Detection of Biofilm Production and Antibiotic Resistance Pattern in Clinical Isolates from Indwelling Medical Devices

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Abstract Microbial biofilms pose great threat for patients requiring indwelling medical devices (IMDs) as it is difficult to remove them. It is, therefore, crucial to follow an appropriate method for the detection of biofilms. The present study focuses on detection of biofilm formation among the isolates from IMDs. We also aimed to explore the antibiogram of biofilm producers. This prospective analysis included 65 prosthetic samples. After isolation and identification of bacteria following standard methodology, antibiogram of the isolates were produced following Kirby–Bauer disc diffusion method. Detection of biofilms was done by tube adherence (TA), Congo red agar and tissue culture plate (TCP) methods. Out of 67 clinical isolates from IMDs, TCP detected 31 (46.3 %) biofilm producers and 36 (53.7 %) biofilm non-producers. *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex were found to be the most frequent biofilm producers. The TA method correlated well with the TCP method for biofilm detection. Higher antibiotic resistance was observed in biofilm producers than in biofilm non-producers. The most effective antibiotics for biofilm producing Gram-positive isolates were Vancomycin and Tigecycline, and that for biofilm producing Gram-negative isolates were Polymyxin-B, Colistin Sulphate and Tigecycline. Nearly 46 % of the isolates were found to be biofilm producers. The antibiotic susceptibility pattern in the present study showed Amoxicillin to be an ineffective drug for isolates from the IMDs. For the detection of biofilm production, TA method can be an economical and effective alternative to TCP method.

Introduction

Biofilm is a microbially derived community characterised by cells that are irreversibly attached to a substratum or interface or to each other. The microbial cells growing in a biofilm are physiologically diverse from planktonic cells of the same organism which, by contrast, are single-cells that may float or swim in a liquid medium [9]. The cells in the biofilm produce extracellular polymeric matrix called polysaccharide intercellular adhesin which protects the cells within the biofilm and also facilitates communication among the cells through biochemical signals such as acyl homoserine lactone in Gram-negative bacteria and oligopeptides in Gram-positive bacteria, in a phenomenon called quorum sensing. Enzymes degrading this extracellular matrix, such as dispersin B and deoxyribonuclease, enable biofilm to spread and colonise new surfaces causing persistent and recurrent infections [14, 19].

Human diseases in which biofilms have been implicated include urinary tract infections, middle ear infections, formation of dental plaque, gingivitis and less common but more lethal processes such as prosthetic valve endocarditis and cystic fibrosis [8]. Biofilms readily develop in indwelling medical devices (IMDs) or foreign bodies, particularly catheter devices, cerebrospinal fluid shunts, intrauterine devices, mechanical ventilators and orthopaedic appliances [3]. These implanted biomedical devices are significant in patient care for diagnostic and therapeutic purposes and due to their routine use in hospitals, critical

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patients easily become vulnerable to microbial colonisation leading to device-related infections (DRIs). Among the DRIs, a majority of infections are catheter-related blood-stream infections, followed by catheter-associated urinary tract infections and ventilator-associated pneumonia [22, 25, 26].

The major concern about biofilm infections is the difficulty in their eradication as interior cells in a biofilm are shielded from immune response of host as well as from the effect of antibiotic agents [17]. Meanwhile in case of mixed bacterial growth, bacteria once deemed antibiotic sensitive can turn resistant on subsequent antibiotic susceptibility test due to the horizontal transmission of plasmid associated drug-resistant genes from resistant bacteria to sensitive bacteria when they become associated within a biofilm [24]. Early detection of biofilm producing organisms is therefore necessary to help manage DRIs and to reduce the spread of antimicrobial resistance. Thus, the present study was done to detect the occurrence of biofilm-forming isolates from IMDs and to explore their antibiotic resistance pattern.

Materials and Methods

This prospective study was conducted in Bacteriology Laboratory of Tribhuvan University Teaching Hospital (TUTH), Kathmandu, Nepal from July 2013 to September 2013. IMDs which included central venous plexus (CVP) tip, endotracheal tube (ET), Foley's catheter tip, femoral tip and ventriculoperitoneal (VP) shunt tube, received in the bacteriology laboratory for culture and sensitivity were included in the study. They were inoculated into blood agar and MacConkey agar (chocolate agar was also used for ET tube) by roll plate method and incubated at 37 °C for 24 h. The isolates were identified by colony morphology, Gram staining and standard biochemical tests as per the guidelines given by American Society for Microbiology (ASM) [12].

