Biofilm is the microbial lifestyle in natural and man-made environments; it may be formed on a wide variety of surfaces, including indwelling medical devices. This structure expresses the importance for public health as the result of its role in several infectious diseases, e.g., bacteremia and sepsis. The most important pathogens related with infections associated with medical devices are coagulase-negative staphylococci, including *Staphylococcus haemolyticus* – bacterial species which express quite often the multidrug resistance. The four clinical multiresistant and methicillin-resistant *S. haemolyticus* were included in the present study. The evaluation of drug susceptibility was performed by using disc-diffusion method and broth microdilution method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. The biofilm formation on the Nelaton catheter and the effect of linezolid, vancomycin, tigecycline and daptomycin on the biofilm formation and disruption of mature structure was based on the method with TTC (2,3,5-triphenyltetrazolium chloride). The adhesion process of *S. haemolyticus* to the Nelaton catheter was inhibited by antibiotics, as follows: linezolid at concentration 0.25-0.5 × MIC, vancomycin – concentration 0.5 × MIC, tigecycline – concentration 0.25-4 × MIC and daptomycin – concentration 0.06-1 × MIC, depending on the isolate. Linezolid inhibited the biofilm formation at concentration between 0.5-1 × MIC, vancomycin – 1-2 × MIC, tigecycline – 0.5-4 × MIC and daptomycin – 0.06-2 × MIC. The concentration of linezolid eradicating the mature biofilm was found to be 1-2 × MIC, vancomycin – 2-8 × MIC, tigecycline – 2-4 × MIC and daptomycin – 0.06-2 × MIC. The most active antibiotic against *S. haemolyticus* biofilm formation and disruption of mature structure seems to be daptomycin.

**Keywords:** biofilm, *Staphylococcus haemolyticus*, adhesion, disruption, antibiotics

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EXPERIMENTAL

Bacterial strains
The four clinical multiresistant isolates of methicillin-resistant *S. haemolyticus* were included in the present study. The strains were stored in trypticasein soy broth (TSB) with 50% glycerol in -72°C.

Determination of drug susceptibility
The evaluation of susceptibility of *S. haemolyticus* to antimicrobial agents was performed according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. The disc diffusion method was used in evaluation of susceptibility to the following antibiotics: cefoxitin, erythromycin, clindamycin, chloramphenicol, tetracycline, ciprofloxacin, rifampicin, gentamicin, sulfamethoxazole/trimethoprim, fusidic acid.

Isolation of bacterial DNA
The DNA Genomic Mini Kit (A&A Biotechnology) was used in isolation of *S. epidermidis* DNA according to the manufacturer guidelines.

Identification of *meca* by PCR
The following sequence of the primer for *meca* was used: 5’-AAAATCGATGGTAAAGGTTGGC-3’ (forward primer), 5’-AGTTCTGCAGTACCGGATTGTC-3’ (reverse primer). For the reaction of PCR the PCR REDTaq® Ready Mix™ PCR Mix with MgCl₂ (Sigma-Aldrich) was used. The final mixture volume of PCR reaction was 25 mL and contained 12.5 mL of REDTaq Ready Mix, 1 mL of each forward and reverse primer (concentration between 0.1-1.0 mM), 1 mL of DNA (50-200 ng) and 9 mL of water. The reaction was performed by using Whatman Biometra thermocycler, while the PCR products were subjected to agarose gel electrophoresis (2% agarose, 1 × TRIS-acetate-EDTA, 120 mV, 40 min). The conditions of 35 cycles of the PCR reaction was following: 94°C – 1 min, 55°C – 1 min, 72°C – 1 min. The gel was stained with ethidium bromide and PCR product for *meca* (533 bp) was visualized using Wilbert Lambert transilluminator by comparison with a molecular size marker – Gene Ruler™ 100bp DNA Ladder (Fermentas).

Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) to the linezolid, vancomycin, tigecycline and daptomycin
The ability of adhesion of the *S. haemolyticus* isolates to the catheter was studied in vitro by TTC (2,3,5-triphenyltetrazolium chloride) method based on the ability of living cells to reduce tetrazolium salt to red formazan precipitates (5). The standardized bacterial suspensions (0.5 according to McFarland standard) in sterile PBS (phosphate-buffered saline) were incubated with the catheter for 2 h at 35-37°C. Nonadherent cells were removed by careful rinsing of catheter fragment with sterile PBS and then resuspended in MHB with one drop of 1% TTC, followed by overnight incubation at 35-37°C. Adhesion process was determined visually by an appearance of red formazan precipitates, both on the catheter surface and in the medium.

Biomaterials
All assays were carried out on the Nelaton catheter made of polichloride vinyl. The catheter was cut aseptically into ca 0.5 cm² fragments and placed into Petri dishes.

Determination of *S. haemolyticus* adhesion to the Nelaton catheter in vitro
The ability of adhesion of the *S. haemolyticus* isolates to the catheter was studied in vitro by TTC method, using two-fold dilutions of antibiotic in Mueller-Hinton broth (MHB) or MHB supplemented with calcium for estimation of susceptibility to daptomycin. Inoculum of bacterial strains was 5 × 10⁷ colony forming units (CFU) per 1 mL. After incubation at 35°C for 18 h, the MICs were assessed visually as the lowest antibiotic concentration showing complete bacterial growth inhibition. In order to determine MBC, 10 mL of the bacterial culture from each well that showed thorough growth inhibition, from the last positive one and from the growth control was streaked onto trypticasein soy agar (TSA). After incubation at 35°C for 24 h, the MBCs were assessed visually as the lowest antibiotic concentrations at which there was no bacterial growth.

Determination of *S. haemolyticus* biofilm formation on the Nelaton catheter in vitro
The ability of formation of biofilm by the *S. haemolyticus* isolates on the catheter was studied in vitro also by TTC method. The standardized bacterial suspensions (0.5 according to McFarland standard) in MHB were incubated with appropriate biomaterial for 24 h at 35-37°C. Nonadherent cells were removed by careful rinsing of catheter fragment with sterile PBS and then resuspended in fresh MHB. Medium changing and catheter washing procedures after overnight incubation at 35-37°C were repeated three times. Finally, one drop of 1% TTC solution was added, followed by overnight incuba-
tion at 35-37°C. Biofilm formation was determined visually on the basis of an appearance of red formazan precipitates both on the catheter surface and in the medium.

The effect of antibiotics on adhesion and biofilm formation by *S. haemolyticus* on the Nelaton catheter *in vitro*

This assay was based on the TTC method described above. In each experiment, several concentrations of antibiotics were used as multiplication of MIC: 0.5; 1.0; 2.0; 4.0; 8.0; 16.0; 32.0; 64.0 × MIC. (I) In order to assay the effect of antibiotics on adhesion, the standardized bacterial suspensions in sterile PBS containing appropriate antibiotic were incubated with the catheter at 35-37°C. Then, a drop of 1% TTC solution was added, followed by overnight incubation at 35-37°C. The minimal concentration of antibiotic inhibited adhesion process was determined visually as the concentration where the red formazan precipitates were not found, both on the catheter surface and in the medium. (II) In order to assay the effect of antibiotic on biofilm formation, the bacterial suspensions in TSB containing antibiotic were incubated with appropriate biomaterial for 72 h at 35-37°C, with medium changing and catheter washing process as described above. Then, a drop of 1% TTC solution was added, followed by overnight incubation at 35-37°C. The minimal concentration of antibiotic inhibited biofilm formation was determined visually as the concentration where the red formazan precipitates were not found, both on the catheter surface and in the medium. (III) In order to assay the effect of antibiotic on biofilm eradication, the mature 72-h biofilms were incubated in the presence of antibiotic for 24-h and then a drop of 1% TTC solution was added, followed by overnight incubation at 35-37°C. The minimal concentration of antibiotic eradicated the mature biofilm was determined visually as the concentration where the red formazan precipitates were not found, both on the catheter surface and in the medium.

Reproducibility of the results: All results were done in triplicate. Representative data are presented.

RESULTS

On the basis of disc-diffusion method the drug-resistance patterns of *S. haemolyticus* strains used in the present study were evaluated (Table 1). All strains possessed the meca gene.

