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TUBE METHOD AND CONGO RED AGAR VERSUS TISSUE CULTURE PLATE METHOD FOR DETECTION OF BIOFILM PRODUCTION BY UROPATHOGENS ISOLATED FROM MIDSTREAM URINE: WHICH ONE COULD BE BETTER?

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ABSTRACT

***Background*: Bacteria tend to live in assemblies called biofilms that aid bacterial virulence. Biofilms contribute to the development of antibiotic resistant urinary tract infection. Therefore, detection of biofilm production by urinary pathogens can assist the physicians to initiate the proper antimicrobial treatment.**

***Methods:* We conducted a prospective study that included patients with suspected urinary tract infection. Collected midstream urine samples were processed by standard microbiological techniques. Detection of biofilm production by the isolated uropathogens was conducted by tissue culture plate method (TCPM), tube method (TM) and Congo red agar (CRA).**

***Results:* A total of 43 (29.7%) isolated uropathogens showed positive biofilm formation by TCPM which was considered the gold standard for biofilm detection. When compared with the TCPM, TM truly identified 40 biofilm producers and 83 non-biofilm producers showing sensitivity and specificity of 93.0% and 81.4% respectively. The CRA truly identified 38 biofilm producers and 77 non-biofilm producers with sensitivity and specificity of 88.4% and 75.5% respectively.**

***Conclusion:* The TM was superior to CRA in biofilm detection and demonstrated better sensitivity and specificity results. Out of the investigated three phenotypic biofilm detection methods, the TCPM was the ideal method for detection of biofilm formation by uropathogens isolated from midstream urine samples. It can be used routinely in the microbiology laboratory with good specificity results and less subjectivity errors.**

PROCÉDÉ DE TUBE ET PLAQUE DE CULTURE DE CULTURE DE TISSU D'AGAR ROUGE CONGO POUR DÉTECTER LA PRODUCTION DE BIOFILM PAR DES UROPATHOGÈNES ISOLÉS À PARTIR D'UNE URINE À MOYENNE FLUX: QUI POURRAIT ÊTRE MIEUX?

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ABSTRAIT

Contexte: Les bactéries ont tendance à vivre dans des assemblages appelés biofilms qui facilitent la virulence bactérienne. Les biofilms contribuent au développement d'une infection des voies urinaires résistante aux antibiotiques. Nous avons détecté des uropathogènes produisant des biofilms isolés à partir de l'urine médiane et évalué trois méthodes phénotypiques in vitro pouvant être appliquées en laboratoire pour la détection de biofilms.

Méthodes: Nous avons mené une étude prospective incluant des patients chez qui une infection des voies urinaires était suspectée. Les échantillons d'urine collectés à mi-chemin ont été traités par des techniques microbiologiques standard. La détection de la production de biofilm par les uropathogènes isolés a été réalisée par la méthode de la culture sur plaque de culture tissulaire (TCPM), la méthode du tube (TM) et l'agar rouge congolais (CRA).

Résultats: Un total de 43 (29,7%) uropathogènes isolés a montré que la formation de biofilm était positive par le TCPM, qui était considéré comme la référence en matière de détection du biofilm. En comparaison avec le TCPM, TM a vraiment identifié 40 producteurs de biofilms et 83 producteurs non-biofilms présentant une sensibilité et une spécificité de 93% et 81,4% respectivement.

L'ARC a vraiment identifié 38 producteurs de biofilms et 77 producteurs de non-biofilms avec une sensibilité et une spécificité de 88,4% et 75,5% respectivement.

Conclusion: La MT était supérieure à l'ARC pour la détection de biofilm et a démontré de meilleurs résultats de sensibilité et de spécificité. Parmi les trois méthodes de détection phénotypiques de biofilms étudiées, le TCPM était la méthode idéale pour détecter la formation de biofilm par des uropthogènes isolés à partir d'échantillons d'urine en cours de route. Il peut être utilisé en routine dans le laboratoire de microbiologie avec de bons résultats de spécificité et moins d'erreurs de subjectivité.

