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Evaluation Of Different Detections Methods Of Biofilm Formation In Urinary Isolates

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Abstract:
Microorganisms growing in a biofilm are associated with chronic and recurrent human infections and are highly resistant to antimicrobial agents. Urinary tract infection (UTI) is one of the leading causes of morbidity encountered in clinical practice. Biofilm produced by the urinary pathogens leads to recurrent and recalcitrant UTI there by contributing to longer stay in hospital and increased cost of treatment. The present study was conducted to evaluate Congo Red Agar method (CRA),TCP,MCRA and Tube Method (TM), in detection of biofilm formation in uropathogens with respect to Tissue Culture Plate method (TCP). A total of 2000 clinical isolates from urine samples will be subjected to different biofilm detection methods. All of the specimens will be collected from patients with nosocomial infections admitted to the L N. Hospital. the TCP method detected biofilm in 137 isolates (45.6%), TM detected biofilm in 118 isolates (39.3%), CRA and MCRA detected biofilm in 33 isolates (11%). The TCP was found to be most sensitive followed by the TM, CRA and the MCRA method. TCP method is the ideal method for detection of bacterial biofilm formation by uropathogens. MCRA method is superior to CRA and not to TCP or TM for detection of the staphylococcal biofilm formation.

Keywords: *TM, CRA, TCP, UTI, Biofilms*

INTRODUCTION

A group of infections that occur in the urinary tract is known as Urinary tract infections (UTIs) and it may be in any part of the urinary tract. It causes more financial burden on society. Commonly occurring UTI is cystitis, which occurs in the bladder, where urine is stored. Other UTIs involve the kidneys (pyelonephritis), bladder (cystitis), prostate (prostatitis), urethra (urethritis) or urine (bacteriuria). Urinary tract infections (UTIs) are one of the most common bacterial infections affecting humans throughout their life span [1]

UTI is the third most common cause of admission to hospitals in India. It has been estimated that about 6 million patients per year are visited worldwide for UTI out of which around 30,000 are treated in the wards. UTI has become the most common hospital-acquired infection, accounting for as many as 35% of nosocomial infections, and it is the second most common cause of bacteremia in hospitalized patients[2,3]. UTI also varies based on severity (i.e., complicated versus uncomplicated). Complicated UTI is the infections in urinary tracts with structural or functional abnormalities or the presence of foreign objects (eg. placing urethral catheter).

Biofilm producing bacteria cause recurrent and chronic UTI there by contributing to longer stay in hospital and increased cost of treatment [4,5]. Biofilm formation is the major virulence determinant of uropathogens. Biofilms promote development of antimicrobial resistance by retarding diffusion of antimicrobials and facilitating plasmid exchange thus enabling dissemination of antimicrobial resistance [6,7]. Detection of biofilm producer strains will guide the clinician in modifying antibiotic therapy for better clinical management [8] and also help in designing adequate control measures as the isolates are also resistant to variety of disinfectants. This emphasizes the need to screen all clinical isolates for biofilm production. Both Gram-positive and Gram-negative bacteria have the capability to form biofilms. Bacteria commonly involved include Staphylococcus epidermidis ATCC 35984, Staphylococcus aureus ATCC 35556,Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 35218 and Staphylococcus epidermidis ATCC 12228 (non-slime producer) There are various methods to detect biofilm production. These include the Tissue Culture Plate (TCP)[9], Tube method (TM)[10], Congo Red Agar method[11] (CRA), MCRA[12].

MATERIALS AND METHODS

A total of 2000 clinical isolates from urine samples will be subjected to different biofilm detection methods. The study will be conducted at the Department of Microbiology, L.N. MEDICAL COLLEGE, Bhopal from June 2019 to March 2020.
Male and female patients of all age groups with symptoms of UTI attending various outpatient departments and admitted in wards of hospital were included in the study.

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Selection of The Isolates:

Isolates will be identified by standard microbiological procedures (Gram staining, colonial morphology, catalase test, cytochrome oxidase reaction, motility, biochemical tests).

