Biofilm forming bacteria isolated from urinary tract infection, relation to catheterization and susceptibility to antibiotics

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In human medicine, it has been estimated that 65% of nosocomial infections are biofilm associated, loading the health care system enormous costs. These biofilm infections are 10 to 1000 times more resistant to the effects of antimicrobial agents. This study aimed at showing the difference between patients with catheter associated urinary tract infection (CAUTI) and those with non catheter associated (UTI) in terms of the type of isolated pathogens, antibiotic susceptibility of isolated pathogens, detection of their ability to form biofilm, and comparing (antibiotic susceptibility of sessile cells) minimal biofilm eradication concentration (MBEC) and (their planktonic counterpart) minimal inhibitory concentration (MIC) for biofilm forming bacteria. The most frequently isolated micro-organisms were Escherichia coli (31.7%) followed by Klebsiella (15%); Staphylococcus aureus; coagulase negative Staphylococcus (CoNS); Enterococcus (11.7%); Proteus (10%); Pseudomonas (6.7%) and the least common was Enterobacter (1.7%). In the catheterized patients, 13 isolates out of thirty bacterial isolates (43.3%) were biofilm forming and 17 isolates (56.7%) were non biofilm forming, while in the non catheterized patients, 9 isolates out of thirty bacterial isolates (30%) were biofilm forming and 21 isolates (70%) were non biofilm forming. Antibiotic sensitivity of the isolated pathogens was done using disc diffusion method which showed that Imipenem and Amikacin were most effective antibiotics against gram-negative isolates while for gram positive isolates, Vancomycin and Ciprofloxacin were most effective. There was no statistical difference between the two groups regarding the isolated pathogens or the antibiotic susceptibility pattern. For the biofilm forming isolates, antibiotic susceptibility of sessile cells MBEC were tested and compared to the MIC of their planktonic counterpart. For gram negative isolates, Amikacin and Imipenem were used and for gram positive isolates, Ciprofloxacin and Vancomycin were used. The difference between MBEC and MIC for tested strain was statistically significant. Therefore, researches on easier methods for diagnosing and quantifying biofilm infection would surely help the fight against biofilm formation. Also for certain infection such as CAUTI, it is advised to test antimicrobial susceptibility in biofilm form MBEC.

Key words: Urinary tract infection, Biofilm.

INTRODUCTION

Urinary tract infection, with its diverse clinical syndromes and affected host groups, remains one of the most common but widely misunderstood and challenging infectious diseases encountered in clinical practice. Antimicrobial resistance is a leading concern and efforts should be made to ensure an appropriate duration of therapy for symptomatic infections (Drekonja and Johnson, 2008). The risk of developing urinary tract infection increases significantly with the use of indwelling devices such as catheters and urethral stents or sphincters (Foxman, 2003). Urinary tract infections
account for an estimated 25 to 40% of nosocomial infections and represent the most common type of these infections (Bagshaw and Laupland, 2006).

Clinical observations have established that, the microbial populations within catheter associated urinary tract infection (CAUTI) frequently develop as biofilms, directly attaching to the surface of catheters (Trautner and Darouiche, 2004). Biofilms are microbial communities of surface-attached cells embedded in a self-produced extracellular polymeric matrix (Donlan and Costerton, 2002). They can cause significant problems in many areas, both in medical settings (For example; persistent and recurrent infections, device-related infections) and in non-medical (industrial) settings (For example, biofouling in drinking water distribution systems and food processing environments) (Flemming, 2002; Fux et al., 2005). A worrying feature of biofilm-based infections is represented by the higher resistance of bacterial and fungal cells growing as biofilms to antibiotics and disinfecting chemicals as well as resisting phagocytosis and other components of the body’s defence system, when compared to planktonic cells (Hoiby et al., 2010).

Traditionally, microbiologists have evaluated the efficacy of an antibiotic (AB) by measuring the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). In virtually all diagnostic laboratories, these measurements are made on freely floating, planktonic, laboratory phenotypes. These assays measure only the concentration of chemotherapeutic agent required to inhibit growth or kill planktonic bacteria. For some antibiotics, the concentration required to kill sessile bacteria may be greater than a thousand times that required to kill planktonic bacteria of exactly the same strain. Thus, even well-chosen treatment based upon laboratory results often merely suppresses an infection until biofilm-associated organisms are reactivated and cause another clinical infection (Olson et al., 2002).