INTRODUCTION

Bacteria tend to live in a community-like assembly called biofilm. Development of bacterial biofilms occurs in a dynamic process that includes bacterial attachment to a particular surface, irreversible binding and formation of a hydrated matrix of polysaccharides and protein (1,2). Surfaces that favor biofilm development include inert surfaces as medical devices and dead tissues as dead bone fragments (1,3). Antibodies are generated in response to the antigens released by the bacteria located in the biofilm. However, these antibodies are unable to kill the bacteria embedded within the biofilm even among people with excellent immune responses (1,4).

Biofilm production aids bacterial virulence through numerous pathogenic mechanisms as it facilitates attachment to solid surfaces, evasion of phagocytosis and gene exchange between the biofilm´s members generating more virulent strains. Moreover, biofilms can protect bacteria from antimicrobial agents resulting in resistant infections that carry a great clinical significance (1,5). The mechanisms by which biofilms escape the effects of antimicrobial agents include: inability of the agent to reach the bacteria present at the deep part of the biofilm, the slowly growing bacteria in the biofilm shows decrease susceptibility to the agents, and some of the bacteria exist in a programmed protected phenotype that is generated in response to surface attachment (6).

Antibiotic resistant urinary tract infection (UTI), either community or healthcare acquired, is a threatening clinical problem faced by treating physicians (7). Biofilms are commonly associated with indwelling devices as urinary catheters leading to resistant UTI. Furthermore, biofilms may attach to urinary tract anatomical structures resulting in chronic and recurrent UTI with increased morbidity and economic burden (5). Therefore, detection of biofilm production by urinary pathogens can assist the physicians to initiate the proper antimicrobial treatment for UTI cases (2).

With the appearance of biofilm associated infections, various laboratory methods for detection of biofilms were developed. Phenotypic detection of biofilm production can be conducted by various techniques as tissue culture plate method (TCPM), tube method (TM) and Congo red agar (CRA) (8).
Previous studies that investigated biofilm formation by uropathogens usually focused on catheterized patients (9-11). This study was performed trying to detect biofilm producing uropathogens isolated from midstream urine and to evaluate three in vitro phenotypic methods (TCPM, TM and CRA) that can be applied in laboratory settings for biofilm detection.

MATERIALS and METHODS

Setting

A prospective study was performed at the Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University from January to October 2018. The study protocol was revised and accepted by our institutional review board.

Sample Collection

During the study period, midstream urine samples were collected from patients showing clinical manifestations of UTI at Mansoura University Hospitals. Urine specimens were transported to the laboratory and processed immediately.

Microbiological Processing

Received urine samples were initially examined by standard microbiological techniques. Urine samples were inoculated on CLED agar plates that were then incubated at 37°C for 24-48 hours. The uropathogens were identified by colonial morphology, Gram staining and biochemical reactions.

Detection of Biofilm Production

Biofilm production by isolated uropathogens in our study was detected by three phenotypic

methods which included TCPM, TM and CRA. Reference strains of *Staphylococcus epidermidis* ATCC 12228 and *Staphylococcus epidermidis* ATCC 31484 were also included as negative and positive control strains respectively. Biofilm production was graded into strong, moderate and non/weak. Strong and moderate results were interpreted as positive biofilm production, while, non/weak results were interpreted as negative biofilm production.

Tissue Culture Plate Method

We used TCPM as the gold standard test for detection of biofilm formation (1,8). A loopful of freshly cultured isolates was inoculated in 10 ml of trypticase soy broth with 1% glucose. The inoculated broth was then kept in the incubator at 37°C for 24 hours. Bacterial suspensions were further diluted 1:100 with fresh medium. Separate wells of a sterile polystyrene tissue culture plate, composed of 96 flat bottom wells, were filled by 200 µl of the prepared bacterial suspension. Similarly, control organisms were put in the tissue culture plate. In addition, only sterile broth was used to ensure sterility and to identify non-specific binding. After incubation at 37°C for 24 hours, the plate was gently tapped to remove the content of the wells followed by washing with 200 µl of phosphate buffer saline. The washing step was repeated four times to remove any free bacteria present in the wells. Sodium acetate (2%) were added to the wells and kept for 30 minutes in order to fix the biofilms formed by bacteria attached to the wells. Staining of the fixed biofilms was conducted using crystal violet (0.1%). After 30 minutes, the wells were thoroughly washed by deionized water to remove any extra stain.

