The Formation of Poly-Microbial Biofilms on Urinary Catheters

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1. Introduction

The number of catheter associated urinary tract infections (CAUTIs) increases every year. The increasing number of CAUTIs bears on fact that urinary catheters became second most often used foreign body inserted into human body. Over 40% of nosocomial infections are infections of urinary tract, especially infections of catheterised patients (Gorman & Jones, 1991). Despite good aseptic management, circa 50% of patients have bacteriuria in first 10–14 days of catheterisation (Morris & Stickler, 1998). The risk of urinary tract infections is significantly higher in long-term inserted catheters (28 days); the percentage of infected catheters in these patients gets near to 100% (Morris & Stickler 1998).

The high number of CAUTIs is associated with biofilm mode of growth of microbes. The biofilm mode of growth is advantageous from several reasons. The artificial surface of the implants facilitates adhesion of bacteria, which can therefore form biofilm. The bacteria in biofilm are protected against drying, mechanical damage and other influences of the outer environment. In the human body the bacteria in biofilm are protected against the immunity system and antibiotic treatment (Stewart & Costerton, 2001). The higher resistance of biofilm bacteria to antimicrobials is a serious problem and the reason of common therapy failure. The extracellular polysaccharide matrix plays the key role in the resistance of biofilm to the antibiotics. It prevents the diffusion of the antibiotics to the bacterial cells, it is the reason of the higher concentration of antibiotic-reducing enzymes in the bacterial surroundings and it partakes on the change of microenvironment in the deeper layers of biofilm. These features play an important role in antibiotics resistance because the low pH reduces effect of some antibiotics (such as aminoglycosides) and the nutrition and oxygen deficiency leads to the growth stasis of bacteria (e.g. the beta-lactam antibiotics become ineffective).

The biofilms grow easily also on the surface of other implants, such as venous, prosthetic of heart valves, orthopaedic devices etc. (Stewart et al., 2001). It’s estimated, that biofilms are associated with about 65 % of nosocomial infections (Licking 1999).

With the inserted catheter, the bacteria can more easily attack urinary tract and urinary bladder (Tunney et al., 1999). There are also other complications that are linked with bacterial colonisation of urinary tract and catheters, e.g. blockage of catheters with crystallic deposits of bacterial origin, generation of gravels and pyelonephritis (Gorman & Tunney, 1997). The obstruction of the urine flow in catheters with crystallic deposits meets circa 50% of long-term catheterised patients; and there is no method of prevention of these deposits nowadays. Except of crystallic deposits that are result of metabolic dysfunction,
there are also crystallic deposits of bacterial origin, caused mainly by urease-positive species of bacteria. The bacteria account to 15-20% of all gravels and these gravels are often connected with biofilm colonisation of long-term inserted urinary catheter or stent.

1.1 Colonisation of urinary catheters with biofilm-positive microbes
Adhesion of bacteria to the catheter depends on many factors, e.g. surface charge, hydrophobicity or hydrophility of the catheter and bacterial cell, on specific genes for adhesion etc. (Liedl, 2001). The risk of infection depends on the length of catheterisation and catheter management.

1.1.1 Intermittent catheterisation and risk of urinary infections
In patients with single or intermittent catheterisation is the risk of UTI significantly lower in comparison with indwelling catheters (Gorman & Jones, 1991). Many studies showed that intermittent catheterisation decreases risk of UTI up to 50% in comparison with indwelling catheterisation and is the preferred method of bladder drainage (Perkas & Giroux, 1993; de Ruz et al., 2000; Larsen et al., 1997; and others). However, intermittent catheterisation can be cause of urethral trauma or stricture, hematuria, epididymitis in men, and other complications. As far as sterile intermittent catheterisation (SIC) and clean intermittent catheterisation (CIC) are concerned, the US National Institute on Disability and Rehabilitation Research published that the CIC does not pose a greater risk of infection than SIC and is much more economic (NIDRR, 1993). Many studies reported CIC to be as safe as SIC (Lemke et al., 2005; King et al., 1992; and others) and the CIC is widely accepted to be appropriate method of catheterisation. However, the SIC is essential in the hospital setting because of the presence of wide spectrum highly antibiotic-resistant pathogens. The bacteria that are present in the bladder during intermittent catheterisation reach only low numbers and the stream of the urine does not allow them to adhere. It is assumed, that most of the bacteria are flushed away with the urine and the rest is killed by immune system.

1.1.2 Long-term catheterisation and risk of urinary infections
The presence of catheter in urinary tract facilitates the bacterial adhesion and colonisation of this niche. The permanent presence of artificial surface help bacteria to colonise the urinary system in the short- and long-term indwelling catheters. In long-term catheterised patients (weeks or longer), e.g. in patients with chronic urinary incontinence, chronic obstruction of urinary tract or neurogenic urinary bladder, the bacteriuria is common; the number of bacteria in one millilitre of the urine is commonly higher that $10^5$ (Mobley & Warren, 1987). Nevertheless, the CAUTI are rarely associated with significant clinical symptoms and more than 90% of these infections are asymptomatic (Tambyah & Maki, 2000). It is widely accepted that such colonisation of the catheter (without signs of pyelonephritis or septicaemia) is not necessary to treat (Warren, 1994). The colonisation of the catheter often cannot be proved by common cultivation of catheterised urine, but it can be proved by the cultivation of extracted catheter. The results of the study of Farsi et al. (1995) show the difference between bacteriuria (present only in 30% of patients) and real colonisation of the catheter (present in 68% of the same set of patients). There are three main ways, how the bacteria can reach the urinary bladder of long-term catheterised patients – bringing the bacterial contamination during insertion of the catheter; extraluminar migration of the bacteria present in urethra; and migration of bacteria in the
lumen of the catheter from contaminated drainage system. The intraluminal invasion to the urinary tract is faster (32-48 hrs) in comparison with extraluminal (72-168 hrs). The intraluminal upstream movement of *Pseudomonas aeruginosa* was 1-2 cm per hour (Nickel et al., 1985).

