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"PREVALENCE OF BIOFILM FORMATION IN UROPATHOGEN'

[1]Himanshu Trivedi, [2]*Dr. Shweta Sao, [3]Dr.Ramnesh Murthy and [4]Dr. Sagrikapradhan

[1]Research Scholar–Dr CV Raman university bilaspur (CG).

[2]Professor and Head Dept of Biotechnology and Microbiology, Dr CV Raman University.

[3]Professor and Head of Microbiology, Microbiology Department, CIMS Hospital Bilaspur Chhattisgarh.

[4]Assistant Professor of Microbiology.

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***Corresponding Author**
Dr. Shweta Sao
Professor and Head dept of Biotechnology and Microbiology, Dr CV Raman University.

ABSTRACT

This study was conducted from February to may 2017 to determine the prevalence, risk factors and Biofilm formation of selected bacterial uropathogens among patients with urinary tract infection (UTI) cases in CIMS Hospital, Bilaspur. A hospital-based cross-sectional survey was conducted to assess the risk factors of UTI. In addition, laboratory based work was conducted to determine the prevalence and bacterial uropathogens using standard procedures. The isolates were identified based on their morphological and biochemical characteristics. Urinarytract infection (UTI) is the most commonly acquired bacterial infection. The purpose of this study was to detect biofilm form ation by uropathogenes isolated from UTIs. The isolated bacteria were tested for biofilm production by tube adherence (TA), congoredagar (CRA) methods. Out of 195 urine specimen, 60 urine samples (30.76%) are culture positive for uropathogen with significant colony count. These 60 uropathogen were processed in the present study for biofilm production, 36 (60%) were culture positive and remaining 29 (48.33%) were culture negative. E. coli was the predominant isolate 45/60 (75%) followed by Staphylococcus aureus 2/60 (3.33%), Klebsiella pneumonia 1/60(1.66%), Pseudomonas aeruginosa 2\60(3.33%), Coagulase negative Staphylococci 2\60(3.33%), Enterococcus faecali 4\60(6.66%), Proteus spp 2\60(3.33), Streptococcus spp 1\60(1.66%), Acinetoobacterspp 2\60(2.33%). The antibiotic resistance was higheramong biofilm producers to commonly used antibiotics ascompared to nonbiofilm producers to its detection by congoredagar method. From this study, we have concluded that TM and CRA method is more qualitative and reliable method to detect biofilm producing microorganisms.

TM and CRA method is used as a general screening method for detection of biofilm producing bacteria in laboratories. Biofilm form at ion is the major virulence determinant of uropathogen, so it is necessary to screen all urinary isolates for biofilm production.

KEYWORDS: Biofilm, Uropathogens, Tube adherence, Congo red agar.

1. INTRODUCTION

Urinary tract infection (UTI) is the most commonly acquired bacterial infection. It poses serious health threat because of antibiotic resistance and high recurrence rate Antibiotic resistance of uropathogens has been known to increase worldwide and biofilm production being the main cause. Biofilm are defined as microbial derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other**.** Bacteria seem to initiate biofilm formation in response to specific environmental conditions, such as nutrient and oxygen availability. Biofilm are the source of persistent infections of many pathogenic microbes. They are responsible for nosocomial infection and also associated with medical conditions including indwelling medical device, dental plaque, upper respiratory tract infection and urogenital infection Both Gram positive and Gram negative bacteria have the capability toproduce biofilm. Biofilm formation allows thestrains to persist for long time in thegenitourinary tract and interfere with bacterialeradication. The microbial biofilms pose aserious health problem as the microorganisms inthe biofilm are difficult to treat withantimicrobial agents. Biofilm production is considered as a marker of clinically relevant infection. (Costerton et.al. 1999 and Reid G.1999) Biofilm are defined as microbial derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. (Donlon et.al. 2002)

Several factors such as exotoxins, surface proteins and extracellular polysaccharides having important roles in virulence of *S. aureus* isolated from mastitis cases have been reported. Furthermore, it has been determined that production of slime factor in *S. aureus* strains causing mastitis was an important virulence factor affecting pathogenesis. It is considered that the first step in mastitis progress is adhesion of *S. aureus* to mammary epithelial cells and slime factor plays an important role for adhesion and colonization. Production of slime factor also plays an important role in antibiotic resistance and it has been reported that slime producing strains are more resistant to antibiotics than non-slime producing strains. (Basegela et.al.1993, Vasudevan P. et.al.2003, Amorena B. et.al 1999).

All microbes like Gram positive and Gram negative bacteria have capacity to synthesized biofilm. Bacteria commonly involved include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Enterococcus faecalis Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa.* (Donlon R.M.2001).

