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ORIGINAL PAPER

A Novel Approach to Study the Effect of Ciprofloxacin on Biofilms of *Corynebacterium* spp. Using Confocal Laser Scanning Microscopy

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Abstract

Non-diphtherial corynebacteria are Gram-positive rods that cause opportunistic infections, what is supported by their ability to produce biofilm on artificial surfaces. In this study, the characteristic of the biofilm produced on vascular and urological catheters was determined using a confocal microscopy for the most frequently involved in infections diphtheroid species. They were represented by the reference strains of *Corynebacterium striatum* ATCC 6940 and *C. amycolatum* ATCC 700207. The effect of ciprofloxacin on the biofilm produced by the antibiotic-susceptible *C. striatum* strain was evaluated using three concentrations of the antimicrobial agent (2×, 4×, and 6× the MIC – the Minimum Inhibitory Concentration). The basis for the interpretation of results was the statistical analysis of maximum points readings from the surface comprising a total of 245 areas of the biofilm image under the confocal microscope. It was observed that ciprofloxacin at a concentration equal to 4×MIC paradoxically caused an enlargement of areas with live bacteria within the biofilm. Biofilm destruction required the application of ciprofloxacin at a concentration higher than 6×MIC. This suggests that the use of relatively low doses of antimicrobial agents may increase the number of live bacteria within the biofilm, and further facilitate their detachment from the biofilm's structure thus leading to the spread of bacteria into the bloodstream or to the neighboring tissues. The method of biofilm analysis presented here provides the original and novel approach to the investigation of the diphtheroid biofilms and their interaction with antimicrobial agents.

Key words: *Corynebacterium amycolatum, Corynebacterium striatum,* biofilm, ciprofloxacin, confocal microscopy

Introduction

Corynebacterium striatum is a member of microbiota of the human skin and mucous membranes but it can also cause opportunistic infections. These infections typically involve immunocompromised patients and are associated with circumstances favoring bacterial translocation from the mucous membranes or the skin through catheters and intubation or resulting from treatment that lead to disruption of tissue integrity. An important feature of *Corynebacterium* species, which promotes infections, is their ability to produce a biofilm on artificial surfaces (Mattos-Guaraldi and Formiga 1991; Kwaszewska et al. 2006; Sousa et al. 2011; Gomes et al. 2013; de Souza et al. 2015; Leal et al. 2016, Qin et al. 2017; Kang et al. 2018). When it occurs, the intravenous biomaterial's surface colonization may lead to a bloodstream infection (Martínez-Martínez et al. 1995; Moore and Norton 1995; Campanile et al. 2009), urinary tract infections (Soriano et al. 2009), infections associated with the implant surface (Merhej et al. 2009) or as the result of intubation can cause respiratory tract infections (Williams et al. 2012; Wojewoda et al. 2012). A subacute and slow process of infection often causes diagnostic problems. Antimicrobial agents are administered, but due to the occurrence of multidrug-resistant strains (Campanile et al. 2009) these infections may cause serious therapeutic problems. Antimicrobials do not destroy bacterial biofilm when applied at a concentration equal MIC and subMICs (Bridier et al. 2011; Gomes et al. 2013).

Revealing the effect of antimicrobial agents on biofilms produced by opportunistic *Corynebacterium* species is particularly important because of poorly understood process of biofilm formation by this group of bacteria and the effect of antimicrobial agents on this bacterial structure. The reference strains of *C. striatum* ATCC 6940 and *C. amycolatum* ATCC 700207 were

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used in the study to investigate their ability to produce biofilm on catheters' surface. *C. striatum* ATCC6940, which is susceptible to all antimicrobial agents, was used to analyze the effects of ciprofloxacin on the resultant biofilm structure. This antimicrobial agent inhibits bacterial DNA synthesis through its effect on the A subunit of gyrase and is used as one of options for treatment of infections caused by these bacteria.

Experimental

Materials and Methods

The in vitro biofilm formation on intravenous, urinary catheter, and glass surface. The inoculum of 0.5 McFarland turbidity was prepared using a 24-hour culture of *C. striatum* ATCC 6940 and *C. amycolatum* ATCC 700207 strains grown on the Columbia Agar medium with 5% sheep blood (bioMérieux), and was subsequently diluted 1:100 with the TSB (Tryptase-Soy Broth, bioMérieux) supplemented with 5% FCS (Fetal Calf Serum, Sigma). The approximately 1 cm fragments, cut lengthwise from vascular (Exeflon) and urological (silicon, RÜSCH) catheters were placed in the medium with bacteria prepared in the LAB-TEK Chamber Slide SYSTEM (Nunc) containers and the incubation was carried out for 48 hours at 37°C under aerobic conditions. After this period, the liquid medium was removed and the biofilm was gently washed with the PBS (Phosphate-Buffered Saline) solution to remove free-floating bacteria. In the next step, SYTO-13 (Sigma) was used for the biofilm staining according to the manufacturer's instructions (1 μg SYTO-13 per 1 ml of the PIPES buffer for 15 minutes at room temperature). In a similar manner, development of the biofilm on the surface of glass (glass coverslip placed in the LAB-TEK Chamber Slide System container) for *C. amycolatum* and *C. striatum* was tested. The biofilm surface was analyzed with a confocal microscope – LSM 5 PASCAL (Zeiss), with 50× magnification, and the images were taken and archived.