After drying, a micro-ELISA reader (at 570 nm wave length) was used to measure the optical densities (OD) of stained bacterial biofilms. Test was carried out in triplicate and average of three OD values was taken. Optical densities values indicated bacterial adherence to the wells and biofilm formation. The OD values were calculated and biofilm production was graded into strong, moderate and non/weak (Table 1) as described in previous studies (2, 12-14).

TABLE 1: GRADING OF BIOFILM FORMATION BY TISSUE CULTURE PLATE METHOD

Optical densities values	Adherence	Biofilm formation
< 0.120	Non	Non/weak
0.120- 0.240	Moderate	Moderate
> 0.240	Strong	Strong

Tube Method

A loopful of the isolated bacteria from overnight cultured media was inoculated in each glass tube containing 10 ml of trypticase soy broth with 1% glucose. The inoculated tubes were then incubated at 37°C. After incubation for 24 hours, tubes were emptied and washed with phosphate buffer saline and left to dry. Crystal violet (0.1%) was used to stain the dried tubes for 15 minutes. Excess stain was then removed by washing the tubes with deionized water. The tubes were then dried in inverted position and examined for biofilm production. Presence of a visible film lining the bottom and the wall of the tube indicated positive result for biofilm production while formation of a stained ring at the air-liquid interface was an evidence of a negative result (5,13,15).

Congo Red Agar Method

Congo red agar is a specially prepared medium composed of brain heart infusion (BHI) broth (37 g/l) supplemented with sucrose (50 g/l), agar No 1 (10 g/l) and Congo red (0.8 g/l). We prepared a concentrated aqueous solution of the Congo red stain that was then autoclaved at 121ºC for 15 minutes. Finally it was added to the autoclaved BHI agar with sucrose at 55ºC. Prepared CRA plates were inoculated with the isolated uropathogens and aerobically incubated at 37°C for 24 hours. Appearance of black dry crystalline colonies on the CRA plates indicated biofilm production while the colonies of biofilm non-producer remained pink or red colored (5,8,16).

Statistical Analysis

In the present study, TCPM was considered the gold standard method of biofilm detection based on the available literature. Accordingly, the data of TCPM were compared with those of TM and CRA. The data were presented as numbers and percentages. Parameters like sensitivity, specificity and positive predictive value (PPV) and negative predictive value (NPV) were calculated for each test by using Greenhalgh´s formulas (17).

RESULTS

A total of 180 midstream urine samples from patients with suspected UTI were processed in our study. Out of the processed 180 urine samples, 145 (80.6%) samples were culture positive. Gram-negative bacteria accounted for 89.0% of the recovered isolates (129/145) while Gram-positive bacteria accounted for 11.0% (16/145). *Escherichia coli* was the commonest isolate encountered in our study (55.9 %)

followed by *Klebsiella pneumoniae* (13.1%) and *Pseudomonas aeruginosa* (10.3%). *Enterococcus faecalis* was the predominant Gram-positive isolate (8.3%) as shown in Table 2.