Reference strains of positive biofilm producer

Staphylococcus epidermidis ATCC 35984,
Staphylococcus aureus ATCC 35556,
Pseudomonas aeruginosa ATCC 27853,
Escherichia coli ATCC 35218 and
Staphylococcus epidermidis ATCC 12228 (non-slime producer)

Tissue culture plate method:

Isolates from fresh agar plates were inoculated in brain heart infusion (BHI) broth with 2% sucrose and incubated for 18–24 h at 37°C in a stationary condition. The broth with visible turbidity was diluted to 1 in 100 with fresh medium. Individual wells of flat bottom polystyrene plates were filled with 0.2 ml of the diluted cultures, and only broth served as a control to check sterility and nonspecific binding of the medium. These plates were incubated for 24 h at 37°C. After incubation, the content of the well was gently removed and then were washed 4 times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating "planktonic" bacteria. Biofilms formed by adherent "sessile" organisms in plate were fixed with sodium acetate (2%) for half an hour and stained with crystal violet (0.1% w/v) for half an hour. Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent bacterial cells usually formed a biofilm on all side wells and were uniformly stained with crystal violet. Optical densities (OD) of stained adherent bacteria were determined with a micro Enzyme-Linked Immuno sorbent Assay auto reader at wavelength of 570 nm (OD 570 nm) and were graded These OD values were considered as an index of bacteria adhering to the surface and forming biofilms. The experiment was performed in triplicate.[13]

Tube method:

BHI broth with 2% sucrose (10 ml) was inoculated with loop full of microorganism from overnight culture plates and incubated for 24 h at 37°C[14]. The tubes were then decanted and washed with PBS (pH 7.3) and dried. Dried tubes were then stained with crystal violet (0.1%) for half an hour. Excess stain was removed, tubes were then dried and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Tubes were examined, and the amount of biofilm formation was scored as absent, moderate or strong. The experiment was performed in triplicate.

Congo Red Agar method

This requires the use of a specially prepared solid medium BHI broth supplemented with 5% sucrose and Congo red. The CRA plate was inoculated with the microorganism from an overnight culture plate and incubated at 37°C for 24–48 h. Positive result was indicated by black colonies with a dry crystalline consistency. The experiment was performed in triplicate.[15]

Modified Congo Red Agar method:

In the MCRA the CRA is modified in the form of changing the concentration of Congo red dye and sucrose, omission of glucose, replacement of BHI Agar by an alternative agar, that is, Blood Base Agar. The MCRA plate was inoculated with organisms from a fresh plate with overnight growth, and then it was incubated for 48 h at 37°C and subsequently 2–4 days at room temperature. Positive result was indicated by black colonies with a dry crystalline consistency. The experiment was performed in triplicate.[16]

RESULTS AND DISCUSSION:

Spectrum of organisms isolated:
From the study total 2000 samples were collected such Staphylococcus epidermidis ATCC 35984 isolates by 300 (10%), Staphylococcus aureus ATCC 35556 isolates by 300 (10%) Pseudomonas aeruginosa ATCC 27853 isolates by 500 (30%) Escherichia coli ATCC 35218 isolates by 500 (40%) and Staphylococcus epidermidis ATCC 12228 isolates by 400 (10%).

Table 1: Spectrum of organisms isolated

Organism	Isolates	Percent
Staphylococcus epidermidis ATCC 35984	300	10%
Staphylococcus aureus ATCC 35556,	300	10%
Pseudomonas aeruginosa ATCC 27853,	500	30%
Escherichia coli ATCC 35218	500	40%
Staphylococcus epidermidis ATCC 12228 (non-slime producer)	400	10%

Organism wise distribution of biofilm production

From the total isolates biofilm producers were distributed as 50,50,50,300,50 to Staphylococcus epidermidis ATCC 35984, Staphylococcus aureus ATCC 35556, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 35218 and Staphylococcus epidermidis ATCC 12228 (non-slime producer) respectively.

Table 2: Organism wise distribution of biofilm production

Organism	Total Isolates	Biofilm producers	Percent
Staphylococcus epidermidis ATCC 35984	300	50	10%
Staphylococcus aureus ATCC 35556,	300	50	10%
Pseudomonas aeruginosa ATCC 27853,	500	50	30%
Escherichia coli ATCC 35218	500	300	40%
Staphylococcus epidermidis ATCC 12228 (non-slime producer)	400	50	10%

Detection of biofilm production by different phenotypic methods

For detection of biofilm production here Tube method (150) and Tissue culture plate method (150) , Congo red agar (100), Modified Congo Red Agar method(100)

Table 3:Detection of biofilm production by different phenotypic methods

Method	Bio film producers	Percent
Congo red agar	100	10%
Tube method	150	40%
Tissue culture plate method	150	40%
Modified Congo Red Agar method	100	10%

Tube method for detection of biofilm formation:

The above picture shows the tubes which were stained with 0.1 % crystal violet Tube A. Non- Biofilm Producers Tube B. Moderate producer of biofilm Tube A. Strong biofilm producer