Therefore, this study aimed at showing the difference between patients with CAUTI and those with non catheter associated urinary tract infection (UTI) in terms of type of isolated pathogens, antibiotic susceptibility of isolated pathogens, detection of their ability of to form biofilm, and comparing antibiotic susceptibility of sessile cells minimal biofilm eradication concentration (MBEC) and their planktonic counterpart minimal inhibitory concentration MIC for biofilm forming bacteria.

**MATERIALS AND METHODS**

The study was performed in the Department of Medical Microbiology and Immunology of Ain Shams University and was conducted on seventy two patients divided into two groups admitted to Ain Shams University Hospitals during the period from November2009 to December 2010 to get the final number of thirty bacterial isolate per group:

**Group 1:** Forty patients had either an indwelling urinary catheter in place at the time of specimen collection and at least one of the following: fever (>38°C), suprapubic tenderness, costovertebral angle pain or tenderness, or the catheter removed within 48 hours prior to specimen collection and had at least one of the following: fever (>38°C), urgency, frequency, dysuria, suprapubic tenderness, or costovertebral angle pain or tenderness.

**Group 2:** Thirty two patients did not have an indwelling urinary catheter in place at the time of specimen collection nor within 48 hours prior to specimen collection and had at least one of the following: fever (>38°C, urgency, frequency, dysuria, suprapubic tenderness, or costovertebral angle pain or tenderness).

All samples were cultured on cystine lactose electrolyte deficient agar (CLED) and blood agar media using 1 µl calibrated loop to detect colony forming unit and incubated aerobically at 37°C for 24 h, count more than 10^5 was considered significant. Identification of isolated organisms was done by conventional microbiological methods (Cheesbrough, 2007).

**Biofilm formation test**

The isolated organisms were tested for their ability to form biofilm, according to Stepanovic et al. (2007). Briefly, flat-bottomed 96-well clear polystyrene tissue culture treated microtiterplate (MP) with a lid (TPP- Switzerland) were inoculated with 200 µl of a bacterial suspension in corresponding to 0.5 McFarland (with further 1:100 dilution). After 24 h incubation at 37°C, the contents of each well were removed by decantation and each well was washed three times with 300 µl of sterile saline. The remaining attached bacteria were heat-fixed by exposing them to hot air at 60°C for 60 min in Fisher isotemp incubator, then150 µl crystal violet (2%) stain was added to each well. After 15 min, the excess stain was rinsed off by decantation, and the plate was washed., 150 µl 95% ethanol was added to each well, and after 30 min, the optical densities (OD) of stained adherent bacterial films were read using a microtiter-plate reader (Tecan Sunrise remote Austria) at 620 nm. The average OD values were calculated for all tested strains and negative controls, the cut-off value (ODc) was established. It is defined as a three standard deviations (SD) above the mean OD of the negative control: ODc=average OD of negative control + (3×SD of negative control). Final OD value of a tested strain was expressed as average OD value of the strain reduced by ODc value (OD= average OD of a strain -ODc); ODc value was calculated for each microtiter plate separately. When a negative value was obtained, it was presented as zero, while any positive value indicated biofilm production. For easier interpretation of the results, strains were divided into the following categories:

1. Non biofilm producer (0) OD ≤ODc
2. Weak biofilm producer (+ or 1) = ODc <OD ≤2×ODc,
3. Moderate biofilm producer (++ or 2) = 2×ODc <OD ≤4×ODc
4. Strong biofilm producer (+++or 3), 4×ODc <OD

**Antibiotic susceptibility testing for planktonic cells**

**Disc diffusion method**

Antibiotic susceptibility of all isolated organisms was done by disc diffusion method, using Muller-Hinton (MH) agar plates. After overnight incubation results were reported and interpretation was done according to clinical and laboratory standards institute (CLSI M100-S20, M100-S20U, guidelines 2010). The antibiotics evaluated were those commonly approved for the treatment of bacterial infections National Committee for Clinical Laboratory Standards.
Minimal inhibitory concentration (MIC)

This was done only for biofilm forming isolates. The antibiotics were selected as the biofilm forming isolates were commonly sensitive to it. For gram negative isolates, Amikacin and Imipenem were used, for gram positive isolates, Ciprofloxacin and Vancomycin were used. MIC was determined by broth micro dilution using 96 wells MP and results were interpreted according to CLSI, guidelines (2010).