Biofilm Detection Methods

The isolates were subjected to biofilm detection by the three phenotypic methods: tube adherence method (TAM), Congo red agar (CRA) method and microtiter/tissue culture plate (TCP) method.

(a) Tube adherence method

Two millilitre (ml) volumes of brain heart infusion (BHI) broth supplemented with 1 % glucose in 12 × 75 mm borosilicate test tubes were inoculated with single colonies and incubated statically for 48 h at 37 °C. The contents were decanted and washed with phosphate-buffered saline (PBS), pH 7.2 and
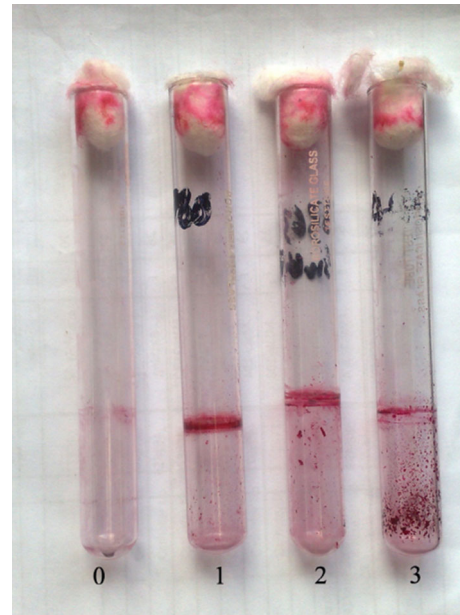

Fig. 1 Tube Adherence method. *0* non-adherent, *1* weakly adherent, *2* moderately adherent, and *3* strongly adherent

dried. One millilitre volume of 1 % safranin solution was added. Each tube was then gently rotated to ensure uniform staining of any adherent material on the inner surface and the contents gently decanted to remove excess stain. The tubes were then washed with distilled water and placed upside down to drain. Presence of an adherent layer of stained material on the inner surface of the tube indicated a positive result (Fig. 1). Presence of stained material at the liquid–air interface alone was not regarded as indicative of slime production. Tubes were examined independently by two different observers and the amount of biofilm formation was scored as 0—absent, 1—weak, 2—moderate, or 3—strong. Experiment was performed in duplicates two times [3, 19, 25].

(b) Congo red agar method

(i) Congo red agar, original (CRAori)

A specially prepared solid medium—brain heart infusion broth (BHI) supplemented with 5 % sucrose and Congo red was prepared. For this, Congo red was prepared as concentrated aqueous solution and autoclaved at 121 °C for 15 min, separately from other medium constituents and was then added when the agar had cooled to 55 °C. Plates were inoculated and incubated aerobically for 24 h at 37 °C. The experiment was performed in duplicates [10, 19] (Fig. 2).

Fig. 2 Congo red agar method showing isolates producing different colours
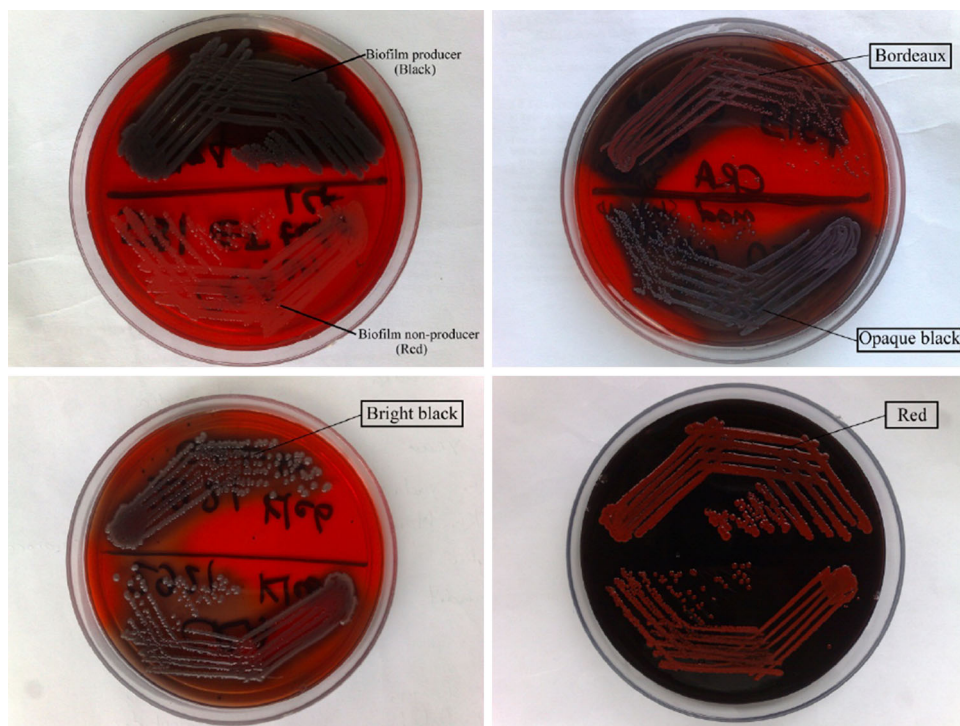
A five-colour reference scale was used to accurately determine all colour variations shown by the colonies. Isolates presenting two tones of black-bright black (BB) and opaque black (OB) were classified as positive for biofilm production whereas red, pink and bordeaux colonies were classified as negative [20].

