A STUDY ON THE DETECTION OF BIOFILM FORMATION BY MULTIDRUG RESISTANT UROPATHOGENIC ISOLATES IN A TERTIARY CARE HOSPITAL

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ABSTRACT

BACKGROUND
Urinary Tract Infections (UTI) are the most common and important nosocomial infections, especially among diabetic patients. Emergence of multi-drug resistance and biofilm formation by these pathogens lead to chronic and recurrent infections. Our study aims at detection of multi-drug resistant uropathogens and biofilm formation by them.

Aims and Objective: Identification and isolation of significant multi-drug resistant uropathogens in diabetic patients. Detection of biofilm formation by these multi-drug resistant uropathogens by TCP, TM and CRA methods and comparison of these three methods for their efficacy.

Settings and Design: This is a hospital-based prospective cross-sectional study carried out among the Diabetic patients suffering from UTI in a tertiary care hospital for a period of six months.

MATERIALS AND METHODS
This study involved 127 Diabetic patients of both Type I and Type II, suffering from UTI. The urine samples collected were processed by standard microbiological techniques and the antibiotic susceptibility pattern of significant uropathogens were assessed. Uropathogens resistant to any three or more of the commonly used anti-microbial agents were considered multi-drug resistant and were assessed for biofilm formation by the following three methods: Tissue Culture Plate (TCP) method, Tube Method (TM) and Congo Red Agar (CRA) method using Pseudomonas aeruginosa ATCC 27853 strain and Staphylococcus epidermidis ATCC 12228 strains as controls. The results were analysed using the standard statistical methods.

RESULTS
In our study, the prevalence of UTI was higher in females (56.69%) and in Type II Diabetic patients (91.34%). Among the isolates, Escherichia coli was the commonest uropathogen. About 52% of the isolates from urine samples were multi-drug resistant showing resistance to Penicillin, Cephalosporins and Sulphonamides. TCP method is found to be the standard method for the detection of biofilm formation. Biofilm formation and multi-drug resistance are found to be more in diabetic patients with poor glycaemic control.

CONCLUSION
Hence, this study reinforces the need for good glycaemic control in diabetic patients to prevent multi-drug resistance and biofilm formation by uropathogens.

KEYWORDS
UTI- Urinary Tract Infection, MDR- Multi-Drug Resistant, TCP- Tissue Culture Plate.


BACKGROUND
Urinary Tract Infections (UTI) are the most common acquired bacterial infections and account for about 25% - 40% of the nosocomial infections.[1] Escherichia coli is the most common pathogen[2] followed by Enterococcus species, Staphylococcus saprophyticus, Proteus species, Klebsiella species, Pseudomonas aeruginosa and Candida albicans. Now-a-days, emergence of multi-drug resistance among these pathogens is of serious concern and could be attributed due to inadequate dosage, improper and widespread use of broad spectrum antibiotics and transfer of anti-microbial resistant genes through plasmids. Biofilm formation by these pathogens worsens the situation further, as it protects them from opsonophagocytosis and antibiotics, thereby enhances its virulence leading to chronic, recurrent infections and sepsis.[3] Biofilms have medical significance, as they decrease their susceptibility to antimicrobial agents.[4]

More than 80% of all infections involve biofilms.[5] A biofilm is an assemblage of microbial cells, irreversibly associated with a surface and encased in a matrix of polysaccharide material.[6] The biofilm consists of layers of cell clusters embedded in a matrix of extracellular polysaccharide called polysaccharide intercellular adhesin (PIA), which consists of b-1, 6-N-acetylglucosamine and is synthesised by N-acetylglucosamine transferase.[7]
Biofilms are associated with indwelling medical devices like catheters, ventilators, implants and infections like dental caries, cystic fibrosis, osteonecrosis, urinary tract infections and eye infections.[8]

Both Gram positive and Gram negative bacteria like Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis and Pseudomonas aeruginosa[9] are capable of biofilm formation.

Biofilm formation can be detected by Tissue Culture Plate Method (TCP), Tube Method (TM) and Congo Red Agar (CRA) Method.[3]

Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial agents than other organisms.[5] In the above perspective, this study proposes early detection of biofilm formation by the pathogens and their association with multi-drug resistance. Through this study, the three methods of biofilm detection are compared for their efficacy. This will guide the clinician for the institution of prompt and effective therapeutic measures for the infections, thereby reducing the morbidity and mortality associated with these infections.

