Evaluation of different detection methods of biofilm formation in the clinical isolates

ABSTRACT

Background: Microorganisms growing in a biofilm are associated with chronic and recurrent human infections and are highly resistant to antimicrobial agents. There are various methods to detect biofilm production like Tissue Culture Plate (TCP), Tube method (TM), Congo Red Agar method (CRA), bioluminescent assay, piezoelectric sensors, and fluorescent microscopic examination. **Objective:** This study was conducted to compare three methods for the detection of biofilms. **Method:** The study was carried out at the Department of Microbiology, Army Medical College, National University of Sciences and Technology, Pakistan, from January 2010 to June 2010. A total of 110 clinical isolates were subjected to biofilm detection methods. Isolates were identified by standard microbiological procedures. Biofilm detection was tested by TCP, TM and CRA. Antibiotic susceptibility test of biofilm producing bacteria was performed by using the Kirby-Bauer disc diffusion technique according to CLSI guidelines. **Results:** The TCP method was considered to be superior to TM and CRA. From the total of 110 clinical isolates, TCP method detected 22.7% as high, 41% moderate and 36.3% as weak or non-biofilm producers. We have observed higher antibiotic resistance in biofilm producing bacteria than non-biofilm producers. **Conclusion:** We can conclude from our study that the TCP method is a more quantitative and reliable method for the detection of biofilm forming microorganisms as compared to TM and CRA methods, and it can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories.

Keywords: biofilms; bacteria; anti-bacterial agents.

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INTRODUCTION

Biofilms are defined as microbially derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. They are embedded in a matrix of extracellular polymeric substances (EPS) they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.[1] Within a biofilm, bacteria communicate with each other by production of chemotactic particles or pheromones, a phenomenon called quorum sensing.[2] Availability of key nutrients, chemotaxis towards surface, motility of bacteria, surface adhesins and presence of surfactants are some factors which influence biofilm formation.[2] Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial agents than planktonic cells. High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antibiotic resistance can increase 1,000 fold.[3] According to a publication by the National Institutes of Health, more than 80% of all infections involve biofilms.[4] Biofilms are associated with many medical conditions including indwelling medical devices, dental plaque, upper respiratory tract infections, peritonitis, and urogenital infections.[5] Both Gram-positive and Gram-negative bacteria have the capability to form biofilms. Bacteria commonly involved include *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa*.[6] There are various methods to detect biofilm production. These include the Tissue Culture Plate (TCP),[7] Tube method (TM),[8] Congo Red Agar method (CRA),[9] bioluminescent assay,[10] piezoelectric sensors,[11] and fluorescent microscopic examination.[12]

Submitted on: 01/03/2011
Approved on: 05/03/2011

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We declare no conflict of interest.

We screened 110 organisms by three different methods, which could be used in a routine clinical laboratory, for determining their ability to form biofilm.

OBJECTIVES

The study was conducted to detect biofilm forming microorganisms isolated from clinical specimens by three different methods (TCP, TM, CRA) and to compare these methods for biofilm detection.

MATERIALS AND METHODS

Place and duration of the study

The study was conducted at the Department of Microbiology, Army Medical College, National University of Sciences and Technology, Pakistan, from January 2010 to June 2010.

Selection of the isolates

A total of 110 clinical isolates were subjected to biofilm detection methods. Organisms were selected on the following criteria: those isolated from the pus, intravenous and urinary catheter tips, urine, sputum and nasobronchial lavage specimens, and those showing increased resistance to commonly available antibiotics by Kirby-Bauer disc diffusion method. Urinary catheter tips, intravenous catheter tips, nasobronchial lavage specimens and few of the pus specimens were related to medical devices (Table 1). All of the specimens were received from patients with nosocomial infections admitted to the hospital.

Isolates were identified by standard microbiological procedures (Gram staining, colonial morphology, catalase test, cytochrome oxidase reaction, motility, biochemical tests). Reference strain 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) were used as control. Biofilm detection was done by the following methods:

Tissue culture plate method

This quantitative test described by Christensen et al.[7] is considered the gold-standard method for biofilm detection.[13] Organisms isolated from fresh agar plates were inoculated in 10 mL of trypticase soy broth with 1% glucose. Broths were incubated at 37°C for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well-flat bottom polystyrene tissue culture treated plates (Sigma-Aldrich, Costar, USA) were filled with 200 µL of the diluted cultures. The control organisms were also incubated, diluted and added to tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37°C for 24 h. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA autoreader (model 680, Biorad, UK) at wavelength 570 nm. The experiment was performed in triplicate and repeated three times. The interpretation of biofilm production was done according to the criteria of Stepanovic et al.[14] (Table 2).