Antibiotic susceptibility of biofilm

For the biofilm forming isolates, antibiotic susceptibility of sessile cells were tested according to (Cernohorska and Votava, 2004, Passerini de Rossi et al., 2009) and compared to the MIC of their planktonic counterpart.

Statistical analysis

Analysis of data was done by IBM computer using statistical program for social science (SPSS) version 12. Quantitative variables were expressed as mean ± standard deviation (SD). Statistical tests included Chi-square test and Spearman correlation coefficient rank test (r) which was used to rank different variables against each other either positively or inversely. Unpaired t-test was used to compare two independent groups as regards quantitative variables in parametric data (SD < 50% mean). Results were considered significant when p value was ≤ 0.05.

RESULTS

In this study, sixty bacterial isolates were obtained from fifty patients divided into two groups. The first group included 23 catheterized patients (8 males, 15 females) giving thirty bacterial isolates and the mean age of group 1 patients was 52 ± 8.8 ranging from 34 to 65. The second group included 27 non-catheterized patients (9 males, 18 females) giving thirty bacterial isolates and the mean age of group 2 patients was 41.5 ± 11.5 ranging from 23 to 66. Out of the sixty isolates the most common isolated pathogens were: Escherichia coli (31.7%); (47.4%) in group 1, (52.6%) in group 2, followed by Klebsiella (15%) (44.4%) in group 1, (55.6%) in group 2, then Staphylococcus aureus, CoNS, (11.7%) (42.9%) in group 1, (57.1%) in group 2, Enterococcus (11.7%) (71.4%) in group 1, (28.6%) in group 2, Proteus (10%) (50% in group 1 and 2, pseudomonas (6.7%) (75%) in group1, (25%) in group 2, and lastly, Enterobacter (1.7%) single isolate in group 2. The difference between two groups was statistically non significant (Figure 1). Out of sixty isolates 38 isolates (63.3%) were non biofilm forming (44.7%) in group1, (55.3%) in group 2 and 22 isolates (35.0%) were biofilm forming [(59.1%) in group1, (40.9%) in group 2 (Figures 2 and 3). Out of 22 biofilm forming isolates 18(81.8%) were weak biofilm forming (50%) in group 1,2 and 4 (18.2%) were moderate biofilm forming all (100%) in group 1 but this difference was statistically non significant (Chi-square test). The highest percent of biofilm formation was detected in CoNS as (57.1%) of CoNS isolates were biofilm forming followed by Pseudomonas (50.0%), Klebsiella (44.4%), Staph (42.9%), E. coli(31.6%) and Enterococci (28.6%) while least biofilm forming was Proteus(16.7%) and Enterobacter (0.0%).

The most effective antibiotics against Gram-negative
isolates were found to be Imipenem and Amikacin and for gram positive isolates Vancomycin and Ciprofloxacin. A statistically significant difference (Unpaired t-test) existed between MIC and MBEC of Vancomycin and Ciprofloxacin exist for gram positive isolate. The isolates that were sensitive or intermediately sensitive in their MIC values became resistant in their MBEC value, and for gram negative isolates the difference between MIC and MBEC of Imipenem and Amikacin was statistically highly significant as for Imipenem isolates that were sensitive or intermediately sensitive in their MIC values became resistant in their MBEC value and for Amikacin isolates that were sensitive or intermediately sensitive in their MIC value became resistant in their MBEC values except for two isolates as the first isolate remained sensitive, while the second became intermediately sensitive (Table 1).

**DISCUSSION**

In this study, the frequency of UTI was greater in women as compared to men as 66% of the patients were females and 34% were males principally owing to anatomic and physical factors. Similar results were shown by kashef et al. (2010) and Al Benwan et al. (2010). This study showed that the most common causative organism of UTI
Table 1. Difference between MIC and MBEC of biofilm forming isolates.