Urinary tract infection (UTI) is a condition in which the urinary tract is infected pathogen causing inflammation (Raju and Tiwari, 2001; Okonk *et al*., 2009). Usually, a UTI is caused by bacteria that can also live in the digestive tract, in the vagina, or around the urethra, which is at the entrance of the urinary tract. Most often these bacteria enter the urethra and travel to the bladder and kidneys (Okonko *et al.,* 2009). Bacteria are the major causative organisms and are responsible for more than 95 % of UTI cases (Ramesh *et al.,* 2008). Clinically important infections usually occur due to gram-negative and gram-positive bacteria, although viruses, fungi, and parasites can also cause infection (Zorc *et al.,* 2005). Common non-bacterial causes of UTI include hemorrhagic cystitis from *adenovirus* and *Candida* infections.Non-bacterial infections are less common and tend to occur more often in immunosuppressed individuals or those with *Diabetes mellitus* cases (Griebling, 2007).

MATERIAL AND METHOD

3.1 Culture Media

Brain Heart Infusion Media (Hi media limited), MacConkey Agar (Hi media limited), Nutrient Agar (Hi media limited), Nutrient Broth (Hi media limited), C etrimide Agar (Hi media limited), Urea Agar Base (Hi media limited), Triple sugar Iron (Hi media limited), Mueller Hinton Agar (Hi media limited), (Hi media limited), Mannitol Salt Agar, (Hi media limited).

Collection of the sample

The study will be carried out in the Department of Microbiology, Chhattisgarh institute of Medical Science, Bilaspur and Department of Microbiology at C.V Raman University during the period of Farvery 2017 to May 2017.

The Mid stream urine samples will be collected in sterile container from patients of all groups, suspected to have a urinary tract infection and transported immediately to the laboratory.

Tube adherence method

This is a qualitative method for biofilm detection. The suspension of the strain to be tested will be poured into a glass tube which contains Brain Heart Infusion broth and incubated at 37 °C for a period of 2 days. Then the supernatant will be discarded and the glass tube stained with 4% solution of crystal violet, washed with distilled water three times and dried. A positive result is defined as the presence of a layer of the stained material which adhered to the inner wall or bottom of the tube. The exclusive observation of a stained ring at the liquid-air interface will be considered as negative.

Congo Red agar method.

The suspension of the tested strains were inoculated into plate which contained a specially prepared solid medium- Brain Heart Infusion broth (BHI) which was supplemented with 5% sucrose and Congo Red. The medium was composed of BHI (37gms/l), sucrose (50 gms/l), agar No.1 (10 gms/l) and the Congo Red stain (0.8 gms/l). Congo Red was prepared as a concentrated aqueous solution and it inoculated and incubated aerobically for 24-48 hours at 37°C. Positive result was indicated by black colonies with a dry crystalline consistency, non biofilm producing strains developed red colonies.

RESULT

Out of 195 urine specimen, 60 urine samples (30.76%) are culture positive for uropathogen with significant colony count. These 60uropathogen wereprocessedinthepresentstudy for biofilm production., 36(60%) wereculturepositiveandremaining 29(48.33%) wereculturenegative. *E.coli*wasthepredominantisolate 45/60(75%) followedby *Staphylococcusaureus* 2/60(3.33%), *Klebsiellapneumonia*1/60(1.66%), ***Pseudomonas aeruginosa*** 2\60(3.33%), ***Coagulase negative Staphylococci*** 2\60(3.33%), ***Enterococcus faecali*** 4\60(6.66%), ***Proteus spp***2\60(3.33**),** ***Streptococcus spp*** 1\60(1.66%), ***Acinetoobacterspp***2\60(2.33%).

Table: 1. ageand sex distribution of UTI Patients (n=60)

Male\female\year	Male	Female	TOTAL
0 – 20	5	8	13
21 – 40	6	21	27
41 – 60	8	3	11
61 above	7	2	9
GRAND TOTAL	26 (43.33)	34 (56.66)	60

As shown as the table no 1. UTI was the more common in female patent (56.66%) in comparison to male patent (43.33). This study UTI was more common 21-40 years of the age.

Table: 2 Bacterial isolates in non biofilm uropathogen

Bacterial isolates	**Total no. of *Isolates (n=60)***	**Congo red (+)**	**Bhi tube (+)**
Escherichia coli	45 (75%)	31(68.88%)	24(53.33%)
Pseudomonas aeruginosa	2 (3.33%)	1	1
Enterococcus faecalis	4(6.66%)	0	2
Coagulase negative Staphylococci	1(1.66%)	0	0
Proteus spp.	2 (3.33%)	0	0
Klebsiella pneumoniae	1 (1.66%)	0	0
Staphylococcus aureus	2 (3.33%)	0	1
Acinetobacter	2 (3.33%)	0	0
Streptococcus	1 (1.66%)	0	0