(ii) Modified Congo red agar (CRAmod)

As CRAori has been shown to have shortcomings in variations in black pigment formation, modification on its agar constituent was employed to improve the outcome on biofilm identity determination. The modifications include reducing the concentration of Congo red dye (0.4 g/L), replacement of sucrose with glucose (10 g/L) and BHI and agar no. 1 by an alternative agar, blood agar base-2 (BAB-2) (40 g/L) [13, 15].

(c) Tissue culture plate method/microtiter plate method

Organisms isolated from fresh agar plates were inoculated in 2 ml of BHI broth. Broths were incubated at 37 °C for 24 h. The cultures were then diluted 1:40 with fresh medium (BHI broth supplemented with 1 % glucose) in the individual wells of sterile 96 well-flat bottom microtiter plate (Terumo, Japan) so that the final volume in each well was 200 μl. The plate was incubated at 37 °C for 24 h.
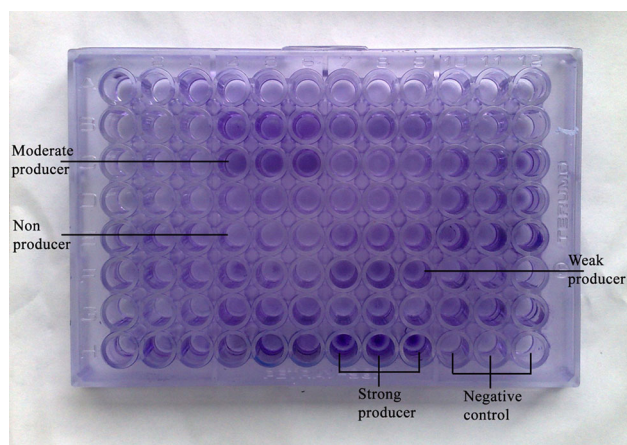

Fig. 3 Tissue culture plate method for biofilm detection

Then, contents of each well were removed by gentle tapping. The wells were washed with phosphate buffer saline (pH 7.2) three times to remove free floating bacteria. Bacteria in biofilm adherent to the wells were stained by 2 % crystal violet for 10–15 min. Excess stain was removed by using distilled water and the plate was kept for drying (Fig. 3). Optical density (OD) of stained bacteria in biofilm was obtained by using enzyme-linked immunosorbent assay (ELISA) plate analyser (AD Touch, apDia$_{nv}$) at wavelength of 492 nm. The experiment

was performed in triplicates two times. Average OD values of each test strain and negative control were calculated. Final OD values of a test strain was expressed as average OD value of the strain reduced by OD cut-off value (OD_c) of negative control. Then the interpretation for biofilm production was done according to the criteria of Stepanovic et al. [23]. OD_c was defined as three standard deviations (SDs) above the mean OD of negative control [2, 4, 6, 18, 21, 23].

The TCP method was considered as the gold-standard for this study and was compared with data from TA and CRA methods. Parameters like sensitivity, specificity, negative predictive value, positive predictive value and accuracy were calculated. True positives were biofilm producers by TCP, TA and CRA methods. False positive were biofilm producers by TAM and CRA method and not by TCP method. False negative was the isolates which were biofilm non-producers by TAM and CRA method but were producing biofilm by TCP method. True negatives were biofilm non-producers by all the three methods.

