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Original Research Article

Biofilm Formation and Antibiotic Resistance of Uropathogens in Pregnant Women with Symptomatic and Asymptomatic Bacteriuria

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ABSTRACT

Introduction: Urinary tract infection is more common in women than men and the susceptibility increases further during pregnancy. Higher incidence of UTI in women can be attributed to factors like shortness of urethra, close proximity of urethra to anus, sexual intercourse pregnancy and catheters. The detection of bacteriuria allows an approach to be made for the prevention of chronic UTI and avoid complications in pregnancy at an early stage. Bacteria adhere to the surfaces, initially in a reversible association and then through irreversible attachment, and eventually develop into an adherent biofilm of highly structured and cooperative consortia .The presence of biofilms also explains the nature of chronic infections that keep recurring after antibiotic treatment ceases. Clearly, biofilm infections in urogenital tissue are associated with significant morbidity and mortality.

Materials & Methods: A total of 100 midstream urine samples from pregnant females were collected aseptically. The samples were cultured on MacConkey agar and incubated at 37°C for 24 hrs. Urine culture yielding colony counts of>10^5organisms/ml, along with >10pus cells/HPF of a centrifuged urine sample were interpreted as diagnostic of bacteriuria. Identification of isolates was performed by colony morphology, gram staining and standard biochemical tests. Antibiotic sensitivity testing against commonly used antibiotics was done by Kirby Bauer disc diffusion method. Extended spectrum Beta lactamase (ESBL) producers were detected by disc potentiation method. Detection of biofilms was done by the Tissue Culture Plate assay described by Christensen *et al* considered as standard test for detection of biofilm formation. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader at wavelength of 570 nm. These OD values were considered as an index of bacteria adhering to surface and forming biofilms.

Results: Out of 100 pregnant females investigated for UTI 30 (30%) had significant bacteriuria. Out of 30% showing significant growth 23 (23%) were symptomatic and 7 (7%) were asymptomatic. Out of 30 urine samples showing significant growth of organisms E.coli was the predominant isolate 18 (60%) followed by Klebsiella pneumoniae 7(23.3%), Enterococcus 2 (6.6%), Pseudomonas aeruginosa2 (6.6%) and Staphylococcus aureus1 (3.33%). Out of 27 gram negative isolates 14(51.8%) were resistant to Amoxicillin, 11(40.7%) to Norfloxacin, 9(33.3%) to Cotrimoxazole, 6(22.2%) to Nitrofurantoin, 4 (14.8%) to Ciprofloxacin, 4 (14.8%) to Nalidixic acid & 2 (7.4%) to Amoxicillin clavulanic acid.

Biofilm production among uropathogens was detected by Tissue culture plate method in 9 of 27 isolates mainly from *E.coli, Pseudomonas and Klebsiella*.

Conclusion: Significant correlation between Biofilm production and multidrug resistance was seen in the study. It is therefore recommended that routine microbiological analysis, antibiotic sensitivity test of mid-stream urine samples and biofilm detection of pregnant females whether symptomatic or asymptomatic should be carried out so as enhance in the administration of drugs for the treatment and management of UTI in pregnancy and prevent further complications.