The longer has the patient catheter, the higher diversity shows the biofilm microflora. Catheter infections of urinary tract are caused most commonly by faecal microflora - gram-negative rods (*Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* sp., *Pseudomonas aeruginosa*, *Proteus mirabilis* etc.) and enterococci (esp. *Enterococcus faecalis*) (Tenke et al., 2006). Less often the urinary infections cause other species of bacteria, e.g. *Staphylococcus epidermidis*, *Streptococcus agalactiae*, and yeasts (*Candida albicans*). Higher pathogenicity of these microbes is caused by the presence of many virulence factors, esp. the ability to form biofilm, the ability to co-aggregate or ability to withstand effect of antibiotics. Some of uropathogenic bacteria, those with hydrophobic surface, adhere better to hydrophobic materials of catheters (e.g. *Enterococcus faecalis*), some other, which are rather hydrophilic, adhere better to hydrophilic surfaces of catheters (e.g. *Escherichia coli*).

The diversity of the microbial biofilm can be shown by the use of sonication techniques, as discussed further. The sonication of catheters followed by isolation, determination and biofilm assessment of particular microbial strains can discriminate particular causative agents of infections of urinary tract and their importance as biofilm-formers in the microbial community of the urinary catheter. The examination of other virulence factors, e.g. different types of motility, urease production etc., also helps with interpretation of importance of particular strains.

### 1.2 Bacterial encrustation and mineralization of biofilm on catheters

Clinical complication of the CAUTIs is obstruction of the urinary flow in the catheters by crystallic deposits. The problem of crystallic deposits meet c. 50% of long-term catheterised patients (Getliffe & Mulhall, 1991); and there is no method of prevention of these deposits nowadays (Stickler et al., 2002). The manipulation with the catheter with crystallic deposits, thus even its removal, traumatizes the mucosa of the urinary bladder and urethra which helps to further bacterial colonisation of the mucosa of urinary tract.

Crystallic deposits can evolve by several mechanisms in the urinary tract, and be of different composition. In practice there are five types of crystallic deposits. There are deposits on the basis of uric acid, calcium oxalate, calcium phosphate, cystine, and magnesium ammonium phosphate (MgNH₄PO₄*6H₂O). Calcium phosphate encrustation may present as brushite (CaHPO₄), hydroxyapatite [Ca₁₀(PO₄)₆(OH)₂], or carbonate-apatite [Ca₁₀(PO₄)₆CO₃] complexes. The first four types listed are often referred to as metabolic encrustation, as they normally result from metabolic dysfunction, whereas magnesium ammonium phosphate encrustation (struvite) has an infectious origin (Tunney et al. 1999, as cited in Gorman & Jones 2003). Urinary stones of microbial origin are often associated with long-term inserted catheters and form approx. 15 – 20% of all urinary stones.

Morris & Stickler (1998) described origin of microbial crystallic deposits by several phases:

- Infection of urinary tract by urease-positive bacteria
- Bacterial adhesion to the catheter surface and biofilm formation
- Increase of the pH of the urine by reason of present bacteria
- Chemical interactions of negatively charged ions of magnesium and calcium
- Crystallization of calcium and magnesium phosphates
One of the factors, leading to the urinary stone formation, is colonisation of the catheter by urease-positive bacteria (Cox et al., 1989; Morris et al., 1999). Urease, main reason of incrustations on the catheters, is produced by approx. 200 bacterial species. From the point of view of the CAUTIs, the most clinically significant are *Proteus vulgaris* (urease produce >90% of strains), *Morganella morganii* (>90%), *Providencia stuartii* (>90%), *Klebsiella pneumoniae* (>60%), *Pseudomonas aeruginosa* (>30%) and *Serratia marcescens* (~29%).

The present urease hydrolyses urea and increases pH of urine. Urine analysis of patients with heavy mineral deposits showed its increased pH. The magnesium ammonium phosphate and hydroxyapatite were present in higher amounts (Keane et al., 1994). Chemically, the change of urine pH and formation of crystallic deposits has several steps (Griffith 1978, as cited in Gorman & Jones, 2003). The urease hydrolyses urea and catalyses formation of ammonium and carbon dioxide (1). The ammonium becomes ammonium ion in neutral or slightly acidic pH (pH of normal urine); which induces alcalization of the urine (2). Carbon dioxide reacts with water and forms carbonic acid (3). Depending on the pH of the urine, the carbonic acid may dissociate (4).

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\begin{align*}
\text{NH}_2\text{CONH}_2 + \text{H}_2\text{O} & \rightarrow 2\text{NH}_3 + \text{CO}_2 \\
\text{NH}_3 + \text{H}_2\text{O} & \rightarrow \text{NH}_4^+ + \text{OH}^- \\
\text{CO}_2 + \text{H}_2\text{O} & \rightarrow \text{H}_2\text{CO}_3 \\
\text{H}_2\text{CO}_3 & \leftrightarrow \text{HCO}_3^- + \text{H}^+ \leftrightarrow \text{CO}_3^{2-} + \text{H}^+
\end{align*}
\]

Production of ammonium leads to increase of pH and precipitation of poorly soluble magnesium and calcium salts in form of magnesium ammonium phosphate, hydroxyapatite, and carbonite apatite, which leads to crystalline formation (Gorman & Tunney, 1997; McLean et al., 1991). The mineral deposits, formed on the basis of microbial infection, are mineralised biofilms, so the process of biofilm formation is also process of crystalline deposits formation. Urethral stents, which enable urinary drainage in patients with obstructive uropathy, meet same problems with crystallic deposits and biofilm formation as urinary catheters, especially in patients with long-term stent drainage. According to Keane et al. (1994), nearly 75% of stents gets obstructed within 24 weeks from insertion. The formation of crystallic deposits and stones on the surface of these devices is the main problem of their management (Choong et al., 2001). These deposits may lead to obstruction of the lumen of catheter or stent, to the retention of urine, bacteriuria, and rarely to other complications, such as pyelonephritis and septicemia. Moreover, the hardness of the crystals of these deposits (c. 5 according to Mohr’s scale) may lead to permanent damage of urethral epithelium.