<table>
<thead>
<tr>
<th>Type of organism</th>
<th>Degree Of Biofilm Formation</th>
<th>Ciprofloxacin µg/ml</th>
<th>Vancomycin µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIC</td>
<td>MBEC</td>
</tr>
<tr>
<td><strong>Gram positive isolates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>Weak</td>
<td>0.5</td>
<td>64</td>
</tr>
<tr>
<td>Enterococci</td>
<td>Weak</td>
<td>2</td>
<td>&gt;64</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Moderate</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>CoNS</td>
<td>Weak</td>
<td>0.125</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Enterococci</td>
<td>Weak</td>
<td>1</td>
<td>&gt;64</td>
</tr>
<tr>
<td>CoNS</td>
<td>Moderate</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Moderate</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>CoNS</td>
<td>Weak</td>
<td>2</td>
<td>64</td>
</tr>
<tr>
<td>CoNS</td>
<td>Weak</td>
<td>(2)</td>
<td>&gt;64</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Amikacin (µg/ml) and Imipinem (µg/ml)</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC</td>
<td>MBEC</td>
</tr>
<tr>
<td>E. coli Weak</td>
<td>4</td>
</tr>
<tr>
<td>Klebsiella Weak</td>
<td>2</td>
</tr>
<tr>
<td>Klebsiella Weak</td>
<td>1</td>
</tr>
<tr>
<td>Proteus Weak</td>
<td>2</td>
</tr>
<tr>
<td>E. coli Weak</td>
<td>4</td>
</tr>
<tr>
<td>Klebsiella Weak</td>
<td>2</td>
</tr>
<tr>
<td>E. coli Weak</td>
<td>2</td>
</tr>
<tr>
<td>Pseudomonas Weak</td>
<td>0.25</td>
</tr>
<tr>
<td>E. coli Weak</td>
<td>1</td>
</tr>
<tr>
<td>E. coli Weak</td>
<td>2</td>
</tr>
<tr>
<td>Klebsiella Weak</td>
<td>1</td>
</tr>
<tr>
<td>Pseudomonas Moderate</td>
<td>32</td>
</tr>
<tr>
<td>E. coli Weak</td>
<td>8</td>
</tr>
</tbody>
</table>

was E. coli (31%) followed by klebsiella (15%), Staphylococcus, Coagulase negative staphylococci CoNS, Enterococcus (11.7%) each, Proteus (3.8%), Pseudomonas (6.7%), and the least common cause being Enterobacter (3.8%). Similar results were reported by Neto et al. (2003), Bgshaw and Lupland, (2006), Savas et al. (2006) and EL-Banoby et al. (2007) who found that E. coli was the most common cause of UTI.

There is a reported difference in prevalence of various uropathogens between patients with indwelling urinary catheters and non catheterized patients. In this study, it was found that E. coli, Klebsiella, S. aureus and CoNS were more often recovered from non catheterized patients while Enterococcus spp. and Pseudomonas spp. were more often recovered from catheterized patients but due to small number of samples the difference was not statistically significant. Similar results were reported by (Savas et al., 2006, ko et al., 2008, Milan and Ivan, 2009) who found that E. coli was the most prevalent organism in both catheterized and non catheterized patient.

As regards the antibiotics used, the present study showed that the most effective antibiotics against Gram-negative isolates were found to be Imipenem and Amikacin and for gram positive isolates Vancomycin and Ciprofloxacin. This is in agreement with Savas et al. (2006) who found that the most effective antibiotics against Gram-negative bacteria were imipenem and meropenem.

Similar results were reported by EL-Banoby et al.(2007) who stated that Amino glycosides were the most common antibiotics to which the organisms were sensitive for nosocomial UTI (46.2%) followed by Monobactam (34.6%), Quinolones (30.8%), and lastly Vancomycin (3.8%). The present study showed no statistically significant difference (Unpaired t-test) between the catheterized and non catheterized UTI patients as regard antibiotic susceptibility. In disagreement with the results of the present study, Ko et al. (2008) noticed that urinary catheters increased antimicrobial resistance of Enterobacteriaceae and rare gram negative bacilli (For example, Acinetobacter spp.) to nearly all antibiotic tested.