The effect of ciprofloxacin on the *C. striatum* ATCC 6940 biofilm produced *in vitro*. The reference strain *C. striatum* ATCC 6940 is susceptible to antimicrobial agents including ciprofloxacin (MIC = 0.125 μg/l). The biofilm was developed on the surface of the LAB-TEK Chaber Sidle System (Nunc) in the TSB with 5% FCS, using an inoculum of 0.5 McFarland turbidity, diluted in the medium (1:100). The incubation was carried out for 48 hours at 37°C under aerobic conditions. After the incubation period, solutions of ciprofloxacin (Ciprofloxacin, Polfa, Poland) were added in three increasing concentrations: MIC2 = 2 × MIC (i.e., 0.25 μg/l); MIC4 = 4 × MIC (0.5 μg/l); MIC6 = 6 × MIC (0.75 μg/l) to three 48-hour independent cultures carried out parallelly in the same conditions. The assays were made in duplicate simultaneously and in the same conditions. We used the preparation of ciprofloxacin used for injections in the treatment of infections. The incubation of the biofilm with the antibiotic solutions was carried out for 2 hours in similar conditions as for the previous biofilms culture. In parallel, control cultures were carried out without the antimicrobial agent. After incubation, the liquid medium was gently removed from over the biofilm, PBS was used for washing and the surface was stained with two dyes (SYTO-13 and propidium iodide).

In order to distinguish live from dead cells within the biofilm, the staining with two dyes was performed. The SYTO-13 staining (1 μg SYTO-13 per 1 ml PIPES buffer for 15 minutes at room temperature) enabled the detection of live cells (live, green fluorescence), whereas propidium iodine (20 μg/ml) enabled the detection of damaged or dead cells, with compromised membranes (dead, red fluorescence). Images were taken using a microscope Axiovert 200M coupled with a camera (magnification 200 times).

The evaluation of ciprofloxacin effect on the growth of *C. striatum* ATCC 6940 biofilm based on the confocal microscope image analysis. The following biofilm parameters were determined: an area occupied by the biofilm (live and dead bacteria), the intensity of biofilm luminosity (live and dead bacteria) and the value of the maxima of luminosity within the space occupied by the biofilm (live and dead bacteria). The measurements were analyzed against the control culture without the antibiotic. The resulting images (in 8-bit grayscale) were analyzed using the program ImageJ v.1.40g, Wayne Rasband, National Institutes of Health, USA.

Interpretation of the biofilm morphology measurements with the quantity of maxima. The algorithm "Find Maxima" allows for determination of the number of sites in the image that differ from the surrounding areas by a given value (in 8-bit grayscale, where 0 = black and 255 = white). In case of a homogeneous biofilm, the number of points differing from the environment is lower than for the heterogeneous biofilm. Due to the fact that in the same image of biofilm, depending on the setting – "the threshold of the difference between the object and the background" may lead to different results, the number of sites differing from the background and from the minimum value of 10 to 255 were analyzed in order to reduce the error that results from the arbitrarily set values, which allow distinguishing between objects and the background. The number of counted maxima readings allowed for an objective assessment of the biofilm parameters, repeatable in all tests.

The analysis included the biofilm surface, which consisted of 245 scanned parts of the image. It covered

the whole analyzed surface of the biofilm observed in the microscope (ca. 1 cm^2) under the influence of each of the multiple MIC values. This allowed for the presentation of the results from maxima readings as a statistical unit. At the same time the uneven formation of biofilm structures on the analyzed surface could be taken into account, which was visible in the "scatter" of the resulting values, and which finally allowed for the statistical evaluation and comparison of effects of various doses of ciprofloxacin on the entire biofilm surface.

The statistical analysis. The statistical analysis of the results was performed using the Statistica 9.0 GB. The compliance of distribution of individual variables within groups with the normal distribution was verified using the Kolmogorov-Smirnov test with application of the Lilliefors' test and the Shapiro-Wilk test. As the distribution of the tested variables significantly differed from the normal distribution, nonparametric tests were used in the further analysis. For comparison of the differences between the tested groups, the Kruskal-Wallis test was used. The results were accepted as statistically significant at the significance level $p \leq 0.05$ and presented as the median (Me), and the average value (x), the minimum value (min) and maximum value (max) of the statistical series.