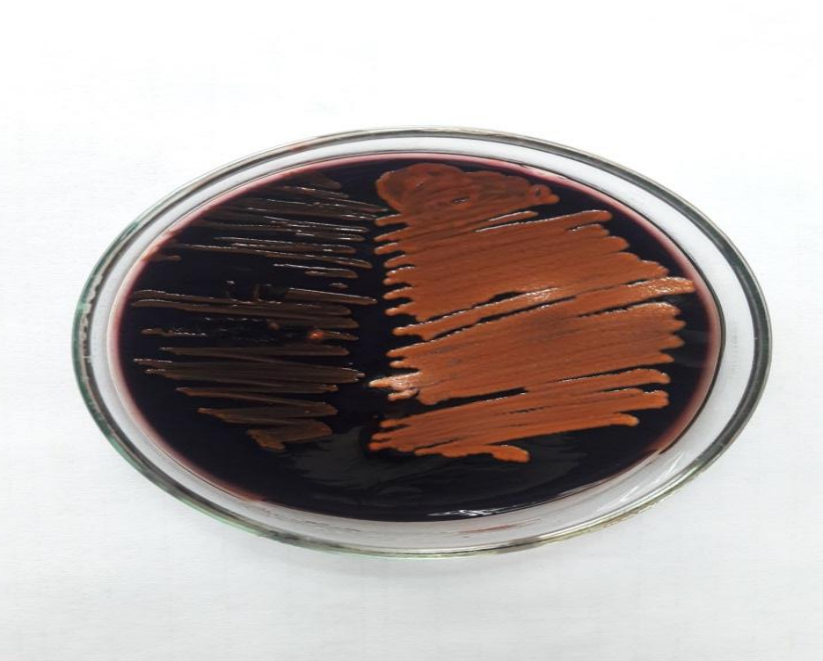

Fig no 1: biofilm produce (left side) non biofilm produce (right side)

- *E.coli* was the most common organism in our study (75%),Pseudomonas auruginosa (3.33%),Enterococcus facials (6.66%) and Proteus spp ,Staphylococcus Spp Acinetobacter organism (3.33%) and other organism in (1.66%).
- We further found that E.coli was the most common organism producing biofilm our study .31 out of 45 (68.88%) isolated of E.coli were is producing by Congo red method.24 out of 45(53.33%) E.coli were produced by BHI method.

Table: 3 Sensitivity pattern of Gram-negative uropathogen and percentage (%)

S no	Antibiotic	*Escherichia coli*	*Pseudomonas aeruginosa*	*Enterococcus faecalis*	*Coagulase negative Staphylococci*	*Proteus spp.*	*Klebsiella pneumoniae*	*Staphylococcus aureus*	*Acinetoobacter*	*Streptococcus*
1	Co trimoxazole	11 (24.44%)	0	2((50%)	0	1(50%)	0	0	0	**0**
2	Ampcillinsul	7(15.56%)	0	2(50%)	0	0	0	0	0	**0**
3	Norfloxacin	16(35.56%)	1(50%)	0	1(100%)	2(100%)	0	0	0	**0**
4	Ofloxacin	12(26.67%)	2(100%)	1(25%)	0	1(50%)	1(100%)	2((100%)	0	**0**
5	Gentamycin	25(55.56)	1(50%)	2(50%)	1(100%)	1(50%)	1(100%)	2(100%)	0	**0**
6	Nitrofuratoin	29(64.44%)	0	3(75%)	1(100%)	1(50%)	1(100%)	1(50%)	0	**0**
7	**Cefuroxime**	**1(2.22%)**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

- As shown as the table num 3 highly reactive antibiotic **nitrifuration** (64.44%) gram negative uropathogen and **gentamycin** is most antibiotic reactive (55.56%) other antibiotic is a commonly reactive .

Table: 4 Age and sex distribution of UTI patient with biofilm production (n=36)

Male\female\year	Male	Female	TOTAL
0 – 20	2(5.55%)	3(8.33%)	5(13.88%)
21 – 40	3(8.33%)	14(45.16)	17(47.22%)
41 – 60	4(11.11%)	3(8.33%)	5(13.88)
61 above	5(13.88%)	2(5.55%)	6(16.66%)
GRAND TOTAL	14(38.88%)	22(61.11%)	36

- Biofilm is most common in patents of 21-40 years of the age found. out of 36 patient, 17(47.22%) were in the 21-40 of age. Second most common age group was 61 years of the age found. out of 36 pateint to the (16.66%).