Important role in the mineral deposit formation have the bacterial capsule and other extracellular polysaccharides. The chemical interactions and polarization between negatively charged biofilm matrix and positively charged calcium and magnesium ions lead to oversaturation of the environment by these ions in the close proximity of the biofilm layer and their subsequent precipitation. These capsular exopolysaccharides may also bind magnesium in the struvite crystals (ammonium magnesium phosphate), which leads to full or partial immersion of struvite crystals in the biofilm matrix (Dumanski et al., 1994; Gorman & Tunney, 1997).
2. Sonication and biofilm protocol

For the better understanding of poly-microbial infections, the cultivation and identification of microbial species is of particular interest. The best way for isolation of wide spectrum of pathogens from urinary catheter biofilm, the sonication seems to be most appropriate method (Hola et al., 2010). The catheter must be aseptically withdrawn into empty sterile test tube and sent immediately for microbial examination. Due to number of microbial species and their different growing speed, the immediate examination is of particular interest. Otherwise the results can be distorted by overgrowing of some fast-growing species (Hola et al., 2010).

2.1 Sonication protocol

The sonication protocol is based on procedure as previously described by Sherertz et al. (1990) for blood stream catheters, with several modifications. The sonication protocol, as it is described here, is used in our laboratory for four years with good results. The sonication of the catheter itself comprises of several subsequent steps, which include sonication, vortexing and diluting. The cut part of the catheter (2 cm; ~7.5 cm$^2$) is sonicated in 5 mL of Brain-Heart Infusion (BHI) for 5 minutes, than vortexed for 2 minutes and sonicated for another 5 minutes. The repeated sonication together with vortexing leads to more accurate results of the procedure. According to our findings, the sonication alone shows worse results (lower number of microbes and lower number of microbial species) in comparison with sonication-vortex-sonication protocol. The suspension is subsequently diluted 10- and 100-times and inoculated to solid media. This step is necessary for accurate quantification and isolation of individual strains (Hola et al., 2010). The set of solid media used in our laboratory comprises of Blood Agar, UriSelect 4 (BioRad), Endo Agar, Blood Agar with 10% of NaCl, Blood Agar with Amikacine (32mg/1L) and Sabouraud Agar with Vankomycine (5mg/1L) and Amikacine (20mg/1L). The quantification is performed on Blood Agar, the UriSelect helps with quantification of mixed cultures and also with species isolation and preliminary identification; the other four media are used for species isolation and preliminary identification; Endo Agar for selective cultivation of most of Gram negative rods, BA with NaCl for selective cultivation of staphylococci, BA with Amikacine for selective cultivation of streptococci and Sabouraud Agar for selective cultivation of yeasts. All isolated strains are identified by the conventional biochemical tests to the species/genus level (Micro-LA-tests, Lachema, CZ and/or API Biomerieux, FR).

2.2 Biofilm protocol

Prior to biofilm production assay, the strains are cultured on Blood Agar and incubated overnight aerobically at 37°C. After verifying purity of the tested strain, several colonies with identical morphology are suspended in sterile physiological saline. The turbidity of the bacterial suspension is adjusted to 0.5 of the McFarland standard (~1.5 x 10$^8$ CFU/ml) using a photometric device. The obtained suspension is vortexed for 1 min and subsequently diluted 1:100 with fresh medium. The inoculum size should be carefully determined, because the size of the inoculum considerably influences the amount of biofilm produced, i.e. biofilm density increases with increasing initial inoculum (Stepanovic et al. 2003).

All strains are cultivated in triplicate in flat-bottomed microtiter tissue culture plates (Fig. 1) in the temperature 37°C for 24 hours in the Brain-Heart Infusion with 4% of glucose (200 μL per well). The choice of the medium depends on planned experiments. For the biofilm formation, the Brain Heart Infusion with 4% of glucose seems to be good choice for most of
the microbial species. The negative control wells contain pure culture medium. After cultivation, the wells of microtiter plates are washed three times with sterile phosphate-buffered saline (PBS; pH 7.2). With every washing step, the wells should be emptied by flicking the plates. The biofilm layer is fixed by air-drying (Stepanovic et al. 2007).

Fig. 1. Biofilm formation assay.