Results

The biofilm on the surface of the vascular, urinary catheters and glass surface. The biofilm produced by *C. striatum* ATCC 6940 (Fig. 1A) on the surface of the vascular catheter (exaflon) was shown as multiple aggregates, with a small area, yet reaching in the central part of the resulting structure the value of 25 µm. The structures in the range of values from 10 to 15 µm prevailed. They were compact with a homogeneous bacterial mass, which might be observed in the cross-section (Fig. 2).

C. amycolatum ATCC 700207 (Fig. 1B) produced the biofilm with a larger area bit still relatively small, with a visible tangled structure, and the peak occupied a small part with a height above 15 µm.

The biofilm produced by *C. striatum* ATCC 6940 (Fig. 3A) on the surface of the urinary silicone catheter formed a large flat surface of the height up to 9 µm. A more diversified structure of a very large area was produced by *C. amycolatum* ATCC 700207 (Fig. 3B). It looked like large granular aggregates reaching the height of up to 35 µm.

The cross-section (Fig. 4) showed the diversified fiber structure with numerous channels, separated by the areas of compact bacterial mass.

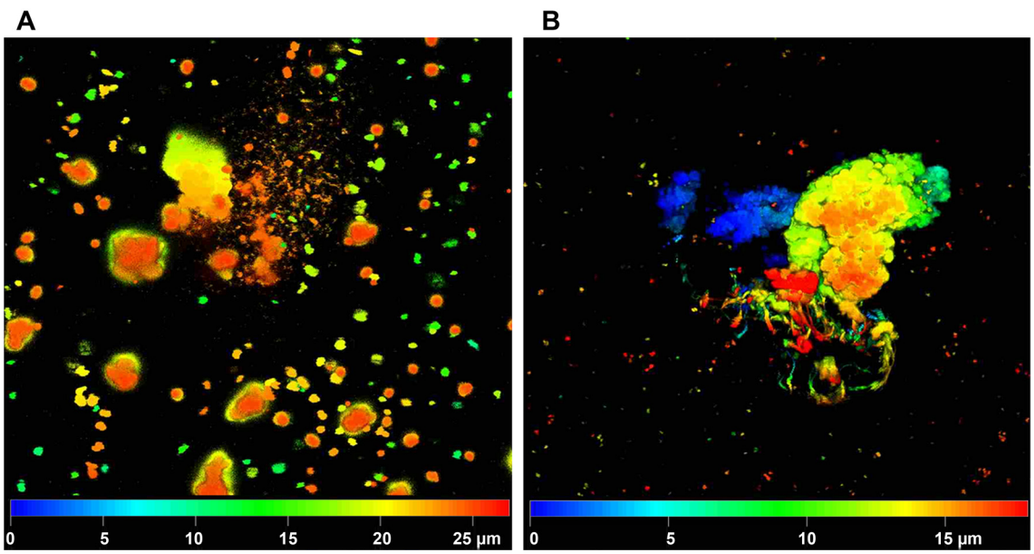

Fig. 1. The biofilms on the surface of the vascular catheter after 48 hours of incubation, stained with SYTO-13 with a marked height of the visible structures, A – *C. striatum* ATCC 6940, B – *C. amycolatum* ATCC 700207 (above 50×).

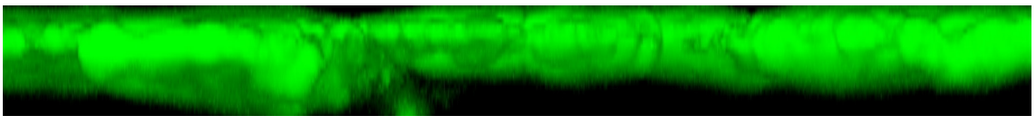

Fig. 2. The cross-section of the biofilm produced on the surface of the vascular catheter after 48 hours, stained with SYTO-13 – *C. striatum* ATCC 6940 (above 50×).