Aims and Objectives
- To identify the significant pathogens with colony count of ≥ 10⁵ CFU/ mL, among the clinical isolates of Urinary Tract Infection in diabetic patients.
- To detect the multi-drug resistance among these significant uropathogens.
- To detect biofilm formation by TCP, TM and CRA methods.
- To compare the above methods in the detection of biofilm formation.

MATERIALS AND METHODS

Study Centre and Duration
This cross-sectional study was conducted in a tertiary care hospital for a duration of six months (August 2016 and January 2017) after obtaining Ethical Clearance from the Institutional Ethical Committee.

Selection Criteria
Diabetic patients presenting with the clinical features of urinary tract infections (UTI) like fever, urgency and frequency of urine, loin or abdominal pain and burning or painful micturition were included in the study.

Diabetic patients without the symptoms of UTI and the non-diabetic patients were excluded.

Collection and Processing of Specimen
Detailed clinical history of diabetic status of the patient was obtained. The patients were instructed to collect about 10 – 20 mL of clean catch midstream urine into a wide-mouth screw cap sterile container after cleaning their external genitalia with soap and water. The urine samples were transported immediately to the Microbiology Laboratory for further processing.

The urine samples were processed by semi-quantitative standard calibrated loop method to assess the significant bacteriuria. The urine samples were processed further with Gram staining, motility by hanging drop method, solid media culture isolation, catalase test, oxidase test, coagulate test and other biochemical reactions. Antibiotic susceptibility pattern of these significant bacterial isolates were assessed by Kirby-Bauer disc diffusion method.

The pathogens resistant to any three or more of the following group of antimicrobials - Penicillins, Cephalosporins, Tetracyclines, Aminoglycosides, Macrolides, Quinolones and Sulfonamides were considered multi-drug resistant isolates.[6]

Biofilm production by these multi-drug resistant uropathogens were assessed by the following methods - Tissue Culture Plate (TCP) method, Tube Method (TM) and Congo Red Agar (CRA) method. Pseudomonas aeruginosa ATCC 27853 and Staphylococcus epidermidis ATCC 12228 were used as controls.

Tissue Culture Plate (TCA) Method
The significant multi-drug resistant uropathogenic isolates were inoculated in 10 mL of trypticase soy broth with 1% glucose and incubated at 37°C for 24 hrs. Then they were diluted 1:100 using fresh trypticase soy broth. About 200 µL of these individual cultures were loaded into the well of tissue culture plate along with the control strains and incubated at 37°C for 24 hrs. Sterile broth was included as a negative control. After incubation, the contents of the wells were discarded and wells were washed 4 times using 0.2 mL of phosphate-buffered saline. The biofilm formed by the pathogens adherent to the wells was fixed with 2% sodium acetate and then stained with 0.1% crystal violet. Then the wells were washed with deionised water and allowed to dry. The optical density of the stained biofilm was measured by ELISA reader at 492 nm wavelength.[5] The results were interpreted as non/weak, moderate and strong based on the criteria of Stepanovic et al.[6,7] as mentioned below:-

Interpretation of Biofilm Production by Tissue Culture Plate (TCP) Method

<table>
<thead>
<tr>
<th>Average OD Value</th>
<th>Biofilm Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 2x ODc</td>
<td>Non/Weak</td>
</tr>
<tr>
<td>&gt;2x ODc ≤ 4x ODc</td>
<td>Moderate</td>
</tr>
<tr>
<td>&gt; 4x ODc</td>
<td>Strong</td>
</tr>
</tbody>
</table>

Optical density cut-off value (ODc) = average OD of negative control + 3x standard deviation (SD) of negative control.

Tube Method (TM)
Tube Method was performed by inoculating a loopful of the multi-drug resistant isolates in 10 mL of trypticase soy broth with 1% glucose in test tubes. They were then incubated at 37°C for 24 hrs. After incubation, the contents were discarded and the tubes were washed with phosphate buffer saline and dried. Then they were stained with 0.1% crystal violet and washed with deionised water and dried in inverted position.[5] Observation of a visible film lining the wall of the tube was regarded as positive for biofilm formation.