The fixed biofilm layer is stained with crystal violet for 15 min at room temperature. After staining, the stain is aspirated with a pipette and excess stain is rinsed off by placing the microtiter plate under running tap water. The washing continues until the water from the plate remains clean. After the microplate is air dried at room temperature, the dye bound to the cells is resolubilized with 150 μL of 95% ethanol per well. Ethanol should be added gently. Thereafter the microtiter plate covered with the lid (to minimize evaporation) is left at room temperature for approx. 30 min (Stepanovic et al. 2007) and the biofilm-positivity is assessed quantitatively by means of optical density (OD) assessment (595 nm). For all tested strains and negative controls, the average OD values are calculated (from the inoculated triplets). The cut-off value (ODc) should be established; the ODc is defined as three standard deviations (SD) above the mean OD of the negative control (5). The OD value of the tested strain is expressed as average OD value of the strain reduced by ODc value (6). ODc value should be calculated for each microtiter plate separately.

\[
\text{ODc} = \bar{\text{OD}}_{\text{negative control}} + 3 \times \text{SD}_{\text{negative control}} \quad (5)
\]

\[
\text{OD} = \bar{\text{OD}}_{\text{tested strain}} - \text{ODc} \quad (6)
\]

For easier interpretation of the results, strains may be divided into the following categories (Stepanovic et al. 2000): strain no producing biofilm (7), strain weakly producing biofilm (8), strain moderately producing biofilm (9) and strain strongly producing biofilm (10). This
categorization should be based on the previously calculated OD values (for this type of data interpretation the OD value of the strain should not be reduced by OD\(_c\) value).

\[
\text{OD} \leq \text{OD}_c
\]

\[
\text{OD}_c < \text{OD} \leq 2 \times \text{OD}_c
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\[
2 \times \text{OD}_c < \text{OD} \leq 4 \times \text{OD}_c
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\[
4 \times \text{OD}_c < \text{OD}
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As the control, biofilm-positive strains deposited in several culture collections may be used, e.g. Staphylococcus epidermidis strains No. ATCC 35981, ATCC 35982, ATCC 35983 and ATCC 35984 or S. epidermidis CCM 7221, deposited in the Czech Collection of Microorganisms in Brno (Christensen et al. 1985; Ruzicka et al. 2004).

3. Antibiotic susceptibility testing

Biofilm, as an important factor of virulence, enables microbes to colonise surfaces and increases their resistance to the antimicrobial agents. For the study of resistance of biofilm isolates to antimicrobials, three assays should be performed, the minimum inhibitory concentration assay (MIC), the minimum biofilm inhibitory concentration assay (MBIC) and minimum biofilm eradication concentration assay (MBEC). The results of these three assays can show the actual susceptibility/resistance of particular strains to antimicrobials. The methods of the minimum biofilm inhibition concentration (MBIC) and minimum biofilm eradication concentration (MBEC) assessment, together with minimum inhibitory concentration assessment, are applicable for the evaluation of the differences in the antibiotic resistance in planktonic and biofilm forms of growth and for the evaluation of differences in the biofilm-positive and biofilm-negative strains (Hola et al., 2004 a).

In our studies we examined coagulase-negative staphylococci and the set of anti-staphylococcal and wide-spectrum antibiotics: penicillin, oxacillin, ampicillin-sulbactam, chloramphenicol, tetracycline, co-trimoxazole, erythromycin, clindamycin, ciprofloxacin, gentamicin, teicoplanin and vancomycin. To the commercially available microtiter plates with serial dilutions of antibiotics covering break-point concentration, we prepared second microtiter plate with serial dilutions of the same antibiotics, which linked up with increasing concentrations of tested antibiotics to cover the MBIC and MBEC values. For the concentrations of diluted antibiotics see Table 1.

3.1 Minimum inhibitory concentration assay

Minimum inhibitory concentration assay (MIC) was proved by the microdilution method according to the European Standards as they are implemented in the Czech Microbiological Standards (Urbášková, 1998). Briefly, fresh 24-hrs culture of the strain cultured on Blood Agar is suspended in physiological saline to the optical density equal to 0,5 according to McFarland Standard. This suspension is inoculated in the wells of microtiter plate with serial (logarithmic) dilutions of tested antibiotics in Mueller-Hinton Broth (commercially available from Trios Ltd., Prague, CZ). The final concentration of cells of the tested strain is equal to 500 000 CFU/ml. After 18 hrs of cultivation the minimum inhibitory concentration is assessed.
Standard antibiotic concentrations (mg/L)

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PEN – penicillin; OXA – oxacillin; AMS – ampicillin-sulbactam; CMP – chloramphenicol; TET – tetracycline; COT – co-trimoxazole; ERY – erythromycin; CLI – clindamycin; CIP – ciprofloxacin; GEN – gentamicin; TEI – teicoplanin; VAN – vancomycine, GC – growth control

Table 1. Used concentrations of antibiotics.

3.2 Minimum biofilm inhibitory concentration and minimum biofilm eradication concentration assays

The resistance/susceptibility was assessed on the hardened-polystyrene pegged plates that fit into standard microtiter plates. These pegged plates enable the biofilm cultivation on all 96 pegs simultaneously, so they prompt and simplify the manipulation with the biofilms (see Fig. 2). For better cell-adhesivity, the surface of the pegged plate was modified by poly-L-lysine (Hola et al. 2004 c). The wells of the microtiter plate were filled with *S. epidermidis* culture (precultured in BHI supplemented with 4% of glucose) and the pegs were submerged in it. The primary adhesion was performed for 90 minutes. Then the pegged
plates were removed into fresh sterile Brain Heart Infusion (BHI) and cultivated at 37 °C for 24 hours.

Fig. 2 – Pegged plate with grown biofilm; the biofilm layer was fixed by drying and stained with crystal violet.

In the MBIC assessment, the biofilm grown on the pegs of the pegged plate was exposed to the action of antibiotics by submerging into the medium with antibiotics compounds (concentrations listed in Table 1). The MBIC value was assessed after 18 hours of cultivation on the basis of presence of turbidity in the wells. After the exposure of the pegs with grown biofilm to the antibiotics (18 hours of cultivation) the pegs were three times washed by the sterile phosphate buffered saline (pH 7,4) and moved into the colorimetric medium, which changes the colour in the presence of living cells. After next 18 hours of cultivation the MBEC was assessed on the basis of colour change of the medium, the presence or absence of turbidity in the well being of no importance (Hola et al., 2004 a).