Another contradictory results reported by Milan and Ivan ,(2009) who evaluated resistance between community-acquired urinary tract infections, nosocomially-acquired urinary tract infections, and found that the highest level of general resistance was among isolates of
nosocomial-acquired UTI and catheter-associated UTI followed by community-acquired UTI isolates. This study found that (43.3%) of isolates of group 1 were biofilm forming while in group 2 (30%) of the isolates were biofilm forming, yet the difference between two groups was statistically non significant. Similar results were found by Watts et al. (2010) who compared the virulence properties of a collection of asymptomatic bacteriuria (ABU) and catheter associated asymptomatic bacteriuria (CA-ABU) nosocomial E. coli isolates and found that the CA-ABU strains displayed no significant difference from the ABU strains in the biofilm formation phenotype; (48%) of CA-ABU strains and (59%) of ABU strains formed a biofilm.

In this study, antibiotic susceptibility of planktomic cells presented as MIC was compared to and their counterpart sessile cells presented by MBEC. Amikacin and Imipenem were chosen for gram negative biofilm forming isolates and Vancomycin, Ciprofloxacin for gram positive biofilm forming isolates. This choice was based upon disc diffusion antibiotic susceptibility as we chose the common sensitive or intermediately sensitive antibiotic. A statistically significant difference in antibiotic susceptibility between planktonic populations and biofilm populations of the same organism was demonstrated in this work.

Similar results were reported by Sepandj et al. (2004) who compared the MIC and MBEC of Ampicillin, Cefazolin, Cefotaxime, Ciprofloxacin, Gentamicin and Tobramycin of eight E. coli strains, Amikacin, Aztreonam, Cefazidime, Ciprofloxacin, Gentamicin, Imipenem, Pipercillin and Tobramycin susceptibility were also tested for eight Pseudomonas strains, isolated from patients with peritoneal dialysis - associated peritonitis. The authors concluded that in their biofilm state, gram-negative bacteria are much less susceptible to antibiotics compared to their antibiotic susceptibility in the planktonic state.

Another agreeing results were reported by Sepandj et al. (2007) who compared the MIC and MBEC of Six samples of Enterococcus faecalis and five isolates of E. faecalis and found that the E. faecalis isolates that were sensitive in their MIC results but demonstrated resistance in their MBEC results to Ciprofloxacin, Vancomycin and Ampicillin. Ampicillin combined with Gentamicin (Gentamicin 1 and 4 mg/ml) resulted in 0 MIC and 2 MBEC resistances. As regards E. faecium, the MIC results were sensitive for Vancomycin only. The MBEC results revealed uniformly poor sensitivity of E. faecium biofilms to antibiotics, including Ampicillin with Gentamicin combinations. Also, Passerini de Rossi et al. (2009) who compared MIC and MBEC of Levofloxacin and Ciprofloxacin for 32 Stenotrophomonas maltophilia isolates and found that S. maltophilia isolates which were sensitive to fluoroquinolones according to their MICs were highly resistant according to the MBEC values.

Another agreeing results were reported by Naves et al. (2010) who studied the in vitro susceptibility of seven E. coli biofilm producing strain in their planktonic and biofilm associated forms to Amoxicillin, Amoxicillin/Clavulanic, Cefotaxime, Gentamycin and Ciprofloxacin and found that E. coli biofilms were much less sensitive than their planktonic counterparts to tested antibiotics. Lastly, Antunes et al. (2010) who compared the MIC and MBEC of Vancomycin for thirty staphylococci isolates found that all isolates presented higher MBEC than the MIC for Vancomycin. But in disagreement with the above results Spoerling and Lewis (2001) who performed a comparative examination of tolerance of biofilms versus stationary and logarithmic-phase planktonic cells with four different antimicrobial agents and concluded that, at least for Pseudomonas aeruginosa, one of the model organisms for biofilm studies, the notion that biofilms have greater resistance than do planktonic cells is unwarranted. They suggested that tolerance to antibiotics in stationary-phase or biofilm cultures is largely dependent on the presence of persister cells.

**RECOMMENDATION**

Current antibiotics have classically been developed to treat infections involving planktonic bacterial populations in acute infection settings and are typically ineffective in the eradication of bacteria in biofilm- associated, persistent infections. The MBEC assay were developed for rapid and reproducible antimicrobial susceptibility testing for bacterial biofilms in the anticipation that the MBEC would be more reliable for selection of clinically effective antibiotics.

Many researches are needed to find easier methods for diagnosing and quantifying biofilm infection, to develop more specific antimicrobial agents and ideal device surfaces that would surely help the fight against biofilm formation.

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