Key Words: Biofilm, Antibiotic resistance, Urinary tract Infection

INTRODUCTION

Urinary tract infection is more common in women than men and the susceptibility increases further during pregnancy. Higher incidence of UTI in women can be attributed to factors like shortness of urethra, close proximity of urethra to anus, sexual intercourse pregnancy and catheters. Ideal pH, temperature and constituents like glucose present in urine also predispose to bacterial growth. [1] There is much to encourage UTI during pregnancy when several anatomical and physiological changes take place in urinary tract. During pregnancy, urethral compression at the pelvic brim by enlarging uterus leads to stasis of urine, incomplete emptying and residual urine which is the single most important factor that can initiate proliferation of microorganisms. [2] Related to it further a number of complications such as Acute and Chronic pyelonephritis, Cystitis, Anemia, Hypertension, Prematurity, Intrauterine growth retardation and increased perinatal mortality are seen. [3] Early treatment of bacteriuria not only could avert the occurrence of pyelonephritis but it would also diminish the risk of prematurity and perinatal mortality. The detection of bacteriuria allows an approach to be made for the prevention of chronic UTI and avoid complications in pregnancy at an early stage. Bacteria adhere to the surfaces, initially in a reversible association and then through irreversible attachment, and eventually develop into an adherent biofilm of highly structured and cooperative consortia. [4] Mature biofilms are typically embedded in copious amounts of extracellular polymer matrix or glycocalyx, which are separated by water-filled channels and voids to allow convective flows that transport nutrients and oxygen from the interface to the interior parts of the biofilm, and remove metabolic wastes. Characteristics are an increased resistance toantibiotic treatment, persistence, evasion of host immune systems (thus exhibiting an altered immune response), expression of different proteins and of quorum-sensing molecules. [5] The presence of biofilms also explains the nature of chronic infections that keep recurring after antibiotic treatment ceases. It is clear, however, that biofilms are associated with urinary tract infections (UTIs) where indwelling devices are not the cause. [4,5] One can imagine the recovery from the symptoms of cystitis following antibiotic treatment, which removes the planktonic bacteria, only to have the symptoms return as a result of regrowth of the planktonic population from a nidus of infection consisting of biofilm bacteria displaying a higher level of resistance to the antibiotic. Chronic biofilm infection with associated tissue destruction in the urethra, bladder, and ureter can result in fibrosis, strictures, stenosis and pyelonephritis. The colonization of the urinary tract with urease-producing bacteria, such as Proteus mirabilis, results in urinary calculi formation in the bladder and kidneys and in further significant health problems associated with infection and obstruction. [5,6] Clearly, biofilm infections in urogenital tissue are associated with significant morbidity and mortality.

Aims & Objective:

To Study the prevalence of pathogens causing UTI among pregnant women. To Isolate and identify the pathogen from urine in pregnant women. To Asses the antibiotic sensitivity pattern and detect ESBL production among gram negative isolates. To study formation of Biofilm among uropathogens using the tissue culture plate method. To compare Biofilm formation and multidrug resistance in symptomatic UTI and asymptomatic Bacteriuria.

MATERIALS & METHODS

A total of 100 urine samples were collected from pregnant women attending the ANC OPD over a period of six months. Subjects comprised of varying ages from 18

to 30 years. Pregnant women of varying gravida and all three trimesters were included. Various symptoms suggestive of UTI were noted down which included Urgency, lower abdominal pain, low backache, burning micturition, fever and dysuria.

I) Collection of Urine –

Urine samples were collected in sterile universal container. The pregnant females were instructed to clean their genital area with soap and water and then collect the mid-stream urine sample after separating the labia. Urine samples were transported to the Microbiology laboratory within 1 hour of collection.

II) Examination of Urine –

1. **Macroscopic examination –** Urine was observed by naked eye for altered colour, presence of turbidity, deposit and findings were recorded.
2. **Microscopic examination –** Urine was centrifuged at 2000 rpm for 15mins in a conical centrifuge tube. The supernatant was discarded and wet preparation of sediment was examined under low and high power to observe Pus cells, RBC, Cast and Crystals and Epithelial cells. More than 10 pus cells per high power field were considered significant.

III) Plating of Urine sample by Standard loop technique:

All the samples were cultured by semiquantitative method. A calibrated loop with an internal diameter of 4mm was used for plating. The samples were cultured on Mac Conkey agar and incubated at 37°C for 24 hrs. Urine culture yielding colony counts of>10^5organisms/ml, along with >10pus cells/HPF of a centrifuged urine sample were interpreted as diagnostic of bacteriuria. Bacterial counts of less than this were considered insignificant and growth of more than 2 types of organisms was considered as contamination. Identification of isolates was performed by colony morphology, gram staining and standard biochemical tests. Antibiotic sensitivity testing against commonly used antibiotics was done by Kirby Bauer disc diffusion method using Clinical Laboratory Standards Institute (CLSI) guidelines. Individual colonies were suspended in peptone water. Turbidity was matched to 0.5 McFarland and using sterile swabs the suspensions were inoculated on Mueller Hinton agar and incubated for 18-24 hrs. For Gram Positive and Gram Negative bacteria the following discs were tested Amoxicillin (25µg), Co-trimoxazole (23.75µg) Nitrofurantoin (300µg), Ciprofloxacin (5µg), Nalidixic acid(30µg), Amoxicillin-clavulanic acid(20µg/10µg) and Norfloxacin(5µg).