3.3 Minimum Inhibitory Concentration in biofilm-positive and biofilm-negative strains

Figure 3 shows average values of MIC of biofilm-negative and biofilm-positive strains of tested antibiotics. The biofilm-positive strains have higher average values of MICs. The median values of MICs of both groups of strains are shown in Table 2. The differences between biofilm-positive and biofilm-negative strains were statistically significant in oxacillin, tetracycline, co-trimoxazole, ciprofloxacin, gentamicin and clindamycin (P ≤ 0,05, n = 88). All strains were susceptible to teicoplanin and vancomycine in both tested groups. Despite the fact, that the MIC value is defined for planktonic form of growth, there is significant difference between biofilm-positive and biofilm-negative strains of microbes (Hola et al., 2004 b).
One of the factors, increasing the resistance of biofilm-positive strains to antibiotics, is the extracellular polysaccharide, in staphylococci presented as polysaccharide intercellular adhesine (PIA). The PIA is inherent compound of the biofilm layer and covers staphylococcal cells as slimy layer. The PIA facilitates bacterial adhesion to solid surfaces and co-aggregation of the bacterial cells. The mechanisms of resistance of cells covered by PIA to antibiotics are not yet fully understood, but it is widely accepted, that they differ from mechanisms of resistance of individual cells (enzyme production, change of bonding place etc.) (Costerton et al., 1995).

Table 2. Mean and median of MICs in biofilm-positive and biofilm-negative strains.

<table>
<thead>
<tr>
<th>Biofilm-positive strains</th>
<th>Mean</th>
<th>SE</th>
<th>Median</th>
<th>Biofilm-negative strains</th>
<th>Mean</th>
<th>SE</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEN</td>
<td>2,516</td>
<td>0,243</td>
<td>2</td>
<td></td>
<td>2,788</td>
<td>0,234</td>
<td>4</td>
</tr>
<tr>
<td>OXA</td>
<td>13,777</td>
<td>2,321</td>
<td>8</td>
<td></td>
<td>6,439</td>
<td>1,432</td>
<td>2</td>
</tr>
<tr>
<td>CMP</td>
<td>5,360</td>
<td>1,258</td>
<td>2</td>
<td></td>
<td>6,535</td>
<td>1,596</td>
<td>2</td>
</tr>
<tr>
<td>TET</td>
<td>4,942</td>
<td>1,446</td>
<td>1</td>
<td></td>
<td>1,695</td>
<td>0,316</td>
<td>0,5</td>
</tr>
<tr>
<td>COT</td>
<td>34,419</td>
<td>4,513</td>
<td>32</td>
<td></td>
<td>17,267</td>
<td>3,428</td>
<td>4</td>
</tr>
<tr>
<td>ERY</td>
<td>11,225</td>
<td>1,211</td>
<td>16</td>
<td></td>
<td>8,826</td>
<td>1,171</td>
<td>16</td>
</tr>
<tr>
<td>CLI</td>
<td>9,540</td>
<td>1,596</td>
<td>8</td>
<td></td>
<td>6,785</td>
<td>1,695</td>
<td>0,125</td>
</tr>
<tr>
<td>CIP</td>
<td>9,426</td>
<td>1,136</td>
<td>16</td>
<td></td>
<td>6,753</td>
<td>0,960</td>
<td>8</td>
</tr>
<tr>
<td>GEN</td>
<td>14,387</td>
<td>1,956</td>
<td>16</td>
<td></td>
<td>5,735</td>
<td>1,824</td>
<td>0,125</td>
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<tr>
<td>TEI</td>
<td>1,953</td>
<td>0,205</td>
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<td></td>
<td>2,680</td>
<td>0,275</td>
<td>2</td>
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<tr>
<td>VAN</td>
<td>1,686</td>
<td>0,074</td>
<td>2</td>
<td></td>
<td>1,802</td>
<td>0,087</td>
<td>2</td>
</tr>
</tbody>
</table>

SE = standard error; PEN = penicillin; OXA = oxacillin; AMS = ampicillin-sulbactam; CMP = chloramphenicol; TET = tetracycline; COT = co-trimoxazole; ERY = erythromycin; CLI = clindamycin; CIP = ciprofloxacin; GEN = gentamicin; TEI = teicoplanin; VAN = vancomycine

Table 2. Mean and median of MICs in biofilm-positive and biofilm-negative strains.
The bonding of the molecules of antibiotic to the negatively charged chemical compounds present in biofilm layer may be the reason of the increased gentamicin resistance. The change of physico-chemical conditions may affect tetracycline and the production of enzymes degrading antibiotics may affect action of oxacillin.

The higher resistance to antibiotics of biofilm-positive strains even in their planktonic form can be explained by influence of weak biofilm layer covering the cells and formation of micro-colonies surrounded by PIA. However, the layer of the polysaccharides is still relatively weak. This, together with the absence of resistance factors that act in the layer of matured biofilm (decreased growth rate inside biofilm layer, strong changes in the inner environment including acidification, lack of oxygen etc.), leads to the observed differences between MIC values of biofilm-positive and values of biofilm-negative strains.

### 3.4 MIC, MBIC and MBEC in biofilm-positive strains

Figure 4 shows average values of MICs, MBICs and MBECs of biofilm-positive strains to tested antibiotics. The minimum concentrations of antibiotics, which are able to penetrate biofilm, in most cases exceed the minimum inhibitory concentrations (MIC) measured for planktonic form of the bacteria by several orders. Comparing the minimum inhibitory concentrations with concentrations affecting the cells in the biofilm (MBIC, MBEC), the all the differences were statistically significant ($P \leq 0.01$). For summary results see Table 3.