Congo Red Agar (CRA) Method
The test isolates along with controls were inoculated onto the Congo red agar medium (Brain heart infusion broth 37 g/dL, Sucrose 50 g/dL, Agar No. 1 10 g/dL and Congo Red Indicator 8 g/dL) the plates were then incubated aerobically at 37°C for 24 hrs.[5] After incubation, isolates showing black crystalline colonies were regarded as biofilm producers.
The results were analysed by using the standard statistical methods.

RESULTS
A total of 127 samples were collected from the diabetic patients suffering from UTI. Out of 127 patients under study, 55 (43.31%) were male patients and 72 (56.69%) were female patients.

Out of 127 urine samples, 100 (78.74%) samples showed microbial growth and 27 (21.26%) showed no growth. Out of 100 positive urine cultures, 92 (92%) were of monomicrobial growth and 8 (8%) were of polymicrobial growth.

A total of 108 uropathogens were isolated from 100 positive urine cultures, 92 (92%) were of monomicrobial growth and 27 (26.87%) showed no growth.

Out of 108 uropathogens, only 67 (62.04%) isolates showed significant count of ≥ 10^5 CFU/mL and were processed further.

Out of 67 significant uropathogenic isolates, 47 isolates were Gram Positive Cocci (GPC) were, 20 (29.85%) were resistant to Gentamicin, 53 (79.10%) were resistant to Nitrofurantoin, 7 (10.45%) were resistant to Cefuroxime, 20 (29.85%) were resistant to Cotrimoxazole, 35 (52.24%) were resistant to Cotrimoxazole. The antibiotic susceptibility pattern of these 67 significant uropathogenic isolates were as follows: around 27 (40.30%) were resistant to Gentamicin, 53 (79.10%) were resistant to Nitrofurantoin, 7 (10.45%) were resistant to Cefuroxime, 20 (29.85%) were resistant to Cotrimoxazole.

The antibiotic resistance pattern observed in Gram negative (GNB) isolates were,

*Escherichia coli* (n= 29)
Nalidixic acid (93.10%) > Norfloxacin (86.21%) > Cotrimoxazole (62.07%) > Gentamicin (41.38%) > Amikacin (17.24%) > Cefuroxime (13.79%) > Nitrofurantoin (3.45%).

*Klebsiella Species* (n= 11, *K. pneumoniae-* 6, *K. oxytoca-* 5)
Nalidixic acid (63.64%), Norfloxacin (63.64%) > Gentamicin (54.55%) > Nitrofurantoin (45.45%), Cotrimoxazole (45.45%) > Amikacin (27.27%) > Cefuroxime (9.09%).

*Pseudomonas Aeruginosa* (n= 2): - Nalidixic acid (100%), Nitrofurantoin (100%) > Cotrimoxazole (50%), Norfloxacin (50%) and all were susceptible to Cefuroxime, Gentamicin and Amikacin.

*Proteus mirabilis* (n= 2)
Nalidixic acid (50%), Norfloxacin (50%), Gentamicin (50%), Cotrimoxazole (50%) and all were susceptible to Cefuroxime, Nitrofurantoin and Amikacin.

*Citrobacter Species* (n= 3)
Nalidixic acid (100%) > Norfloxacin (66.67%), Nitrofurantoin (66.67%) > Gentamicin (33.33%), Cotrimoxazole (33.33%) and all were susceptible to Cefuroxime.

The antibiotic resistance pattern of Gram Positive Cocci (GPC) were,

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th><em>Escherichia coli</em> (n= 29)</th>
<th><em>Klebsiella sp.</em> (n= 11)</th>
<th><em>Pseudomonas sp.</em> (n= 2)</th>
<th><em>Proteus sp.</em> (n= 2)</th>
<th><em>Citrobacter sp.</em> (n= 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin</td>
<td>41.3%</td>
<td>54.5%</td>
<td>0%</td>
<td>50%</td>
<td>33.33%</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>93.10%</td>
<td>63.64%</td>
<td>100%</td>
<td>50%</td>
<td>33.33%</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>86.21%</td>
<td>63.64%</td>
<td>50%</td>
<td>0%</td>
<td>66.67%</td>
</tr>
<tr>
<td>Amikacin</td>
<td>17.24%</td>
<td>27.27%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>3.45%</td>
<td>45.45%</td>
<td>100%</td>
<td>0%</td>
<td>66.67%</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>13.79%</td>
<td>9.09%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>20.69%</td>
<td>18.18%</td>
<td>0%</td>
<td>100%</td>
<td>33.33%</td>
</tr>
<tr>
<td>Cotrimoxazole</td>
<td>62.07%</td>
<td>45.45%</td>
<td>50%</td>
<td>50%</td>
<td>33.33%</td>
</tr>
</tbody>
</table>