TABLE 2: SPECTRUM OF ISOLATED UROPATHOGENS

Bacterial isolate	Number	Percentage (%)
Escherichia coli	81	55.9%
Klebsiella pneumoniae	19	13.1%
Pseudomonas aeruginosa	15	10.3%
Enterococcus faecalis	12	8.3%
Proteus mirabilis	10	6.9%
Acinetobacter baumannii	4	2.8%
MSSA	3	2.1%
MRSA	1	0.7%
Total	145	100%

MSSA=Methicillin sensitive *Staphylococcus aureus*, MRSA=Methicillin resistant *Staphylococcus aureus*

Out of 145, 43 (29.7%) isolates demonstrated positive biofilm formation by TCPM which is postulated to be the gold standard for biofilm detection. *Enterococcus faecalis* isolates showed the highest biofilm production (75.0%) followed by *Escherichia coli* (32.1%), *Klebsiella pneumoniae* (21.1%), *Pseudomonas aeruginosa* (20%) and *Proteus mirabilis* (10%). None of the isolated *Acinetobacter baumannii, Methicillin sensitive Staphylococcus aureus or Methicillin resistant Staphylococcus aureus* strains produced biofilm as shown in Table 3.

TABLE 3: BIOFILM PRODUCTION AMONG ISOLATED UROPATHOGENS BY TCPM

Bacterial isolate	Total isolates	Biofilm producers (%)
Escherichia coli	81	26 (32.1%)
Klebsiella pneumoniae	19	4 (21.1%)
Pseudomonas aeruginosa	15	3 (20.0%)
Enterococcus faecalis	12	9 (75.0%)
Proteus mirabilis	10	1 (10.0%)
Acinetobacter baumannii	4	0
MSSA	3	0
MRSA	1	0
Total	145	43 (29.7%)

MSSA=Methicillin sensitive *Staphylococcus aureus*, MRSA=Methicillin resistant *Staphylococcus aureus*

In the current study, the TCPM, TM and CRA detected biofilm formation in 29.7% (43/145), 40.7% (59/145) and 43.4% (63/145) of the isolates respectively as demonstrated in Table 4. Eight isolates were found positive only by TM while 14 isolates were found positive only by CRA. None of the isolates were only TCPM positive.

When compared with the TCPM, TM truly identified 40 biofilm producers and 83 non-biofilm producers (Table 5), while, CRA truly identified 38 biofilm producers and 77 non-biofilm producers (Table 6).

TABLE 4: DETECTION OF BIOFILM PRODUCTION AMONG ISOLATED UROPATHOGENS BY DIFFERENT METHODS

Method	Total isolates	Biofilm producers (%)	Non-biofilm producers (%)
TCPM	145	43 (29.7%)	102 (70.3%)
TM	145	59 (40.7%)	86 (59.3%)
CRA	145	63 (43.4%)	82 (56.6%)

TCPM=Tissue culture plate method, TM=Tube method, CRA=Congo red agar

TABLE 5: COMPARISON OF TM WITH TCPM FOR BIOFILM DETECTION

TM	TCPM		Total
	Positive	Negative	
Positive	40	19	59
Negative	3	83	86
Total	43	102	145

TCPM=Tissue culture plate method, TM=Tube method

TABLE 6: COMPARISON OF CRA WITH TCPM FOR BIOFILM DETECTION

CRA	TCPM		Total
	Positive	Negative	
Positive	38	25	63
Negative	5	77	82
Total	43	102	145

TCPM=Tissue culture plate method, CRA=Congo red agar

The performance characteristics of TM and CRA when compared with TCPM, which is the gold standard for biofilm detection, were demonstrated in Table 7. The TM showed a sensitivity of 93.0%, specificity of 81.4%, PPV of 67.8% and NPV of 96.5% while the CRA had a sensitivity of 88.4%, specificity of 75.5%, PPV of 60.3% and NPV of 93.9%.

In the present study, the TCPM detected 13 (9.0%) isolates as strong biofilm producers and 30 (20.7%) as moderate biofilm producers. The TM detected strong and moderate biofilm formation in 10.3% and 30.3% of isolates respectively, while, CRA detected strong and moderate biofilm formation in 11.0% and 32.4% of isolates respectively as shown in Table 8.