As shown in Table 2, on the basis of MIC the studied strains were susceptible to linezolid (LZD), vancomycin (VA), tigecycline (TGC) and daptomycin (DA). The MBC/MIC ratio showed the bactericidal effect for vancomycin and daptomycin or bacteriostatic effect for linezolid and tigecycline.

The power of anti-adherent and anti-biofilm activity of the antibiotics was assessed on the basis

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Table 1. The drug resistance patterns of *Staphylococcus haemolyticus*.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Drug resistance pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FOX, E, CC, CIP, GM, SXT</td>
</tr>
<tr>
<td>2</td>
<td>FOX, CIP, RA, GM</td>
</tr>
<tr>
<td>3</td>
<td>FOX, E, CC, TE, C, CIP, GM, SXT</td>
</tr>
<tr>
<td>4</td>
<td>FOX, E, CC, GM</td>
</tr>
</tbody>
</table>


Table 2. The minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and MBC/MIC ratio of linezolid, vancomycin, tigecycline and daptomycin for *Staphylococcus haemolyticus*.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (mg/L)</th>
<th>MBC (mg/L)</th>
<th>MBC/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LZD</td>
<td>1</td>
<td>8-48</td>
<td>8-48</td>
</tr>
<tr>
<td>VA</td>
<td>2</td>
<td>2-8</td>
<td>1-4</td>
</tr>
<tr>
<td>TGC</td>
<td>0.06-0.25</td>
<td>2-8</td>
<td>8-64</td>
</tr>
<tr>
<td>DA</td>
<td>0.5-1</td>
<td>1-4</td>
<td>2-4</td>
</tr>
</tbody>
</table>

LZD - linezolid, VA - vancomycin, TGC - tigecycline, DA - daptomycin.
of the ratio between MIC values and minimal concentration of a given antibiotic inhibiting the biofilm formation or eradicating the mature structure. According to Table 3, the adhesion process of S. haemolyticus to the Nelaton catheter was inhibited by antibiotics, as follows: linezolid – 0.25-0.5 × MIC, vancomycin – 0.5 × MIC, tigecycline – 0.25-4 × MIC and daptomycin – 0.06-1 × MIC, depending on the isolate. The somewhat higher concentrations of antibiotics were necessary for the inhibition of biofilm formation; linezolid inhibited this process at concentration between 0.5-1 × MIC, vancomycin – 1-2 × MIC, tigecycline – 0.5-4 × MIC and daptomycin – 0.06-2 × MIC. The concentration of linezolid eradicating the mature biofilm was found to be 1-2 × MIC, vancomycin – 2-8 × MIC, tigecycline – 2-4 × MIC and daptomycin – 0.06-2 × MIC.

**DISCUSSION AND CONCLUSION**

Nowadays, the biofilm associated infections are one of the most important problems in medicine. The 65% of nosocomial infections are assessed to be related with biofilm formation. The many methods have been studied to inhibit the biofilm formation or even disruption of the mature structure (1, 2, 4). One of the methods used in prevention of biofilm formation is the incorporation of the antibiotic or non-antibiotic agents into biomaterials. According to the literature data (8-10) some antibiotics: imipenem, being an inhibitor of cell wall synthesis, or the inhibitors of bacterial protein synthesis as aminoglycosides, fluoroquinolones, oxazolidinones, streptogramins, have been found to influence on the biofilm formation by several bacterial species. Moreover, also the non-antibiotic chemical compounds are promising agents inhibiting the formation of biofilm structure, e.g., EDTA, usnic acid, surfactin or ovotransferin (10-12).

As presented here, linezolid (oxazolidinone), vancomycin (glycopeptide), tigecycline (glycylcycline) and daptomycin (lipopeptide) affected various steps of biofilm development by methicillin resistant S. haemolyticus, i.e., adhesion, formation and eradication of mature biofilm structure formed. It is generally accepted, that these antibiotics are very often the “drug of choice” in the treatment of infections caused by multidrug resistant staphylococci, especially S. aureus.