IV) Demonstration of Extended spectrum beta lactamase (ESBL) by Double Disc diffusion test:

Principle:

Most ESBL will be inhibited by β-lactamase inhibitor Clavulanic acid, hence restoring susceptibility to cephalosporin. Thus increase in zone size in presence of inhibitor compared with cephalosporin alone by 5mm or more is indicative of ESBL producing strain. ESBL producing strains were considered multidrug resistant as they are resistant to all beta lactam antibiotics.

Method:

A lawn culture of the test strain is exposed to the disc of a later generation cephalosporin, e.g. Cefotaxime (30µg) or Ceftazidime (30µg), and a disc of Co-amoxiclav (20µg amoxicillin/10µg clavulanic acid) placed at a distance of 30mm apart. After overnight incubation, if the strain has an extended spectrum β-lactamase, the inhibition zone around the cephalosporin disc is enhanced on the sides nearest to the co-amoxiclav disc.

V) Detection of Biofilms –

Tissue Culture Plate method –

The TCP assay is most widely used and was considered as standard test for detection of biofilm formation. In present study all isolates were screened for their ability to form biofilm by TCP method. Isolates from fresh agar plates were inoculated in Tryptic Soy Broth media and incubated for 24 hour at 37°C in stationary condition and diluted 1 in100 with fresh medium. Individual wells of sterile, polystyrene, 96 well-flat bottom

tissue culture plate wells were filled with 0.2 ml aliquots of the diluted cultures and only broth served as control to check sterility and non-specific binding of media. The tissue culture plates were incubated for 18-24 hours at 37°C. After incubation content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2 mL of phosphate buffer saline (PBS pH 7.2) to remove free-floating 'planktonic' bacteria. Biofilms formed by adherent organisms stained with crystal violet (0.1%). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent organisms usually formed biofilm on all side wells and were uniformly stained with crystal violet. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader wavelength of 570 nm (OD570 nm). These OD values were considered as an index of bacteria adhering to surface and forming biofilms. Experiment was performed in triplicate and repeated three times, the data was then averaged and standard deviation was calculated. The mean OD value obtained from media control well was deducted from all the test OD values.

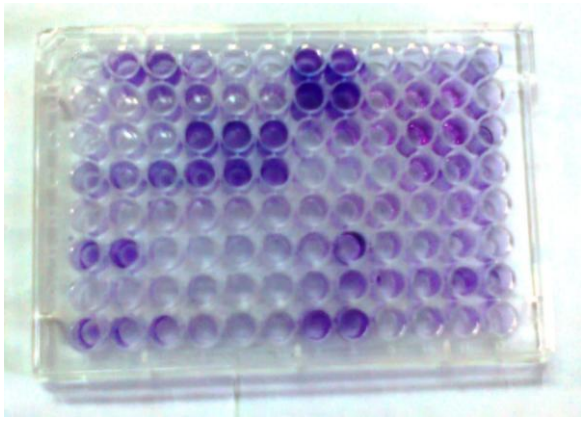

Figure 3 :-
Screening of biofilm producers by TCP method: Strong, moderate and non slime producers differentiated with crystal violet staining in 96 well tissue culture plate.

Table 1: Classification of Bacterial adherence by TCP method.		
Mean OD Values	Adherence	Biofilm Formation
<0.183	Non	Non/weak
0.183-0.238	Moderate	Moderate
>0.238	Strong	High

RESULTS

Out of 100 pregnant females investigated for UTI 30 (30%) had significant bacteriuria. Out of 30% showing significant growth 23 (23%) were symptomatic and 7 (7%) were asymptomatic. (Fig 1 &2,3,4)

Out of 30 urine samples showing significant growth of organisms E. coli was the predominant isolate 18 (60%) followed by Klebsiella pneumoniae 7(23.3%), Enterococcus 2 (6.6%), Pseudomonas aeruginosa2 (6.6%) and Staphylococcus aureus1 (3.33%). (Table 2)