The antibiotic resistance pattern of Gram Negative Bacilli were,

<table>
<thead>
<tr>
<th>Antibiotic Used</th>
<th>No. of Isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin</td>
<td>27</td>
<td>(40.30%)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>53</td>
<td>(79.10%)</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>52</td>
<td>(77.61%)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>18</td>
<td>(26.87%)</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>14</td>
<td>(20.90%)</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>7</td>
<td>(10.45%)</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>20</td>
<td>(29.85%)</td>
</tr>
<tr>
<td>Cotrimoxazole</td>
<td>35</td>
<td>(52.24%)</td>
</tr>
</tbody>
</table>

Out of 108 uropathogens were isolated from 100 samples. Out of 108 uropathogens, only 67 (62.04%) isolates showed significant count of ≥ 10^5 CFU/mL and were processed further.

Out of 67 significant uropathogenic isolates, 47 isolates were Gram Negative Bacilli (29 were *Escherichia coli*, 11 were *Klebsiella sp.*, 5 were *Pseudomonas sp.*, 2 were *Proteus sp.*, 3 were *Citrobacter sp.* and 2 were *Staphylococcus aureus*, 4 were *Staphylococcus epidermidis*, 2 were *Staphylococcus saprophyticus* and 7 were *Enterococcus sp.*).
**Staphylococcus aureus (n=7)**
Nalidixic acid (85.71%), Norfloxacin (85.71%) > Cotrimoxazole (71.43%) > Nitrofurantoin (28.57%) > Cloxacillin (14.29%), Gentamicin (14.29%), Amikacin (14.29%), Cefuroxime (14.29%).

**Staphylococcus epidermidis (n=4)**
Norfloxacin (75%), Amikacin (75%) > Cloxacillin (25%), Cotrimoxazole (25%), Cefuroxime (25%) and all were susceptible to Nitrofurantoin and Gentamicin.

**Staphylococcus saprophyticus (n=3)**
Norfloxacin (100%) > Cloxacillin (50%), Cotrimoxazole (50%), Cefuroxime (50%) and all were susceptible to Nitrofurantoin and Gentamicin.

**Enterococcus sp. (n=7)**
High level Gentamicin (85.71%) > Nalidixic acid (71.43%), Norfloxacin (71.43%), Amikacin (71.43%) > Nitrofurantoin (42.86%).

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th><strong>Staphylococcus aureus</strong> (n=7)</th>
<th><strong>Staphylococcus epidermidis</strong> (n=4)</th>
<th><strong>Staphylococcus saprophyticus</strong> (n=2)</th>
<th><strong>Enterococcus sp.</strong> (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin</td>
<td>14.29%</td>
<td>0%</td>
<td>0%</td>
<td>85.71%</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>85.71%</td>
<td>50%</td>
<td>50%</td>
<td>71.43%</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>85.71%</td>
<td>75%</td>
<td>100%</td>
<td>71.43%</td>
</tr>
<tr>
<td>Amikacin</td>
<td>14.29%</td>
<td>75%</td>
<td>50%</td>
<td>71.43%</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>28.57%</td>
<td>0%</td>
<td>0%</td>
<td>42.86%</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>14.29%</td>
<td>25%</td>
<td>50%</td>
<td>42.86%</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>14.29%</td>
<td>25%</td>
<td>50%</td>
<td>42.86%</td>
</tr>
<tr>
<td>Cotrimoxazole</td>
<td>71.43%</td>
<td>25%</td>
<td>50%</td>
<td>42.86%</td>
</tr>
</tbody>
</table>

Table 6. Antibiotic Resistance % of Gram Positive Cocci

Out of 67 uropathogenic isolates, 35 were found to be resistant to Penicillins, Cephalosporins, Sulphonamides, Aminoglycosides and Fluoroquinolones; were identified as multi-drug resistant isolates (MDR). These isolates were subjected to Biofilm detection.

**Comparison of Detection Methods of Biofilm Formation**
Out of 35 MDR isolates, 16 (45.71%) were found to produce biofilm by Tissue Culture Plate (TCP) method, 15 (42.86%) by Tube Method (TM) and 15 (42.86%) by Congo Red Agar (CRA) method.