It was found that the above drugs are able to prevent the adhesion of S. haemolyticus cells. However, the higher concentrations were necessary in inhibition of biofilm formation or in disruption of mature structure. In some of the cases these concentrations were higher than MIC values estimated against the planktonic cells. It was found that linezolid and daptomycin expressed the best antibiofilm properties, both in formation and in disruption of the mature structure. These agents were able to eradicate of S. haemolyticus biofilm even in the subinhibitory concentrations in opportunity to vancomycin and tigecycline. However, it has to be

**Table 3.** The influence of linezolid, vancomycin, tigecycline and daptomycin on adhesion, biofilm formation and eradication of mature structure of Staphylococcus haemolyticus.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Minimal concentration of antibiotic (mg/L)</th>
<th>Multiplication of MIC values</th>
</tr>
</thead>
<tbody>
<tr>
<td>LZD</td>
<td>Adhesion 0.25-0.5</td>
<td>0.25-0.5</td>
</tr>
<tr>
<td></td>
<td>Biofilm formation 0.5-1</td>
<td>0.5-1</td>
</tr>
<tr>
<td></td>
<td>Eradication of biofilm 1-2</td>
<td>1-2</td>
</tr>
<tr>
<td>VA</td>
<td>Adhesion 1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Biofilm formation 2-4</td>
<td>1-2</td>
</tr>
<tr>
<td></td>
<td>Eradication of biofilm 4-16</td>
<td>2-8</td>
</tr>
<tr>
<td>TGC</td>
<td>Adhesion 0.03-0.125</td>
<td>0.125-2</td>
</tr>
<tr>
<td></td>
<td>Biofilm formation 0.125–0.25</td>
<td>0.5-4</td>
</tr>
<tr>
<td></td>
<td>Eradication of biofilm 0.125-1</td>
<td>2-4</td>
</tr>
<tr>
<td>DA</td>
<td>Adhesion 0.03-1</td>
<td>0.06-1</td>
</tr>
<tr>
<td></td>
<td>Biofilm formation 0.03-1</td>
<td>0.06-2</td>
</tr>
<tr>
<td></td>
<td>Eradication of biofilm 0.03-2</td>
<td>0.06-2</td>
</tr>
</tbody>
</table>

LZD - linezolid, VA - vancomycin, TGC - tigecycline, DA - daptomycin.
remembered that linezolid expresses bacteriostatic effect – it inhibits only the growth of the bacterial cell but does not kill the population, in contrast to daptomycin (being a bactericidal drug).

It was also found that vancomycin requires higher concentrations both in biofilm formation and in eradication of the structure. This observation confirms that antibiotics inhibiting the cell wall synthesis are less active against biofilm formation in opposite to antibiotics inhibiting the protein synthesis. The previous data showed that even antibiotics which do not express the antibacterial activity against the species can decrease the ability of biofilm formation, e.g., azithromycin against biofilm of Pseudomonas aeruginosa as the result of inhibition of the alginate synthesis (13). However, it has to be noted that the addition of the other chemical compounds can increase the anti-biofilm activity of antibiotics, e.g., vancomycin + heparin or minocycline + EDTA (10, 14).

The mechanism of influence of antibiotics on biofilm formation is still unknown. However, it has been found that some of the antibiotics (dicloxacillin) decreased the synthesis of extracellular molecule - poly-N-acetylglucosamine (PNAG) and changed the hydrophobicity of bacterial cell surface of S. epidermidis (15). In opposite, Rachid et al. (16) showed that tetracyclines increased the expression of ica operon involved in synthesis of polysaccharide intracellular adhesine (PIA). Recently, a capsular polysaccharide (CP) was proposed to be an important virulence factor of S. haemolyticus (17). However, the presence of ica operon has been reported but to date its contribution in these species to biofilm formation is unclear (17).

As it was mentioned above, one of the factors responsible for the low activity of antibiotics against the bacterial strains embedded in biofilm is slow penetration of the drugs into the structure. The data of Stewart et al. (18) showed that daptomycin can readily penetrate thick S. epidermidis biofilms in minute or two what is much shorter than the duration of antibiotic exposure which is typically tens of hours.

Daptomycin effects the processes involved in the biofilm formation and caused disruption of the mature structure at comparable concentrations. The most active antibiotic against S. haemolyticus biofilm formation and disruption of mature structure seems to be daptomycin.

REFERENCES


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