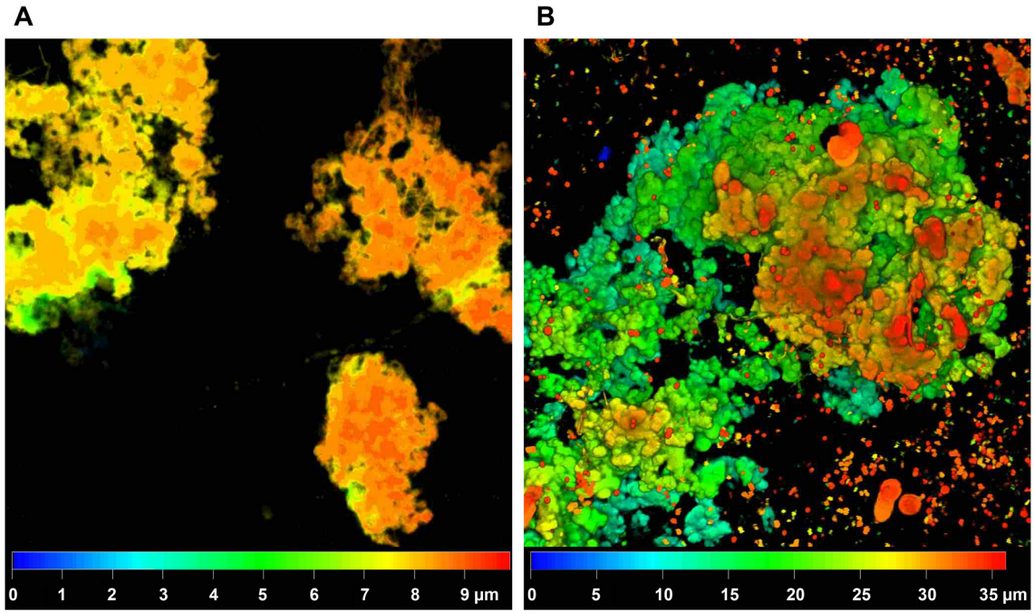

Fig. 3. The biofilm produced on the surface of the urological catheter after 48 hours of incubation, stained with SYTO-13 with a marked height of the visible structures (above 50×), A – *C. striatum* ATCC 6940, B – *C. amycolatum* ATCC 700207.

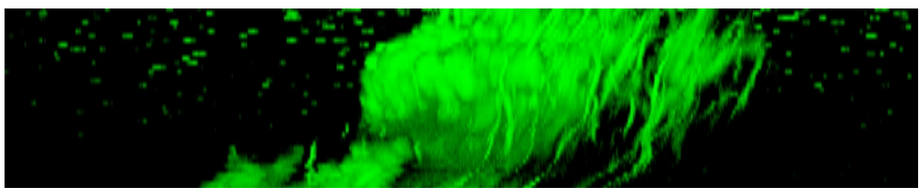

Fig. 4. The cross-section of the biofilm produced on the surface of the urological catheter after 48 hours of incubation, stained with SYTO-13 – *C. amycolatum* ATCC 700207 (above 50×).

Both the strains of *C. striatum* ATCC 6940 and *C. amycolatum* ATCC 700207 produced similar structures on the glass surface (Fig. 5). The biofilm on the glass surface formed small clusters and differed from the biofilm on the catheters. The positively charged surface of the glass did not contribute to the formation of the biofilm by *C. striatum* and *C. amycolatum* in contrast to the negatively charged surface of the catheters.

The effects of ciprofloxacin on biofilm – the confocal microscope image analysis. The effects of ciprofloxacin used in various concentrations on the biofilm produced by *C. striatum* ATCC 6940 were shown in the microscopic image (Fig. 6).

The area of dead cells (red) became progressively bigger along with the increasing doses of the antibiotic in comparison with the green-stained living cells of the biofilm. Yet, a visual inspection of the image under the microscope did not disclose the results that appeared in the drawings based on a statistical evaluation of the biofilm morphology measurements with the number of maxima. Figure 7 shows statistically significant differences of the area occupied by live bacteria for MIC6

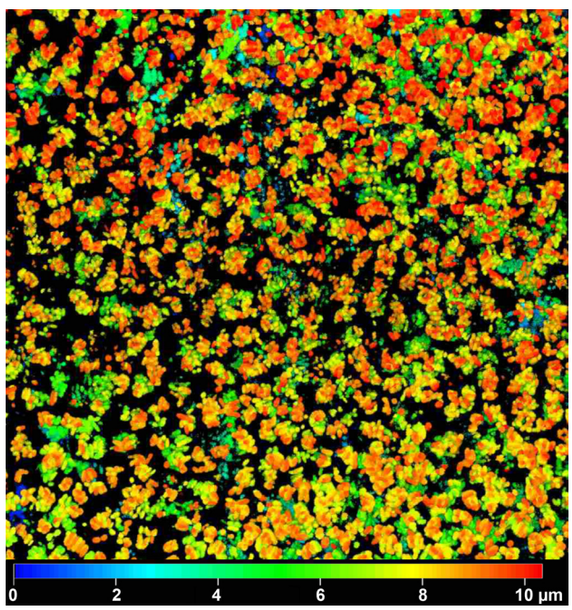

Fig. 5. The biofilm produced on the surface of the glass after 48 hours of incubation, stained with SYTO-13 with a marked height of the visible structures *C. striatum* ATCC 6940 (above 50×).