<table>
<thead>
<tr>
<th>Detection Method</th>
<th>Total MDR Isolate</th>
<th>Biofilm Producers</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue Culture Plate</td>
<td>35</td>
<td>16</td>
<td>45.71%</td>
</tr>
<tr>
<td>Tube method</td>
<td>35</td>
<td>15</td>
<td>42.86%</td>
</tr>
<tr>
<td>Congo red agar</td>
<td>35</td>
<td>15</td>
<td>42.86%</td>
</tr>
</tbody>
</table>

Table 7. Comparison of Biofilm Production by Various Methods

![Chart 1. Distribution of Significant Uropathogenic Isolates](image1)

![Figure 1. Biofilm Formation by TCP Method](image2)

![Chart 2. Overall Antibiotic Resistance Pattern](image3)
DISCUSSION
In this study, we found that the prevalence of UTI were more in females (56.69%) when compared to males (43.31%). This was correlated with the finding of Getenet Beyene et al.[2] which tells that females (64.9%) have high risk of UTI than males (35.1%). It also correlates with the study by Arul Prakasham KC et al.[10] and Devanand Prakash et al.[11] This strengthens the view that UTI infections are more common among female population.

In this study, UTI were common among type II diabetic patients (91.34%) than type I diabetic patients (8.66%). It correlates with the study done by Ramanath Katta Venkatesh et al.[12] which tells that 43% of diabetic patients have UTI. It also correlates with the study of Hamdan Z. Hamdan et al.[13] and S Niveditha et al.[1]

In our study, the polymicrobial pattern of growth was found to be 8%. It was around 2.9% in the study done by J. Janifer et al.[14]

In this study Gram negative bacilli (73.15%) were more common than Gram positive cocci (26.25%), which correlates with the finding of Devanand Prakash et al.[11] that out of 155 bacterial uropathogens, 140 (90.32%) were Gram negative and 15 (9.68%) were Gram positive isolates.

In this study, Escherichia coli (47.22%) was the commonest isolate among the uropathogens. This coincides with the study done by Subramanian Pramodhini et al.[1] where E. coli was found to be the most frequently isolated uropathogen (70%) and S Niveditha et al.[1] study also signifies E. coli as the most frequently isolated organism.

In this study, the antimicrobial resistance pattern observed was Nalidixic acid (79.10%) > Norfloxacine (77.61%) > Cotrimoxazole (52.24%) > Gentamicin (40.30%) > Amikacin (26.87%) > Nitrofurantoin (20.90%). This is similar to the pattern observed by Subramanian Pramodhini et al,[1] which reads as follows: Nalidixic acid (84%) > Norfloxacine (72%) > Cotrimoxazole (72%) > Gentamicin (40%) > Nitrofurantoin (40%) > Amikacin (20%).

In this study, Escherichia coli (28.57%) and Enterococcus sp. (28.57%) were found to be most common biofilm producing organisms. It coincides with the study of Subramanian Pramodhini et al.[1] which shows 63% were Escherichia coli.

In this study, out of the 35 MDR isolates 16 (45.71%) were biofilm producers by Tissue Culture Plate (TCP) method, 15 (42.86%) by Tube Method (TM) and 15 (42.86%) by Congo Red Agar (CRA) method. This correlates with the findings by Munesh Kumar Gupta et al.[15]

In this study, we found that the rate of biofilm formation was higher in the patients with poor glycaemic control compared to those with good glycaemic control correlating with the finding of May Sewify et al.[16]

CONCLUSION
The current study concludes that UTI is more common in female diabetic patients than male diabetic patients. Escherichia coli is the most common uropathogenic isolate. The uropathogens were more resistant to Nalidixic acid, Norfloxacine and fluoroquinolones and many are found to be multi-drug resistant isolates. Escherichia coli and Enterococcus sp. are the most common biofilm forming multi-drug resistant uropathogens. Tissue culture plate method is found to be the standard method for the detection of biofilm formation. Biofilm formation and multi-drug resistance are found to be more in diabetic patients with poor glycaemic control. Hence, this study reinforces the need for good glycaemic control in diabetic patients in preventing emergence of multi-drug resistance and biofilm formation by uropathogens. It also emphasises initiation of early and appropriate anti-microbial therapy to reduce the mortality and morbidity associated with UTI in diabetic patients.

REFERENCES