Tabalenum: 5. sensitivity pattern of Gram-negative uropathogenwith UTI patient percentage (%)

Num	Antibiotic	*Escherichia coli (N=32)*	*Pseudomonas aeruginosa* (n=1)	*Enterococcus faecalis* (n=2)	*Coagulase negative Staphylococci (n=0)*	*Proteus spp. (n=0)*	*Klebsiella pneumonia (n=0)*	*Staphylococcus aureus (n=1)*	*Acinetoobacter (n=0)*	*Streptococcus* (N=0)
1	Co trimoxazole	7(19.44%)	0	1(50%)	0	0	0	0	0	0
2	Ampcillinsul	4(11.11%)	0	2(100%)	0	0	0	0	0	0
3	Norfloxacin	9(25%)	1(100%)	0	0	0	0	0	0	0
4	Ofloxacin	7(19.44)	1(100%)	1(50%)	0	0	0	1(100%)	0	0
5	Gentamyc in	15(41.66)	0	3(150%)	0	0	0	1(100%)	0	0
6	Nitrofuratoin	16(44.44%)	1(100%)	2(100%)	0	0	0	1(100%)	0	0
7	Cefuroxime	1(2.7%)	0	0	0	0	0	0	0	0

As shown as the table num 5 highly reactive antibiotic **nitrifuration** (44.44%) gram negative uropathogen and **gentamycin** is most antibiotic reactive (41.66%). Other antibiotic is a commonly reactive antibiotic these are reactive.

DISCUSSION

Urinary tract infections are serious health threat with respect to antibiotic resistance which, biofilm production being the prime cause. Biofilms play a significant role in colonization during infection, providing an opportunity for the bacteria to develop drug resistance.

In our study, As shown as the Table no 1. UTI was the more common in female patent (56.66%) in comparison to male patent (43.33) This study UTI was more common 21-40 years of the age. In our study Tablenum 2. Our study showed the E.coli was the most common organism in our study (75%), Pseudomonasauruginosa (3.33%), Enterococcus facials (6.66%) and Proteus spp, Staphylococcus Spp Acinetobacter organism (3.33%) and other organism in (1.66%).

We further found that ***E.coli*** was the most common organism producing biofilm our study .31 out of 45 (68.88%) isolated of E.coli were is producing by Congo red method.24 out of 45(53.33%) *E.coli* were produced by BHI method.. Similar studies showed 54% and 44.85% of biofilm production by uropathogens from UTI **(Hassan *et al.*, 2011 and Abdagire *et al.*, 2014)**.

Knobloch *et al*. did not recommend the CRA method for biofilm detection in their study. Out of 128 isolates of S. aureus, CRA detected only 3.8% as biofilm producers as compared to microtiter plate which detected 57.1% as biofilm producing bacteria**.** **Ruzika *et al.*** showed that TM is better for biofilm detection than CRA**.**

As shown as the **Table num 3** highly reactive antibiotic **nitrifuration** (64.44%) gram negative uropathogen and **Gentamycin** is most antibiotic reactive (55.56%) other antibiotic is a commonly reactive. similar studies **Rewatkar and Wadher (2013)** found that the most effective antibiotics Gram Negative bacteria were ofloxacin and Gentamycin and Norfloxacin was most effective bacteria**.**

Tabalenum 4 in our study showed the Biofilm is most common in patents of 21-40 years of the age found. Out of 36 patient, 17(47.22%) were in the 21-40 of age. Second most common age group was 61 years of the age found. out of 36 pateint to the (16.66%)

Tabalenum 5- As shown as the table num 5 highly reactive antibiotic **nitrifuration** (44.44%) gram negative uropathogen and **gentamycin** is most antibiotic reactive (41.66%) similer studies to **Rewatkar and Wadher (2013)** found that the most effective antibiotics Gram

Negative bacteria were ofloxacin and Gentamycin and Norfloxacin was most effective bacteria**.**

Baqai *et al.* tested TM to detect biofilmformation among uropathogens, according to their results, 75% of the isolates exhibited biofilm formation**. Melo *et al.*** found allowed an easy and quantitative classification of the staphylococcal isolates. Matching results from both CRA test were obtained with 81 (87%) of the strains screened**.**

In current study antibiotic sensitive was higher among biofilm producers to commonly used antibiotics as compared to non biofilm producers. This may be because bacterial biofilms are often associated with long term persistence of organism in various environments, increases bacterial growth rate in a biofilm formation

CONCLUSION

We further found that E.coli was the most common organism producing biofilm our study .31 out of 45 (68.88%) isolated of *E.coli* were is producing by Congo red method. 24 out of 45(53.33%).

This study concluded that the ability of Biofilm formation and *E.coli* was the most frequently isolated uropathogen 45(75%). This finding was in close association will other studies. Biofilm formation is the major virulence determinant of uropathogen, so it is necessary to screen allurinary isolates for biofilm production.

The Congored method, present ssignificant relation when compared with non biofilm and UTI patient with biofim producers are the most effective antibiotics (Nitrofuratoin, Gentamycin, Norfloxacin).

From this study, we have concluded that tube adherence and congo red agar method is more qualitative and reliable method to detect biofilm producing microorganisms. tube adherence and congo red agar method is used as a general screening method for detection of biofilm producing bacteria in laboratories.

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