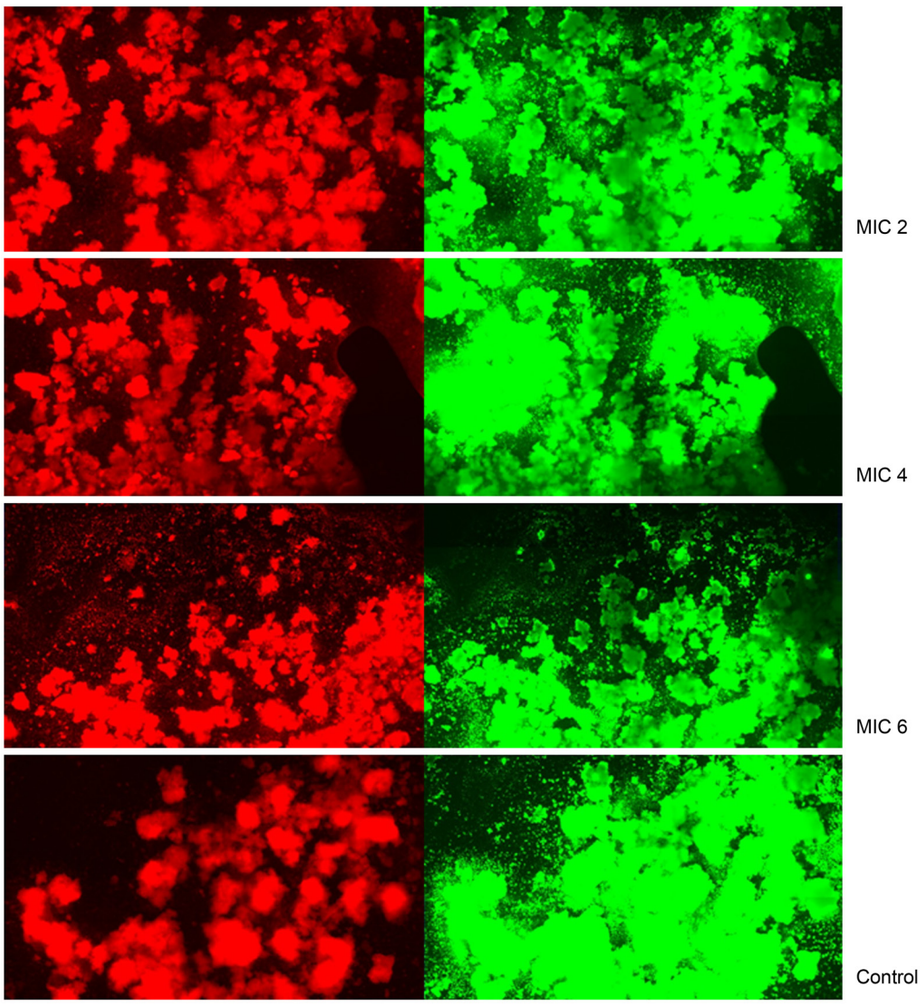

Fig. 6. Biofilms exposed to ciprofloxacin (MIC2 = 0,25 μg/l; MIC4 = 0,5 μg/l; MIC6 = 0,75 μg/l and control samples without antibiotic) – biofilm surface appearance: dead bacterial cells (red) and live bacterial cells (green) (above 200×).

against the control biofilm. In contrast, the percentage of biofilm surface containing live bacteria after treatment with ciprofloxacin MIC4 was higher than after treatment with the MIC2 concentration what was indicated by "flattenings" and the formation of a biofilm on a larger area compared to the control culture (Fig. 8 and Fig. 9). It can be assumed that a detachment of dead bacteria and the unveiling of deeper areas of biofilm live bacteria occurred as a result of the antibiotic activity, which was also confirmed by the values gained by the ratio of the area occupied by live bacteria to the area of dead bacteria after treatment with ciprofloxacin (Fig. 9). Paradoxically, the biofilm exposed to ciprofloxacin at a concentration equal to 4×MIC demonstrated the extension of the area in spite of the greater concentration of the antibiotic. It was also confirmed by the ratio of luminosity of living bacteria to dead bacteria (Fig. 10).

Discussion

The ability of opportunistic *Corynebacterium* species to produce biofilm has already been demonstrated in numerous articles (Soriano et al 1993; Gomes et al. 2009; Frolova et al. 2014) on the other biofilm-producing microbes, such as: *Staphylococcus* spp., *Enterococcus* spp., *Streptococcus* spp., *Micrococcus* spp., which often accompanied infections associated with *Corynebacterium* (Cordero-Ampuero et al. 2007; Dowd et al. 2008; Kania et al. 2008; Wolcott et al. 2009). A special organs and tissues where the biofilm is developed and *Corynebacterium* spp. participates in mixed infections are surgical wounds (Kathju et al. 2009), sinuses in chronic sinusitis that may contain rare species, e.g. *Corynebacterium argentoratense* (Kania et al. 2008), and periprosthetic knee and joint infections (Cordero-Ampuero et al. 2007). Lipophilic bacteria on the skin,