Out of 27 gram negative isolates 14(51.8%) were resistant to Amoxycillin, 11(40.7%) to Norfloxacin, 9(33.3%) to Cotrimoxazole, 6(22.2%) to Nitrofurantoin, 4 (14.8%) to Ciprofloxacin, 4(14.8%) to Nalidixic acid& 2 (7.4%) to Amoxicillin-clavulanic acid. The gram positive isolates Enterococcus and Staphylococcus aureus showed good sensitivity to all antimicrobial drugs. (Table 3)

E.coli isolates showed multidrug resistance in which there was resistance to three or more drugs and were ESBL producers. These strains were found to show increased Biofilm production.

Biofilm production among uropathogens was detected by Tissue culture plate method in 9 of 27 isolates mainly from *E.coli, Pseudomonas and Klebsiella*. (Table 3).

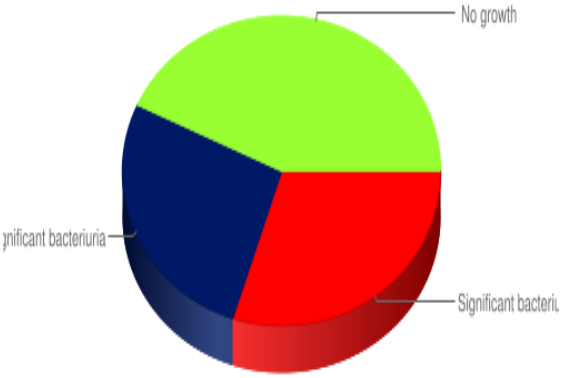

Figure 1. Detection of bacterial growth in urine cultures of pregnant women. Significant bacteriuria (30%), Non significant bacteriuria (27%), No growth (43%).

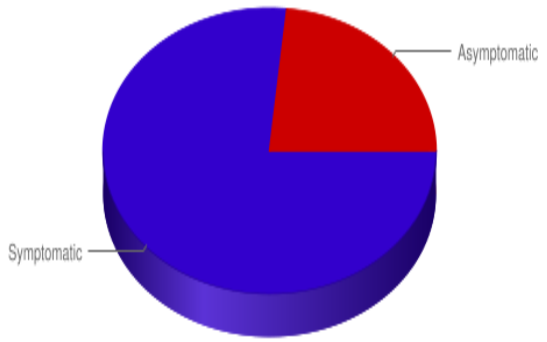

Figure 2. Showing Symptomatic bacteriuria (23%) and Asymptomatic bacteriuria(7%) of total Significant bacteriuria(30%).

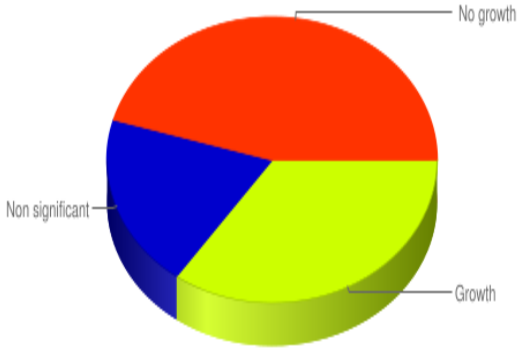

Figure 3. Symptomatic Group showing Growth(23%),Non significant (13%), No growth (30%)

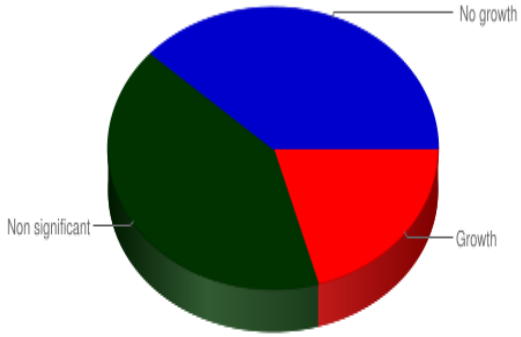

Figure 4. Asymptomatic group showing Growth(7%),Non significant(14%), No growth(13%).