![Fig. 4. Comparison of MIC, MBIC and MBEC values (log).](image-url)

PEN – penicillin; OXA – oxacillin; AMS – ampicillin-sulbactam; CMP – chloramphenicol; TET – tetracycline; COT – co-trimoxazole; ERY – erythromycin; CLI – clindamycin; CIP – ciprofloxacin; GEN – gentamicin; TEI – teicoplanin; VAN – vancomycin; MIC – yellow; MBIC – green; MBEC – blue

The results show, that the MIC values did not correspond with the values that are able eradicate the biofilm. The biofilm layer act as a barrier for antibiotic diffusion to the cells, e.g. glycopeptides, with their large molecules have very low effect on staphylococci in the biofilm layer, because their large molecules cannot penetrate the biofilm layer (König et al., 2001). Another mechanism of resistance is chemical bonding of the positively charged antibiotics to the negatively charged compounds of the biofilm layer (aminoglycosides) (Lewis, 2001). The diffusion barrier formed by the biofilm layer acts also in the opposite
direction – the enzymes such as beta-lactamases cannot diffuse from the close proximity of the bacterial cells, so the concentration of these enzymes in the bacterial surrounding is relatively high (Stewart, 1996). The accumulation of waste products and defec tion of nutrients may lead to the change of physico-chemical conditions in micro-colonies. Such environment decreases efficiency of aminoglycosides. These entire factors act in combination, which enhances their effect (Lewis, 2001) and similar mechanisms can be found also for other antimicrobial compounds.

<table>
<thead>
<tr>
<th></th>
<th>MIC Mean</th>
<th>Median</th>
<th>MBIC Mean</th>
<th>Median</th>
<th>MBEC Mean</th>
<th>Median</th>
</tr>
</thead>
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<tr>
<td>PEN</td>
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<td>931</td>
<td>1024</td>
<td>936</td>
<td>1024</td>
</tr>
<tr>
<td>OXA</td>
<td>11.1</td>
<td>4</td>
<td>219</td>
<td>256</td>
<td>2420</td>
<td>2048</td>
</tr>
<tr>
<td>AMS</td>
<td>5.6</td>
<td>2</td>
<td>34.2</td>
<td>32</td>
<td>1979</td>
<td>2048</td>
</tr>
<tr>
<td>CMP</td>
<td>8.27</td>
<td>2</td>
<td>37.8</td>
<td>4</td>
<td>698</td>
<td>512</td>
</tr>
<tr>
<td>TET</td>
<td>6.68</td>
<td>0.5</td>
<td>25.3</td>
<td>1</td>
<td>1002</td>
<td>128</td>
</tr>
<tr>
<td>COT</td>
<td>16.9</td>
<td>4</td>
<td>289</td>
<td>16</td>
<td>15639</td>
<td>2048</td>
</tr>
<tr>
<td>ERY</td>
<td>14.9</td>
<td>16</td>
<td>4096</td>
<td>4096</td>
<td>4096</td>
<td>4096</td>
</tr>
<tr>
<td>CLI</td>
<td>9.17</td>
<td>16</td>
<td>2283</td>
<td>4096</td>
<td>2340</td>
<td>4096</td>
</tr>
<tr>
<td>CIP</td>
<td>9.96</td>
<td>16</td>
<td>922</td>
<td>256</td>
<td>3377</td>
<td>4096</td>
</tr>
<tr>
<td>GEN</td>
<td>10.4</td>
<td>0.25</td>
<td>80.8</td>
<td>64</td>
<td>182</td>
<td>128</td>
</tr>
<tr>
<td>TEI</td>
<td>2.02</td>
<td>2</td>
<td>10.4</td>
<td>8</td>
<td>558</td>
<td>512</td>
</tr>
<tr>
<td>VAN</td>
<td>1.64</td>
<td>2</td>
<td>7.09</td>
<td>4</td>
<td>209</td>
<td>256</td>
</tr>
</tbody>
</table>

PEN – penicillin; OXA – oxacillin; AMS – ampicillin-sulbactam; CMP – chloramphenicol; TET – tetracycline; COT – co-trimoxazole; ERY – erythromycin; CLI – clindamycin; CIP – ciprofloxacin; GEN – gentamicin; TEI – teicoplanin; VAN – vancomycine

Table 3. Average MIC, MBIC and MBEC values.

The results of our studies confirm the importance of biofilm-positive bacteria as causative agents of biofilm infections of catheters and implants and indicate increased risk of failure of conventional antimicrobial therapy caused by increased resistance of such strains.

4. Poly-microbial biofilms and their composition

In our studies we presented the difference in results of microbial assessment based on use of pre-cultivation and sonication techniques (Hola et al., 2010). Our results showed that the sonication technique is more reliable for examination of biofilm infections of catheters, because it detects wider number of microbial species. Another advantage of sonication technique is quantification of isolated microbes, which can be very helpful for the treatment of the infection and for more detailed knowledge about mixed-species biofilm community. The sonication technique also solves problem of over-growing of some fast-growing microbes, such as Pseudomonas aeruginosa. The over-growing is often present in “classic” pre-cultivation technique and can suppress growth of other species and thus lead to the lower sensitivity of these techniques. From all above-mentioned reasons, the infection can be misinterpreted as single- or dual-species infection only (Hola et al., 2010).
The CAUTI are rarely single-species. More often these infections are poly-microbial. The number of isolated strains from one catheter/stent ranges between one and seven, where the mono-species infection is present only in 16.2% of catheters. Most of the CAUTI are three-species biofilms – c. 30%, less often two- and four-species biofilms (see Fig. 5).