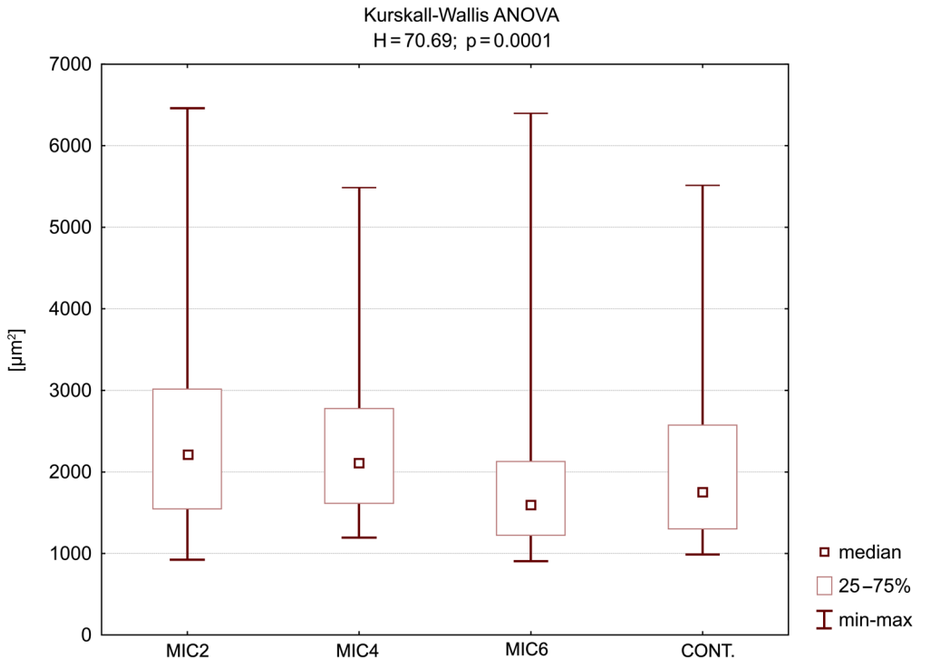

Fig. 7. The area occupied by the live bacteria forming the biofilm after treatment with ciprofloxacin at three concentrations (MIC2 = 0,25 µg/l; MIC4 = 0,5 µg/l; MIC6 = 0,75 µg/l) and control samples without the antibiotic.

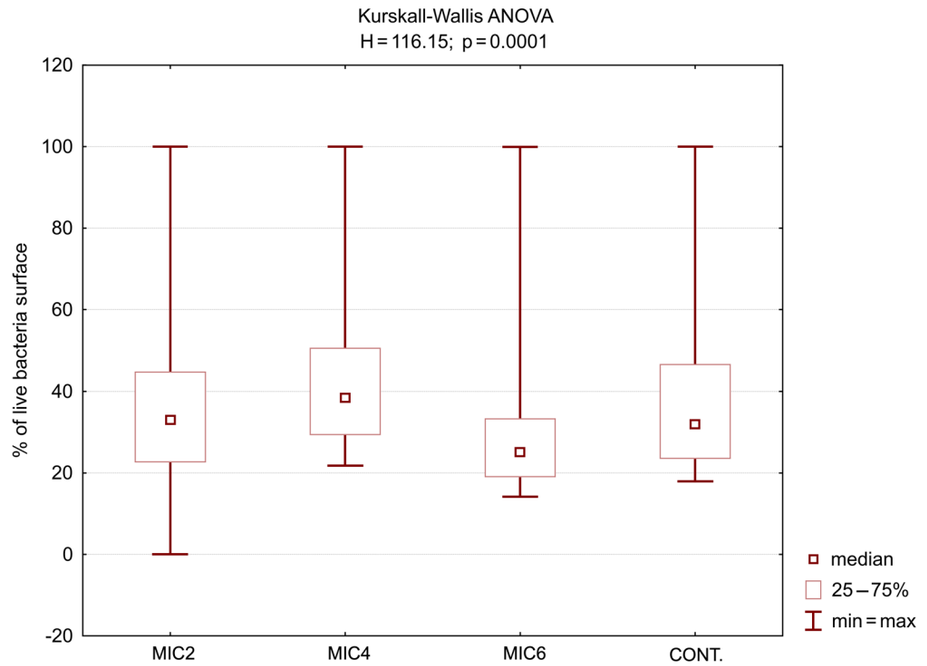

Fig. 8. The percentage of the biofilm surface with live bacteria after exposure to ciprofloxacin at three concentrations (MIC2 = 0,25 µg/l; MIC4 = 0,5 µg/l; MIC6 = 0,75 µg/l) and control samples without the antibiotic.

especially *Corynebacterium jeikeium* (Kwaszewska et al. 2006) are also involved in the development of multi-species biofilms.

The complex nature of biofilm formed by *Corynebacterium* spp. is confirmed by its detection on the surface of polyps in the nose along with other species found in the oropharynx (Zernotti et al. 2010).