All isolates were subjected to antibiotic susceptibility following Kirby–Bauer disc diffusion method and interpreted as per CLSI recommendations [5]. Antibiotic discs (MAST, UK) used were Amoxycillin (10 μg), Cefixime (5 μg), Ceftriaxone (30 μg), Ceftazidime (30 μg), Cefoxitin (30 μg), Cefotaxime (30 μg), Cefepime (30 μg), Norfloxacin (10 μg), Ciprofloxacin (5 μg), Levofloxacin (5 μg), Gentamicin (10 μg), Amikacin (30 μg), Meropenem (10 μg), Polymyxin-B (300 units), Colistin Sulphate (10 μg), Cefoperazone–Sulbactam (75/30 μg), Piperacillin–Tazobactam (100/10 μg), Co-trimoxazole (25 μg), Cloxacillin (30 μg), Nitrofurantoin (300 μg), Tigecycline (15 μg), Vancomycin (30 μg), Teicoplanin (30 μg), Chloramphenicol (30 μg) and Amoxicillin–clavulanate (20/10 μg). Isolates showing resistance to three or more than three commonly used antibiotics were taken as multidrug-resistant (MDR) organisms [1]. Biofilm producer *Staphylococcus epidermidis* ATCC 35984 and biofilm non-producer *S. epidermidis* ATCC 12228 were used as the positive and negative controls, respectively. Data were analysed using Microsoft Excel 2013 and represented as frequency distribution and percentage. Ethical permission was obtained from the Institutional Review Board at the Institute of Medicine, Tribhuvan University, Kathmandu.

Results and Discussion

A total of 65 samples with significant bacterial growth were collected during the study period. One ET sample showed polymicrobial growth resulting in a total of 67 isolates. Table 1 shows device-wise distribution of samples and the corresponding type and number of organisms isolated.

TCP method detected **31** (46.3 %) biofilm producers and 36 (53.7 %) biofilm non-producers. Biofilm producing Gram-negative organisms ($n = 20$) were *Klebsiella pneumonia*e (30 %), *Burkholderia cepacia* complex (30 %), *Pseudomonas aeruginosa* (15 %), *Acinetobacter calcoaceticus baumannii* complex (10 %), *Escherichia coli* (10 %) and *Citrobacter fruendii* (5 %) and biofilm producing Gram-positive organisms ($n = 11$) were *Enterococcus faecalis* (54.5 %), *Staphylococcus aureus* (27.3 %) and *S. epidermidis* (18.2 %). Strong biofilm production by TCP method was shown by *P. aeruginosa* in CVP tube. *K. pneumoniae, Bc* complex and *P. aeruginosa* were shown to be the most frequent biofilm producers by the TCP method (Table 2).

Similarly, tube method showed 37 (55.2 %) biofilm producers and 30 (44.8 %) biofilm non-producers. Strong biofilm production was caused by *Bc* complex on catheter tube. By CRA method, the CRAori showed 31 (46.3 %) isolates as biofilm producers and 36 (53.7 %) isolates as biofilm non-producers whereas CRAmod showed 35 (52.2 %) isolates as biofilm producers and 32 (47.8 %) biofilm non-producers. Sensitivity, specificity and accuracy of the tube method were 80.7, 66.7 and 73.1 %, respectively. For CRA method, sensitivity, specificity and accuracy remained low and were 44.8, 52.6 and 49.3 % for CRAori and 44.8, 42.11 and 43.3 % for CRAmod, respectively (Table 3).

When an IMD is used, its susceptibility to microbial contamination and biofilm formation is determined by several factors such as number and type of organisms to which the device is exposed, duration of use of the device, composition and flow rate of the medium in or on the device, device material construction, conditioning films on the device, antimicrobial drug concentration and ambient temperature [7]. The polymicrobial growth found in ET tube in this study constituted *K. pneumoniae* as biofilm producer and *Bc* complex and *Acb* complex as non-biofilm producers. Biofilms on IMDs might initially be composed of a single species, but longer exposures inevitably lead to multispecies biofilms [22].