Tube method

Described by Christensen et al.,[8] this is a qualitative method for biofilm detection. A loopful of test organisms was inoculated in 10 mL of trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37°C for 24 h. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak/none, 2-moderate and 3-high/strong. The experiment was performed in triplicate and repeated three times.

Congo Red Agar method

Freeman et al.[9] have described a simple qualitative method to detect biofilm production by using Congo Red Agar (CRA) medium. CRA medium was prepared with brain heart infusion broth (Oxoid, UK) 37 g/L, sucrose 50 g/L, agar No. 1 (Oxoid, UK) 10 g/L and Congo Red indicator (Oxoid, UK) 8 g/L. First Congo Red stain was prepared as a concentrated aqueous solution and autoclaved (121°C for 15 minutes) separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose at 55°C.[5] CRA plates were inoculated with test organisms and incubated at 37°C for 24 h aerobically. Black colonies with a dry crystalline consistency indicated biofilm production.[5] The experiment was performed in triplicate and repeated three times.

Antibiotic susceptibility test of biofilm producing bacteria was done on Mueller Hinton agar (Oxoid, UK) using the following antibiotic discs: ampicillin, cotrimoxazole, ciprofloxacin, aztreonam, meropenem, cefoperazone-sulbactam, chloramphenicol, vancomycin, erythromycin, amoxicillin-clavulanic acid, oxacillin, linezolid, penicillin. All antibiotic discs were obtained from Oxoid, UK. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus*

Table 1. Correlation of biofilm production of isolates and sensitivity test with the patient clinical condition and type of medical device used

Organism	Biofilm production	Pt. clinical condition/Type of infection	Type of medical device	Sensitivity to following antibiotics
E. coli	Strong	Urine retention due to stroke	Foley's urinary catheter	Amikacin, meropenem, cefoperazone-sulbactam
S. epidermidis	Strong	Care of a metastatic terminal disease	Urinary catheter	Vancomycin, linezolid, ciprofloxacin
E. coli	Strong	For relief of bladder outlet obstruction due to urethral stricture	Foley's urinary catheter	Aztreonam, meropenem
E. faecalis	Moderate	Neurogenic bladder	Foley's urinary catheter	Vancomycin, linezolid, cotrimoxazole
E. coli	Strong	Benign prostatic hypertrophy	Foley's urinary catheter	Ciprofloxacin, aztreonam, meropenem
E. coli	Strong	Post-operation to	Foley's urinary catheter monitor output	Aztreonam, meropenem, ciprofloxacin
S. epidermidis	Strong	Urologic surgery	Foley's urinary catheter	Vancomycin, linezolid, ciprofloxacin
K. pneumoniae	Strong	Urine retention due to calculus disease	Foley's urinary catheter	Cotrimoxazole, ciprofloxacin, ceftriaxone, meropenem
E. coli	Strong	Urine retention due to cerebrovascular disease	Foley's urinary catheter	Amikacin, ceftriaxone, meropenem
K. pneumoniae	Strong	Hip fracture surgery	Foley's urinary catheter	Ciprofloxacin, ceftriaxone, meropenem
E. coli	Strong	Post-operation to monitor output	Foley's urinary catheter	Meropenem, amikacin, aztreonam, ceftriaxone
S. epidermidis	Strong	Care for traumatic spinal cord injury	Foley's urinary catheter	Vancomycin, linezolid, cotrimoxazole, ciprofloxacin
S. epidermidis	Moderate	Neurogenic bladder	Foley's urinary catheter	Linezolid, ciprofloxacin, cotrimoxazole
E. coli	Strong	Total abdominal hysterectomy surgery	Foley's urinary catheter	Ceftriaxone, meropenem
S. epidermidis	Moderate	Bladder outlet obstruction due to urethral stricture	Foley's urinary catheter	Vancomycin, linezolid, cotrimoxazole, ciprofloxacin
E. coli	Strong	Urologic surgery	Foley's urinary catheter	Meropenem, ceftriaxone
E. coli	Strong	Benign prostatic hypertrophy	Foley's urinary catheter	Ciprofloxacin, aztreonam, meropenem
K. pneumoniae	Strong	Palliative care in a incontinent impaired patient	Foley's urinary catheter	Aztreonam, meropenem
S. epidermidis	Strong	Monitoring of central venous pressure in an acutely ill patient	Central venous catheter	Linezolid, ciprofloxacin

(Cont.)