Moreover, Gomes et al. (2009) reported the biofilm development by a nontoxic strain of *Corynebacterium diphtheriae* on the surface of polyurethane catheter

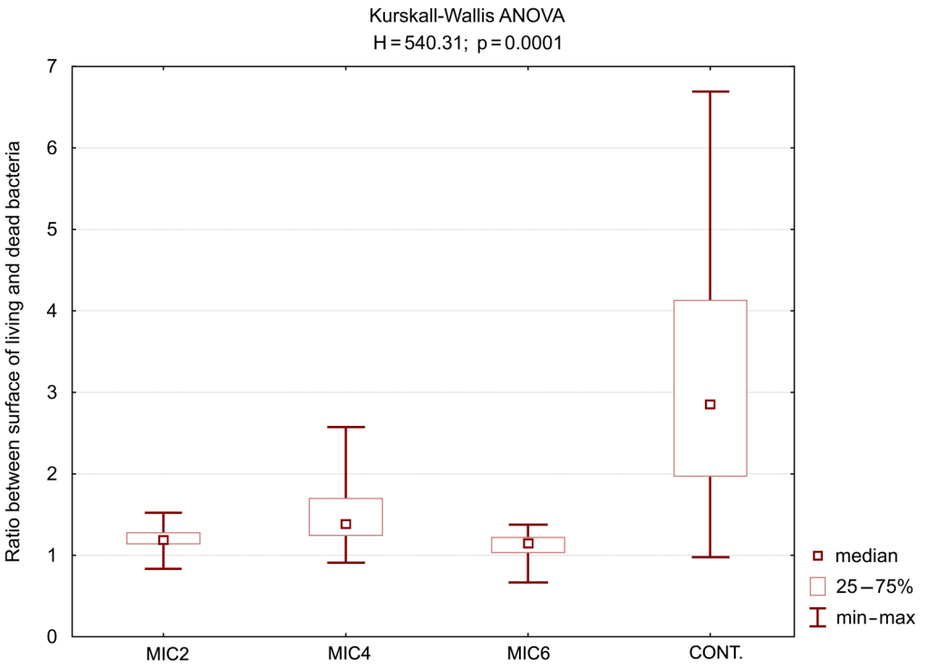

Fig. 9. The ratio of the area occupied by the live bacteria to the surface of dead bacteria within the biofilm after treatment with ciprofloxacin at three concentrations (MIC2 = 0,25 μg/l; MIC4 = 0,5 μg/l; MIC6 = 0,75 μg/l) and control samples without the antibiotic.

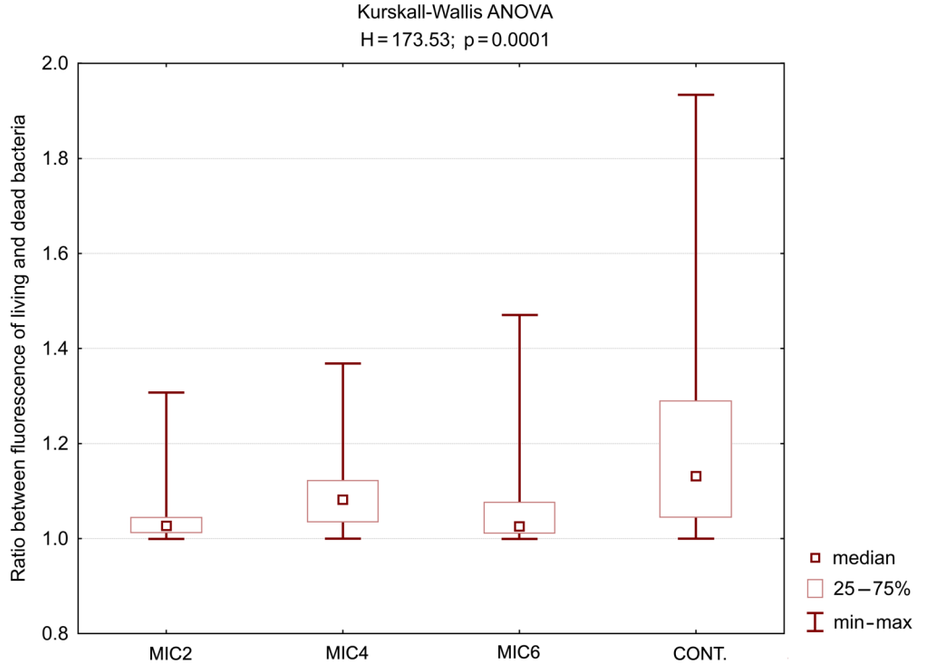

Fig. 10. The ratio of light intensity of live bacteria to dead bacteria after treatment with ciprofloxacin at three concentrations (MIC2 = 0,25 μg/l; MIC4 = 0,5 μg/l; MIC6 = 0,75 μg/l) and control samples without the antibiotic.

in a patient with cancer. The *in vitro* studies on the biofilm did not show high capacity of the *C. diphtheriae* strain to develope biofilms on polystyrene; they were formed more intensely on the glass surface. This strain also showed aggregation and adherence to the surface of HEp-2 cells.