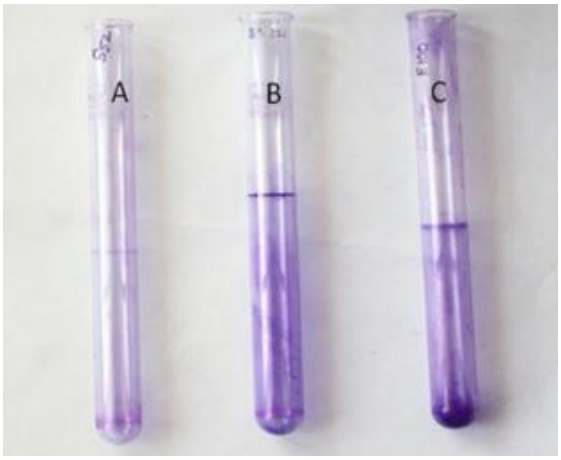

Figure 1: Tube method for detection of biofilm formation

Tissue culture plate method for detection of biofilm formation

The microtiter plate with flat bottom, shows the calorimetric changes. The stain used was 0.01% crystal violet, the wells showing violet colour with OD the values • 0.12 indicate the strains producing biofilms and the unstained wells, with OD values < 0.12 indicate that he strains which do not produce biofilms. A. Non-biofilm producers B. Moderate C. High

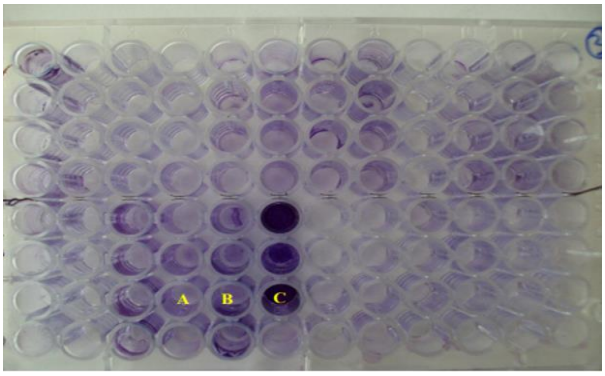

Figure 2: Tissue culture plate method for detection of biofilm formation

Congo red agar method for detection of biofilm formation:

A. Pink colonies were not considered as biofilm producers
B. Colonies showing dark pink colour were considered as mild to moderate biofilm producers
C. Black crystalline colonies were strong biofilm producers The above picture shows Congo red agar plate, with black crystalline colonies, indicating the strains which are strong biofilm producers and pink colonies which do not produce biofilms

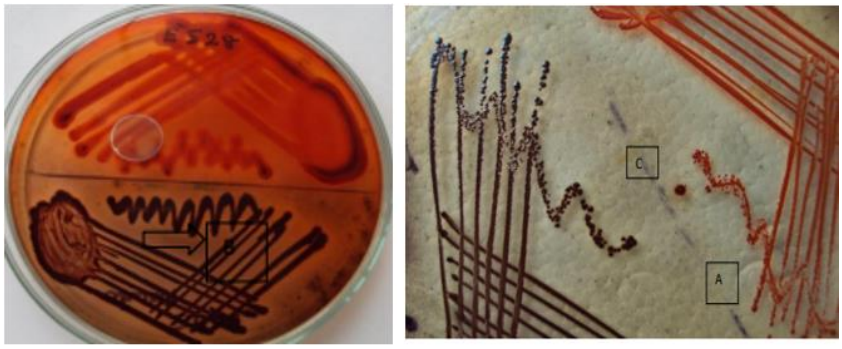

Figure 3: Congo red agar method for detection of biofilm formation

Modified Congo Red Agar method

In this study we use modified Congo red agar for biofilm formation. In vitro slime production ability on the published Congo red agar by in diffuse black pigment in the agar with growth of black pigmented colonies but pigmentation decreased with time. In the present study the modified Congo red agar (MCRA) was optimized to get strong black pigmentation at 48hrs incubation and then for 2-4 days room temperature. Black colored colonies with dry crystalline consistency interpretedas positive biofilm producing strains. Red coloured colonies- interpreted as negative for biofilm production

Figure 4: Modified Congo Red Agar method for detection of biofilm formation

CONCLUSION:

UTI is considered as the most common bacterial infection worldwide causing significant morbidity and loss of work place productivity. UTIs have become a serious health threat with 250 millions of cases reported annually with much recurrence rate and chronicity. There is an increasing trend in the antimicrobial resistance among uropathogens and is attributed to formation of biofilms. There are accurate biofilm detection methods like molecular methods. A suitable method which is

cost effective easy to do and requiring less technical expertise is the need of the hour. The TCP was found to be most sensitive followed by the TM, CRA and the MCRA method. TCP method is the ideal method for detection of bacterial biofilm formation by uropathogens. MCRA method is superior to CRA and not to TCP or TM for detection.

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