Table 1. Correlation of biofilm production of isolates and sensitivity test with the patient clinical condition and type of medical device used

Organism	Biofilm production	Pt. clinical condition/Type of infection	Type of medical device	Sensitivity to following antibiotics
S. epidermidis	Moderate	Total parenteral nutrition	Central venous catheter	Vancomycin, linezolid, cotrimoxazole, ciprofloxacin
K. pneumoniae	Strong	Long term IV antibiotics	Intravenous catheter	Aztreonam, cefoperazone-sulbactam, meropenem
S. epidermidis	Strong	To correct electrolyte imbalance, fluid replacement	Intravenous catheter	Vancomycin, linezolid, Ciprofloxacin
S. aureus	Moderate	Blood transfusion	Intravenous catheter	Rifampicin, ciprofloxacin vancomycin
S. epidermidis	Strong	Total parenteral nutrition	Central venous catheter	Linezolid, cotrimoxazole
S. epidermidis	Strong	Monitoring of central venous pressure in a patient in ICU	Central venous catheter	Vancomycin, ciprofloxacin
K. pneumoniae	Strong	Pneumonia in a patient on ventilator	Endotracheal tube	Meropenem, ceftriaxone, aztreonam
P. aeruginosa	Strong	Ventilator associated pneumonia	Endotracheal tube	Cefoperazone-sulbactam, amikacin, meropenem
S. epidermidis	Moderate	Prosthetic joint infection	Artificial joint	Vancomycin, linezolid, ciprofloxacin
S. epidermidis	Strong	Acute renal failure/ peritoneal dialysis/ cellulites, peritonitis	PD Catheter	Vancomycin, linezolid, cotrimoxazole, ciprofloxacin
S. aureus	Moderate	Prosthetic joint infection	Artificial joint	Rifampicin, linezolid, vancomcin, erythromycin

Table 2. Interpretation of biofilm production

Average OD value	Biofilm production
≤ ODc / ODc < ~ ≤ 2x ODc	Non/weak
2x ODc < ~ ≤ 4x ODc	Moderate
> 4x ODc	Strong

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

aureus ATCC 29213 were used as control strains. Antibiotic susceptibility test was performed by using the Kirby-Bauer disc diffusion technique according to CLSI guidelines.[15]

RESULTS

Among 110 isolates, TCP, the standard method, detected 25 as strong and 45 as moderate biofilm producers. The majority of the organisms associated with biofilm production were *S. epidermidis* (37.1%) followed by *E. coli* (27.1%), *K. pneumoniae* (15.7%), *S. aureus* (11.4%), *E. faecalis* (4.2%) and *P. aeruginosa* (4.2%). Biofilm producing bacteria were isolated from urine (30%) followed by urinary catheter tips (25.7%), pus (12.8%), sputum (11.4%), intravenous catheter tips (10%) and nasobronchial lavage specimens (10%). Strong biofilm production was caused by *E. coli* and *S. epidermidis* on Foley's urinary catheter, mainly in immunocompromised patients, sensitive predominantly to meropenem, aztreonam, vanomycin and linezolid. *S. epidermidis* was responsible for strong biofilm

production in patients with intravenous catheters, sensitive mostly to linezolid and vancomycin (Table 1).

By TM, the number of strong biofilm producers were 21, moderate were 33 and weak or non-biofilm producers were 56. Very different results were observed by the CRA method, with which only four isolates showed black colonies with crystalline appearance (Table 3).

We have observed higher antibiotic resistance in biofilm producing bacteria than non-biofilm producers. By the standard method (TCP), biofilm producing bacteria include the strong (25) and moderate biofilm producers (45), and non-biofilm producing bacteria include non-biofilm producers (40) (Tables 4 and 5).