![Fig. 5. Number of strains isolated from one catheter.](image)

In these mixed-species biofilm communities, several microbial species are very often present, although the species composition of catheter is variable. Such species are *Escherichia coli* (present in 76.5% of poly-microbial catheter infections), *Enterococcus* sp. (at least one species present in 76.5% of poly-microbial catheter infections), *Candida* sp. (at least one species present in 64.7% of poly-microbial catheter infections) and *Klebsiella* sp. (at least one species present in 41.1% of poly-microbial catheter infections). The composition of other microbial species in the biofilm community is variable (Hola et al., 2008). Up to now we isolated 47 different microbial taxa from urinary tract catheters. Most often we isolated *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis* and *Candida albicans*. These microbial species presented over 65% of total microbial isolates. For the full list of microbial taxa present in poly-microbial CAUTIs see Table 4.

The ability to form biofilm is present in most of our isolates. Very often, circa in 70% of the isolates, we can prove strong biofilm production. Only very low number of strains isolated from IUC is not able to form biofilm (less than 5%). The biofilm formation also differs among particular microbial species. Some species show high ratio of biofilm-positive strains whereas other show lower. The differences in the biofilm formation among microbial species were statistically significant (ANOVA, p = 0.0031). The highest ratios of strong biofilm-positive strains have species *Enterococcus faecalis* (95%), *Proteus mirabilis* (94%), *Candida tropicalis* (91%) and *Staphylococcus aureus* (100%). Low ratio of strong biofilm-positive strains had e.g. *Escherichia coli* (35%).
### Microbial taxa present in poly-microbial CAUTIs

<table>
<thead>
<tr>
<th>Acinetobacter baumannii</th>
<th>Klebsiella sp. (other)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burkholderia cepacia</td>
<td>Kluyvera cryocrescens</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>Morganella morganii</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>Ochrobactrum anthropi</td>
</tr>
<tr>
<td>Candida krusei</td>
<td>Pantoea agglomerans</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>Pantoea sp.</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>Proteus mirabilis</td>
</tr>
<tr>
<td>Citrobacter koseri</td>
<td>Proteus vulgaris</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>Providencia rettgeri</td>
</tr>
<tr>
<td>Citrobacter sp. (non-freundii, non-koseri)</td>
<td>Providencia stuartii</td>
</tr>
<tr>
<td>Corynebacterium sp.</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>Enterococcus faecium</td>
<td>Pseudomonas sp.</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>Ralstonia pickettii</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>Raoultella terrigena</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>Serratia marcescens</td>
</tr>
<tr>
<td>Enterobacter dissolvens</td>
<td>Serratia odorifera</td>
</tr>
<tr>
<td>Enterobacter kobei</td>
<td>Streptococcus agalactiae</td>
</tr>
<tr>
<td>Enterobacter sp. (other)</td>
<td>Streptococcus sp. (alpha-haemolytic)</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Streptococcus pyogenes</td>
</tr>
<tr>
<td>Hafnia alvei</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>Klebsiella ornithinolytica</td>
<td>Staphylococcus epidermidis</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>Staphylococcus haemolyticus</td>
</tr>
<tr>
<td>Klebsiella ozanae</td>
<td>Staphylococcus hominis</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.** List of microbial taxa isolated from poly-microbial CAUTIs in St. Anne’s University Hospital during years 2007-2010.

The strong biofilm forming strains seem to be responsible for biofilm production in mixed-species biofilms. These species seem to be primary colonisers and co-aggregate with other species or just provide refuge to other species that are only weak biofilm-producers building up the mixed-species biofilm community.

The other virulence factors, which can be present in bacteria, play important role in the mixed-species biofilms. These virulence factors affect the microenvironment in the biofilm niche, e.g. urease production increases pH of the biofilm layer; the production of beta-lactamases protect whole mixed-species community etc.

Also presence of microbes in different stages and forms plays important role in the mixed-species biofilm formation, for example the *Candida* species form pseudohyphae in their biofilm mode of growth; the strains of the genus *Proteus* may profit from close contact with each other, because in the formation of parallel cells they are capable of faster movement on

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the catheter surface and they produce higher amount of extra-cellular polysaccharides (Stickler & Hughes, 1999), which protects the microenvironment in the biofilm layer.

5. Prevention of biofilm infections of urinary tract

Progress in the area of prevention of urinary catheter-associated infections is very limited and the preventive procedures used nowadays rather only prolong the “abacterial window” then really prevent the infection. There are only few effective preventive strategies available for prevention of CAUTIs. These include avoiding unnecessary catheterisation, selecting alternative catheterisation procedures, maintaining the closed drainage system, and eliminating bacterial colonisation of the patient (Jacobsen et al., 2008).

Every from above-mentioned preventive strategies are bound onto well-informed personnel, which plays the key role in the prevention of biofilm infections of urinary tract. The prolongation of the catheterisation or even unnecessary flat catheterisation are the first steps which can be changed in the course of prevention of the CAUTIs. More attention to the selective and limited catheter use can lead to reduction of the number of CAUTIs. Once it is determined that a patient requires urinary catheterisation, the risk of developing CAUTI is affected by the duration of the catheterisation (Jacobsen et al., 2008). To reduce the risk of infection; the urinary catheter should be changed approx. every 8 days (Rudra & Rudra, 2002) and drainage bags should be emptied minimally every 4 hours to prevent bacteria reaching the lumen of catheter (Newman, 1998). To the minimization of inappropriate prolongation of the catheterisation may help various reminder systems (Blodgett, 2009; Jacobsen et al., 2008).

The use of a closed drainage system rather than open collection container, reduces the incidence of bacteriuria to approximately 50% at 11 days of continuous catheterisation in comparison with 95% presence of significant bacteriuria in patients with open catheter drainage for 96 hours (Trautner & Darouiche, 2004). The drainage system should be dependent at all times. The presence of the drainage tube and/or collection bag above the level of the urinary bladder is associated with an increased risk of CAUTIs, as well as the presence of the drainage tube below the level of the collection bag (Maki & Tambyah, 2001). The differences in bacterial colonisation of the urinary tract with intermittent catheterisation and with indwelling catheters are discussed in chapter 1.1.1 and 1.1.2.