Table 2: Spectrum of urinary pathogens from urine of pregnant women

Pathogen	No. Of Positive culture	Percentage
Escherichia coli	18	60
Klebsiellapneumoniae	07	23.3
Enterococcus feacalis	02	6.6
Pseudomonas aeruginosa	02	6.6
Citrobactersp	01	3.3
Total	30	100

Table 3: Comparison of Biofilm formation & Multidrug resistance in Symptomatic & Asymptomatic Pregnant females with UTI

Organism	Biofilm positive	ESBL Positive	Symptomatic UTI	Asymptomatic bacteriuria
Escherichia coli (18)	05 (27.7%)	06(33.3%)	14(77.7%)	04(22.2%)
Klebsiella pneumonia (7)	03 (42.8%)	05(71.4%)	05(71.4%)	02(28.5%)
Enterococcus faecalis(2)	0	0	02(100%)	0
Pseudomonas aeruginosa (2)	01 (50%)	02(100%)	02(100%)	0
Citrobacter sp.(1)	1(100%)	1(100%)	00	01(100%)
Total (30)	9 (30%)	14 (46.6%)	23 (76.6%)	07(23.3%)

DISCUSSION

The prevalence of UTI among pregnant women was 30% which was high compared to other studies (Fig 1). Studies suggest a prevalence of 2-12% among pregnant women which is slightly higher than from the west (4-7%). [6-10] Incidence of asymptomatic bacteriuria was 7% in this study though other studies have shown a high prevalence of asymptomatic bacteriuria. [7,11] Prevalence of symptomatic bacteriuria was 23%. (Fig 2,3) Various presenting symptoms were urgency, burning sensation during micturition, lower abdominal pain, fever, lower backache. Fever was the commonest symptom. Frequency of urination normally increases during pregnancy, lower abdominal pain, low backache are common complaints in pregnancy hence not significant parameters unless associated with fever as discussed by other authors also. [10] Observations suggest that a proportion of patients with asymptomatic bacteriuria go on to develop symptomatic infection. The development of symptomatic disease may ultimately protect patients from complications, since symptomatic disease leads to treatment. [13] Treatment of bacteriuria early in pregnancy decreases incidence of pyelonephritis to 2-3%, about 90% risk reduction and also decreases premature delivery. [12]

Antibiotic choice in asymptomatic bacteriuria should focus on coverage of common uropathogens rather than eradication of bacteriuria. Some authors suggest that the positive effect of antibiotic therapy is medicated by an alternate mechanism, such as modification of vaginal flora. [12] Nitrofurantoin, however does not alter vaginal flora and hence was chosen as the drug of choice as it also showed good sensitivity.

In this study, 18(60%) were E.coli isolates which was the commonest isolate as seen in other studies [5] (Table 2). Amongst the total isolates ESBL producers were 46.6%. These were multidrug resistant isolates. This was similar to other studies which showed high prevalence rate of ESBL (MDR) strains. [14,15] Table 3 shows correlation between various isolates producing biofilms and multidrug resistant. Biofilm formation was seen in 30% isolates which was lower compared to ESBL producing strains 46.6%. All the ESBL producing strains were Biofilm positive. Biofilm formation indicates persistence of infection and it has prognostic value. Hence the present study showed significant correlation between biofilm formation and multidrug resistance as described in other studies. [16-18]

The human vagina and the bacterial communities that reside therein represent a finely balanced mutualistic association. [19,20] Women are more prone to UTIs due to the proximity of the urethra, vagina, and rectum. The retrograde ascent of bacteria from the perineum is the most common cause of acute cystitis in pregnant women. [21]

CONCLUSION

This study showed high prevalence of UTI among pregnant women. Significant bacteriuria was observed in both asymptomatic and symptomatic pregnant women. *E. coli* was the commonest isolate and was found to be MDR. Hence urine examination should be an integral investigation of antenatal care. Screening for bacteriuria and treatment with antibiotics is essential to prevent further complications later. Biofilms endows bacteria with several advantages, such as acquisition of antibiotic tolerance, expression of several virulence factors and increased resistance against phagocytosis and other host defence mechanisms. Periodic studies are recommended to confirm the findings of this study and also monitor any changes in the susceptibility patterns of uropathogens causing UTI in pregnant women.

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