Table 3. Screening of the isolates for biofilm formation by Tissue Culture Plate, Tube Method and Congo Red Agar methods

	Biofilm formation	TCM n (%)	TM n (%)	CRA n (%)
No. of isolates (110)	High	25 (22.7)	21 (19)	4 (3.6)
	Moderate	45 (41)	33 (30)	7 (6.3)
	Weak/none	40 (36.3)	56 (51)	99 (90)

Table 4. Resistance pattern (%) of biofilm producing Gram-positive bacteria

Antimicrobial agent	Biofilm producing Gram-positive organisms %	Non-biofilm producing Gram-positive organisms %
Penicillin	100	100
Rifampicin	70	30
Ciprofloxacin	40	10
Erythromycin	40	20
Cotrimoxazole	30	25
Linezolid	0	0
Vancomycin	0	0

Table 5. Resistance pattern (%) of biofilm producing Gram-negative bacteria

Antimicrobial agent	Biofilm producing Gram-negative organisms %	Non-biofilm producing Gram-negative organisms %
Ampicillin	100	100
Ciprofloxacin	95	50
Cotrimoxazole	90	83
Aztreonam	90	50
Amikacin	64	37
Ceftriaxone	58	33
Cefoperazone-sulbactam	36	0
Meropenem	0	0

Table 6. Diagnostic parameters of Tube method and Congo Red Agar method for biofilm detection

Screening method	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
TM	73	92.5	94	66	80
CRA	11	92	73	37	41

Statistical analysis of tissue culture plate, tube and Congo Red Agar methods

The TCP method was considered the gold-standard for this study and compared with data from TM and CRA methods. Parameters like sensitivity, specificity, negative predictive value, positive predictive value and accuracy were calculated. True positives were biofilm producers by TCP, TM and CRA method. False positive were biofilm producers by TM and CRA method and not by TCP method. False negative were the isolates which were non-biofilm producers by TM and CRA but were producing biofilm by TCP method. True negatives are those which were non biofilm producers by all the three methods. Sensitivity and specificity of TM was 73% and 92.5%, respectively. For CRA method, sensitivity and specificity remained low and were 11% and 92%, respectively (Table 6).

DISCUSSION

Biofilm producing bacteria are responsible for many recalcitrant infections and are notoriously difficult to eradicate. They exhibit resistance to antibiotics by various methods like restricted penetration of antibiotic into biofilms, decreased growth rate and expression of resistance genes.[16] There are various methods for biofilm detection.[7-12] In this study we evaluated 110 isolates by three screening methods for their ability to form biofilms.

In our study, we have found that the majority of biofilm producing bacteria was from urinary catheter tips (26.3%). Similarly, Donlan[6] reported in his study the association of biofilm producing bacteria with urinary catheters.

In the TCP method, the number of isolates showing biofilm formation was 70 (64.7%), and non or weak biofilm producers were 40 (36.3%). Regional data from India also showed that out of 152 isolates tested, the number of biofilm producers identified by TCP method was 53.9 %, and non-biofilm producers were 46%.[13] We have performed the TCP method by addition of 1% glucose in trypticase soy broth. Addition of sugar helps in biofilm formation.[16,17] This was also reported by studies conducted by Mathur et al.[13] and Bose et al.[18]

Tube method detected 49% isolates as biofilm producers and 51% as non-biofilm producers. By this method, three isolates were found to be false positive and 19 were false negative. TM is 73% sensitive, 92.5% specific and 80% accurate for biofilm detection. This method correlated well with TCP for identifying strong biofilm producers, but it was hard to differentiate between moderate, weak and non-biofilm producers due to the changeability in the results detected by different observers. In accordance with the preceding studies, TM cannot be suggested as general screening test to identify biofilm producing isolates.[8,13]

In another study, Ruzicka et al.[19] noted that out of 147 isolates of *S. epidermidis*, TM detected biofilm formation in 79 (53.7%) and CRA detected in 64 (43.5%) isolates. They showed that TM is better for biofilm detection than CRA.[19] Baqai et al.[20] tested TM to detect biofilm formation among uropathogens. According to their results, 75% of the isolates exhibited biofilm formation.[20] With the CRA method, 11 were found to be biofilm producing bacteria and 99 as non-biofilm producers. The CRA method showed very little correlation with the other methods and parameters of sensitivity (11%), specificity (92%) and accuracy (41%) were very low. By this method, three isolates were found to be false positive and 62 were false negative. Knobloch et al.[21] 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 TCP which detected 57.1% as biofilm producing bacteria.[21]

CONCLUSION

We can conclude from our study that TCP is a quantitative and reliable method to detect biofilm forming microorganisms. When compared to TM and CRA methods, and TCP can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories.

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