TABLE 7: PERFORMANCE CHARACTERISTICS OF TM AND CRA FOR BIOFILM DETECTION WHEN COMPARED WITH TCPM

Method	Sensitivity	Specificity	Positive predictive value	Negative predictive value
TM	93.0%	81.4%	67.8%	96.5%
CRA	88.4%	75.5%	60.3%	93.9%

TM=Tube method, CRA=Congo red agar

TABLE 8: GRADING OF BIOFILM FORMATION BY DIFFERENT METHODS

Biofilm formation	TCPM	TM	CRA
Strong	13 (9.0%)	15 (10.3%)	16 (11.0%)
Moderate	30 (20.7%)	44 (30.3%)	47 (32.4%)
Non/weak	102 (70.3%)	86 (59.3%)	82 (56.6%)
Total	145 (100%)	145 (100%)	145 (100%)

TCPM=Tissue culture plate method, TM=Tube method, CRA=Congo red agar

DISCUSSION

Antimicrobial resistant bacteria continue to be a major challenge for treating physicians. The ability to produce biofilm is considered one of the main causes of antimicrobial resistance. Bacteria embedded in the biofilm can survive a higher concentration of antimicrobials up to 1500 folds than those needed to eliminate free bacteria (18).

In the present study, we processed midstream urine samples and then investigated the ability of isolates to form biofilm by three in vitro phenotypic methods that can be used in most laboratory settings. A total of 145 bacterial isolates were recovered from the urine samples in our study. Gram-negative bacteria found to be the predominant uropathogens constituting 89% of the total isolates (129/145). *Escherichia coli* was the most prevalent organism isolated from the urine samples (55.9%) followed by *Klebsiella pneumoniae* (13.1%) and *Pseudomonas aeruginosa (10.3%).* Similarly, other studies reported *Escherichia coli* and *Klebsiella pneumoniae* as the predominant uropathogens (2,5,11,19). In our study, *Enterococcus faecalis* was the most prevalent Gram-positive bacteria (8.3%) that was consistent with the work of Noor et al., and Ruchi et al., who reported that *Enterococcus species* were the commonest Gram-positive isolates in their studies (5,20). On the contrary, Panda and his colleagues reported that *Staphyloccocus species* were the predominant Gram-positive isolated uropathogens (2).

The recovered 145 bacterial isolates were further subjected to TCPM, TM and CRA methods for phenotypic detection of biofilm production. The TCPM, the gold standard method, detected biofilm formation in 43 out of 145 bacterial isolates (29.7%). The highest biofilm production was found among *Enterococcus faecalis* as 9 out of 12 isolates (75%) were biofilm producers. In accordance with our results, Ruchi and his colleagues reported that 27% of isolated uropathogen showed biofilm formation by the TCPM and that 71.4% of *Enterococcus faecalis* isolates were biofilm producers (5). Our results were higher than those reported by Ira et al., who found that 53% of isolated *Enterococcus species* produced biofilm (21). In another study by Panda and his colleagues, TCPM detected biofilm production in 137 out of 300 (45.6%) isolated uropathogens which were higher than our findings (2).

In the present study, *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* produced biofilm in 32.1%, 21.1% and 20% respectively. These results were considerably lower than those of Niveditha et al., who reported that *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* produced biofilm in 60%, 63% and 100% respectively (11). Higher results than ours were also reported by Abdallah and his colleagues who found that 44.4% of *Klebsiella species* and 50% of *Pseudomonas species* were biofilm producers (7). There is no clear explanation for such variations in these studies. Though, this might be attributed to the different methodology as Niveditha et al., analyzed only urine samples from catheterized patients while Abdallah et al., compared biofilm formation in midstream and catheterized urinary specimens. Therefore, the pattern of biofilm production in these studies could be different from our study in which we only analyzed midstream urine samples. In agreement with this explanation, Ruchi and his colleagues who also analyzed midstream urine samples reported that *Escherichia coli* and *Klebsiella pneumoniae* produced biofilm in 27.1% and 16.7% of the isolates respectively which were consistent with our results (5).