One of the goals of this study was to develop an experimental model corresponding to *in vivo* conditions

during biofilms formation by *Corynebacterium* spp. on the catheter surface. Fragments of hospital vascular and urinary catheters were used in the research. The reference strains, representative of the species responsible for most infections caused by diphtheroids (*C. striatum* and *C. amycolatum*) were used in the experiments. These strains formed biofilms that differ in their structures and cross-sections, depending on the type of catheter. It results not only from the properties of the strains themselves, although this difference was also visible, but also from different chemical structure of the catheter surfaces (silicone, polyurethane). The image of the biofilm in the confocal microscope clearly draws attention to the compact nature of the bacterial mass in the cross-section of the vascular catheter, in comparison to clear fiber nature with visible water channels on the urinary catheter with its tendency to tear up.

An appropriate eradication method of the arising biofilm should be based on the knowledge of effects of various antimicrobial agents on its structure. Biofilms' important property is low susceptibility to antibiotics as well as to antiseptics and disinfectants, which makes the structure of the biofilm very difficult to destroy during the antibiotic therapy (König et al. 2002; Bridier et al. 2011; Williams et al. 2012).

Corynebacterium urealyticum, known for its potential to cause urinary tract infections, especially in catheterized and long-term hospitalized patients, has large capacity for adherence. Soriano et al. (2009) studied the biofilm produced by this species and investigated the effects of antimicrobial agents (ciprofloxacin, moxifloxacin, vancomycin and erythromycin) on biofilms produced in the "artificial urine" (formulation based on tryptic soy broth) by three strains of *C. urealyticum.* The MIC values of antibiotics recorded in this research were 2–8 times higher for the biofilm than for the planktonic forms. The study confirmed that only high concentrations of antibiotics could destroy the biofilm.

Different methods were used to determine the effects of antibiotics on biofilms in the *in vitro* assays. They were often associated with technical constraints related to the specific way in which biofilm were formed (Donlan 2002). The sensitivity to antibiotics was detected using the minimum inhibitory concentration by a method with serial dilutions technique of liquid growth medium (Frolova et al. 2014).

The investigation of antibiotic's effects on biofilms with the use of techniques that could damage their structure (e.g., sonication) may lead to misinterpretations, since it provokes bacteria to change the nature of their growth, shifting the cells (of the disintegrated biofilm) to the metabolism of vegetative forms (incubation in liquid media).

In this study, a method that did not damage biofilm was applied, and experiments were performed with the use of a confocal microscopy. This experimental model imitated the natural conditions. The statistical development of the data enabled a more accurate comparison of results.

The image acquired in confocal microscopy can also be very helpful in the direct diagnostics of the biofilm formed on the mucous membranes, e.g., in chronic sinusitis (Psaltis et al. 2007). To obtain detailed information on how the biofilm surface changes under the influence of ciprofloxacin, the evaluation of its effects was performed based on parameters of the biofilm such as: the size of the area occupied by live and dead bacteria and the intensity of luminosity of fluorescent dyes bound to bacteria. The later may be the indicator of altered cell surfaces after contact with the antimicrobial agent.

The statistical analysis of the results obtained here led to interesting conclusions. Namely, the number of live bacteria within the biofilm produced by *C. striatum* decreased, but paradoxically, its size and surface increased and the biofilms became flatter (compare Fig. 6 and Fig. 7).

The evaluation of the biofilm surface using the confocal microscopy was possible owing to the multiple scanning of successive areas of the surface examined. This approach to the analysis of the whole biofilm area allowed for the statistical analysis when the settings of the image were based on a constant value of the focus.

To the best of our knowledge, the method and microscope images analysis used in this study for investigation of the activity of antimicrobial agents on biofilms produced by opportunistic *C. striatum* ATCC 6490 has not been previously published. Moreover, this method could also be applied to investigate the biofilms of other bacterial species and their capacities to survive in the presence of antimicrobial agents.

Conclusions

The *in vitro* evaluation of the ciprofloxacin effect on the biofilm produced by *C. striatum* ATCC 6490 revealed that its potential destruction would require an application of the antibacterial agent at a concentration higher than MIC6. Ciprofloxacin at a concentration equal to the value MIC4 caused a paradoxical increase of the surface of live bacteria within the biofilm. This suggests that the use of low doses of antibiotics may promote formation of a higher quantity of live bacteria in the biofilm. It could, in turn, promote detachment of planktonic bacteria from the biofilm structure and lead to their spread into the bloodstream or the neighboring tissues.

The applied method provides the original and novel approach to the investigation of the diphtheroid biofilms and their interaction with antimicrobial agents.

Moreover, the development of the method useful for comparison of the effects of changes in the biofilm structure under the influence of antimicrobial agents would also be helpful to study the properties of biofilms produced by other bacterial species and to investigate options of their eradication.

Conflict of interest

Authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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