueller Hinton broth, as recommended by the EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines and ISO 20776-1:2019 (Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices-Part 1: Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases). Biofilm MIC assays were performed when wells contained approximately 10^4 to 10^6 bacteria growing as a biofilm following conditions developed from the procedure described above. By using laser scanning confocal microscopy, we have established that biofilms are produced at this level in wells. These biofilms can then be used for the assessment of antimicrobial activities using the microdilution method. The strains were tested for susceptibility to a Furagin soluble. The Antimicrobial susceptibility testing results were evaluated based on the minimal concentrations that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates' growth.

The data were subjected to statistical processing using the STATISTICA 10.0 application software package. Quantitative statistics were described using the Shapiro-Wilk test. When distributing a characteristic other than normal, calculated by the median (Me), the lower 25th (LQ), and upper 75th quartile (UQ).

RESULTS

Uncomplicated UTI

38 strains of *E.coli* isolated from patients with uncomplicated UTI had the ability to form BF.

MIC_{90} of antibiotics was 16 times higher in *E.coli* isolates selected from the biofilm population than the MIC_{90} of isolates from the planktonic cultures.

Furagin soluble MIC_{50} and MIC_{90} values for planktonic E.coli isolates were 4 and 8 µg/ml. Furagin soluble MIC_{50} and MIC_{90} values for biofilm E.coli isolates were 32 and 128 µg/ml, 87% of the biofilm *E.coli* isolates were susceptible to Furagin soluble (Table 1).

Complicated UTI

34 strains of *E.coli* isolated from patients with complicated UTI had the ability to form BF.

MIC_{90} of antibiotics was 8 times higher in *E.coli* isolates selected from the biofilm population than the MIC_{90} of isolates from the planktonic cultures.

Furagin soluble MIC_{50} and MIC_{90} values for planktonic *E.coli* isolates were 2 and 4 µg/ml. Furagin soluble MIC_{50} and MIC_{90} values for biofilm *E.coli* isolates were 16 and 32 µg/ml, 100% of the biofilm *E.coli* isolates were susceptible to Furagin soluble (table 2).

DISCUSSION

Previous studies [8-10] have shown that the cells in a biofilm were more resistant to antimicrobial agents compared to free-floating cells. The use of antibiotics for the routine treatment of UTI is generally able to eliminate planktonic cells, while biofilm cells can spread further. Biofilm formation is an important virulence factor of *E.coli*.

E.coli strains in both uncomplicated and complicated UTI were able to produce biofilm. 87% of the biofilm *E.coli* isolates in uncomplicated UTI were susceptible to Furagin soluble against 100% of the biofilm *E.coli* isolates in complicated UTI. MIC_{90} was higher for biofilm forms in both uncomplicated and complicated UTI (16 times and 8 times, accordingly), though all within achievable therapeutic urine concentration range. Furagin soluble has lower MIC as compared with nitrofurantoin [11] and high *E.coli* sensitivity even in biofilm forms.

CONCLUSION

There is a moderate difference between antibiotic resistance of planktonic and biofilm form *E.coli* causing complicated and uncomplicated UTI against Furagin soluble. Results suggest that Furagin soluble can be successfully utilized in clinical practice not only for treating cystitis and pyelonephritis [12,13] but also for treating and preventing catheter-related and other complicated UTI, where the likelihood of biofilm formation is much higher.

Table 1: *E.coli* isolates' susceptibility to Furagin soluble from patients with uncomplicated UTI.

Furagin soluble	S, %	I, %	R, %	MIC_{50} µg/ml	MIC_{90} µg/ml	Geometric mean MIC, µg/ml	Min / max	Quartile
Planctonic form	100	0	0	4	8	3,39	1/8	2; 8
BF form	87	0	13	32	128	29,21	8/128	16; 64

Table 2: *E.coli* isolates' susceptibility to Furagin soluble from patients with complicated UTI.

Furagin soluble	S, %	I, %	R, %	MIC_{50} µg/ml	MIC_{90} µg/ml	Geometric mean MIC, µg/ml	Min/ max	Quartile
Planctonic form	100	0	0	2	4	2,08	1/8	2;2
BF form	100	0	0	16	32	12,02	2/64	8;16

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