A total of 145 isolates were tested for biofilm production by TCPM, TM and CRA in the current study. We chose these in vitro methods

because they can be performed in most laboratory settings. The TCPM detected biofilm production in 43 isolates (29.7%), the TM detected biofilm production in 59 isolates (40.7%) while the CRA detected biofilm production in 63 isolates (43.4%). Parallel detection pattern was reported by Ruchi et al., who detected biofilm production in 27% of isolated uropathogens by TCPM, 37.9% by TM and 40.8% by CRA (5). Similar pattern was also reported by Turkyilmaz and his colleagues who studied biofilm production in *Staphylococcal species* and detected biofilm in 50.5% by TCPM, 55.5% by TM and 61.1% by CRA (22).

Out of the tested 145 urine samples in our study, 43 isolates were biofilm producers and 102 isolates were non-biofilm producers according to the results of TCPM. The TM truly identified 40 biofilm producers and 83 non-biofilm producers showing a sensitivity of 93% and specificity of 81.4%. Ruchi et al., reported slightly better sensitivity and specificity results for the TM (94.5% and 83% respectively) (5). In other studies conducted by Hassan et al., and Panda et al., the TM had sensitivity results of 73% and 81% respectively and specificity results of 92.5% and 95.1% respectively (2,8). Ira et al., reported that TM sensitivity and specificity were 61% and 68% respectively which were considerably lower than our results (21).

The CRA, in the present study, truly identified 38 biofilm producers and 77 non-biofilm producers demonstrating a sensitivity of 88.4% and specificity of 75.5% which were lower than those of TM. Ira et al., reported a CRA specificity of 77% which was close to our results (21). Studies conducted by Ruchi et al., Hassan et al., and Panda et al., demonstrated better specificity results for the CRA than ours (81%, 92% and 93.9% respectively) (2,5,8). Similarly, the CRA sensitivity reported by Ruchi and his colleagues was 94.5% that was better than the one reported by us (5).These variations in the reported sensitivity and specificity of TM and CRA can be explained by the subjective errors during interpretation of these phenotypic qualitative tests. Moreover, inter-batch variation of the used media can affect their results.

In the current study, the TCPM and TM detected strong biofilm formation in 9% and 10.3% of isolates respectively. These results were similar to those of Panda et al., who reported that 11% and 10.7% of the isolates demonstrated strong biofilm formation when tested by TCPM and TM respectively (2). Similarly, Mathur and his colleagues reported that 14.4% and 11.8% of the isolates demonstrated strong biofilm formation when tested by TCPM and TM respectively (23). However, other studies reported considerably higher results as up to 52% of the isolates showed strong biofilm formation by TCPM and TM (24, 25). The CRA, in our study, detected strong and moderate biofilm formation in 11% and 32.4% of isolates respectively that was higher than the results reported by other studies (2, 23, 24). Overall, we found that TM and CRA correlated well with TCPM regarding strong biofilm detection but not for moderate and non/weak biofilm detection. This could be accredited to the subjective assessment used in TM and CRA in comparison to the objective grading scheme used in TCPM.

The CRA is a simple qualitative screening method for biofilm detection with an advantage of remaining viable colonies that can be beneficial for further studies (5). In the present study, CRA was more rapid and easier than other phenotypic tests. However, the CRA demonstrated lower sensitivity and specificity results than those of TM. The TCPM remains to be the gold standard phenotypic test for detection of biofilm production and it was the most specific test in the present study. It was also an easy test to perform in the laboratory and it detected the biofilm production in both qualitative and quantitative ways. Moreover, the interpretation of the TCPM results is conducted by ELISA reader which eliminates the subjective errors seen with other phenotypic tests.

CONCLUSION

Biofilm producing bacteria are important etiological agents of UTI in non-catheterized patients. Incomplete clearance of infection caused by biofilm production can lead to chronic UTI with a worse outcome. Therefore, detection of biofilm formation in such cases is important as it allows for better antimicrobial choice by the treating doctors. The TM was superior to CRA in biofilm detection and demonstrated better sensitivity and specificity results. Out of the investigated three phenotypic biofilm detection methods, the TCPM was the ideal method for detection of biofilm formation by uropathogens isolated from midstream urine samples. It can be used routinely in the microbiology laboratory for biofilm detection with good specificity results and less subjectivity errors.

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