There are many ways of surface treatment of catheters, which have been examined during last decade. These techniques of catheter surface treatment should prevent bacterial adhesion to the artificial surface ant thus prevent formation of biofilm infection. These procedures include e.g. incorporation of the antimicrobial compound into the catheter material (without chemical bond), increase of surface concentration of antimicrobial compounds by means of catheter soaking, chemical bonding of antimicrobial compounds to the surface of the catheter, chemical bonding of antimicrobial compounds in polymer structure of the material or use of new anti-adherent coatings. These procedures lead to significantly higher concentrations of antibiotic, which can act directly in the place of origin of the biofilm focus (Jansen & Peters, 1991).

These methods, such as antimicrobial-impregnated urinary catheters rather only prolong the “abacterial window” then really prevent the infection and the colonising microflora one day anyway appears. Study performed in patients with acute spinal cord injury, who received long-term urinary catheters, showed that the silver-coated catheters delayed but did not prevent the onset of bacteriuria (Schaeffer et al., 1988). The in vitro laboratory study of
colonisation of different types of catheters showed no differences among silicone and silver-coated catheters (Hola et al., 2009) The same problem is in antibiotic impregnation of urinary catheters - the onset of bacterial colonisation is later, but is always present (Darouiche et al., 1999; Guay, 2001; Johnson et al., 1999). In general, the antimicrobial-coated urinary catheters may be beneficial in hospitalized patients that undergo short-term bladder colonisation (Trautner & Darouiche, 2004). Additionally, all antibiotic-impregnated urinary catheters have same problem - the subinhibitory levels of the antimicrobial agent that is eluted into the urine may induce resistance in the resident organisms, especially in patients with prolonged catheter use (Stickler, 2002).

The consensus of antimicrobial treatment of CAUTIs is, that systemic antibiotics are not recommended in general for patients with asymptomatic bacteriuria (Warren, 1994). The systemic dosage of antibiotic should be used only in cases with clear indication of antimicrobial therapy (signs of septicaemia, pyelonephritis etc.).

6. Treatment of biofilm infections of urinary tract

The antibiotic treatment cannot efficiently affect bacteria embedded in the biofilm layer. In vivo the antibiotics can suppress symptoms of the infection by the eradication of planktonic cells, but they fail in the eradication of the cells embedded in the biofilm. After antibiotic treatment the biofilm can act as the focus of the infection and cause recurrence of the infection. It is well known, that biofilm-associated infections commonly persist, until the colonised surface is removed from the patient’s body (Stewart & Costerton, 2001).

Comparison of the antibiotic resistance of planktonic and biofilm form of microbes causing CAUTIs showed, that bacterial biofilms may survive several orders higher concentrations of antibiotic (Hola et al. 2004 c; Souli & Giamarellou, 1998; Mah & O’Toole, 2001). It is obvious, that the presence of biofilm on the urinary catheter leads to therapy failure. There are many mechanisms of biofilm resistance against antibiotics, which supply and overlap.

Some mechanisms of biofilm resistance were discussed above. The most important type of the biofilm resistance is the diffusion barrier formed by biofilm matrix (Ishida et al., 1998). The penetration potential differs among different antibiotics and depends also on the infectious agents present in the biofilm layer (Vrany et al., 1997).

The chemical bonding of antibiotics and increased concentration of antibiotic-degrading enzymes in the close proximity of bacterial cells are other two mechanisms, which can suppress action of beta-lactam antibiotics (Lewis, 2001; Stewart, 1996).

Another mechanism is based on changes in the biofilm layer, the absence of nutrients and decreased levels of oxygen. These conditions may lead to the starvation of cells in the biofilm layer. The starving cells grow more slowly or don’t grow at all. Such slowly growing cells show increased resistance to beta-lactams (Spoering & Lewis, 2001; Schierholz & Beuth, 2001). The accumulation of waste products, which changes physico-chemical properties inside the biofilm layer, decreases efficiency of aminoglycosides and tetracyclines (Lewis, 2001).

Spatial heterogeneity of the cells in the biofilm layer is another important form of biofilm resistance. The spatial heterogeneity is important survival strategy, because minimally part of the cells, which represent wide scale of different metabolic states, have always chance to survive every metabolically targeted attack (Costerton et al., 1999). Because of these unique and changing properties of the biofilm-positive microbes, it is extremely difficult to find simple antimicrobial compound, which would be capable of getting over most of strategies.
of biofilm resistance. Despite the antibiotic treatment, the infections of the implants often persist until the device is removed (Schierholz & Beuth, 2001). To the particular recommendations for the treatment of biofilm infection of uroprosthetic system belong replacement of colonised catheter (and by this way removal of the biofilm nidus) and, if necessary for successful treatment, increased dosage of antibiotics. On the other side, if the patient has no signs of septicaemia or pyelonephritis, the colonisation of the catheter is not necessary to be treated (Warren, 1994).

7. Acknowledgment

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Urinary tract infections (UTIs) are among the most common bacterial infections worldwide, and they are also the leading cause of hospital-acquired infections. Therefore, the appropriate management of UTIs is a major medical and financial issue. This book covers different clinical manifestations of UTI, with special emphasis on some hard-to-treat diseases, and special conditions in respect of treatment; antibiotic resistance and the available alternative strategies for the prevention and treatment of UTIs and it deals with urinary tract infections in children. The aim of this book is to give a summary about the different aspects of the diagnosis, management and prevention of urinary tract infections for all medical disciplines.

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