For TAM, in this study, the number of true positives and true negatives were higher and the number of false positives and false negatives were lower than for CRA method. This ultimately increased the parameters like sensitivity, specificity and accuracy for TAM than the CRA method. Four of the TCP negative *E. coli* isolates produced black colonies on CRAmod but red colonies on CRAori. This reduced the specificity and accuracy of CRAmod than CRAori in this study, which otherwise gave the same net result as CRAori. In fact, CRAmod has been considered superior to CRAori in

Table 1 Device-wise distribution of samples and the corresponding type and number of organisms isolated

Indwelling medical device	No. of samples	E. coli	K. pneumoniae	P. aeruginosa	B. cepacia complex	Acb complex	C. fruendii	E. faecalis	S. aureus	S. epidermidis	Total
Foley's tip	50	12	5	1	5			14	9	4	50
CVP tip	8		2	2	2				1	1	8
ET tube	5	1	1		1	3	1				7
Femoral tip	1								1		1
VP shunt	1			1							1
Total	65	13	8	4	8	3	1	14	11	5	67

E. coli Escherichia coli, C. fruendii Citrobacter fruendii, K. pneumoniae Klebsiella pneumoniae, E. faecalis Enterococcus faecalis, P. aeruginosa Pseudomonas aeruginosa, S. aureus Staphylococcus aureus, B. cepacia complex *Burkholderia cepacia* complex, *S. epidermidis Staphylococcus epidermidis, Acb* complex *Acinetobacter calcoaceticus baumannii* complex

Table 2 Result for biofilm production by the three methods

Isolates	Total	Biofilm production							
		CRAori		CRAmod		TAM		TCP	
		+	−	+	−	+	−	+	−
Gram-negative	37	15	22	19	18	23	14	20	17
E. coli	13	9	4	13	0	5	8	2 (10 %)	11
K. pneumoniae	8	6	2	6	2	5	3	6 (30 %)	2
B. cepacia complex	8	0	8	0	8	7	1	6 (30 %)	2
P. aeruginosa	4	0	4	0	4	4	0	3 (15 %)	1
Acb complex	3	0	3	0	3	1	2	2 (10 %)	1
C. fruendii	1	0	1	0	1	1	0	1 (5 %)	0
Gram-positive	30	16	14	16	14	14	16	11	19
E. faecalis	14	11	3	11	3	7	7	6 (54.5 %)	8
S. aureus	11	4	7	4	7	5	6	3 (27.3 %)	8
S. epidermidis	5	1	4	1	4	2	3	2 (18.2 %)	3
Total	67	31	36	35	32	37	30	31	36

Table 3 Results of biofilm production by considering TCP method as gold-standard

Statistical parameters	TAM	CRAori	CRAmod
True positives	25	13	13
False positives	12	18	22
False negatives	6	16	16
True negatives	24	20	16
Sensitivity	80.7 %	44.8 %	44.8 %
Specificity	66.7 %	52.6 %	42.1 %
Positive predictive value	67.6 %	41.9 %	37.1 %
Negative predictive value	80.0 %	55.6 %	50.0 %
Accuracy	73.1 %	49.3 %	43.3 %

terms of permanent formation of intense black pigment in biofilm producers [15]. There was a good association between the TAM and the TCP method than between the CRA method and the TCP method in this study. In a similar study, the TCP method showed 64.7 % biofilm producers and 36.3 % biofilm non-producers [11]. They also showed good correlation between TAM and TCP method but very little correlation between CRA and TCP methods.

All of the biofilm positive as well as biofilm negative Gram-negative bacterial isolates were resistant to Amoxicillin but sensitive to Polymyxin-B and Colistin Sulphate. For all other drugs, antibiotic resistance associated with biofilm producers was greater than that with biofilm non-producers. Only two of the biofilm producing Gram-negative isolates were resistant to Tigecycline whereas none of the biofilm non-producing Gram-negative isolates were resistant to Tigecycline (Table 4). All of the biofilm positive and biofilm negative Gram-positive isolates were sensitive to Tigecycline and Vancomycin. For all other drugs, antibiotic resistance associated with biofilm producers was greater than that with biofilm non-producers (Table 5). All of the biofilm-forming Staphylococcal isolates in this study were found to be methicillin resistant which coincides with the result of Mariana et al. [15].

The higher antibiotic resistance pattern showed by biofilm producing bacteria than biofilm non-producers could

Table 4 Antibiotic resistance pattern in Gram-negative isolates

Antibiotics	Biofilm positive isolates (n = 20)	%	Biofilm negative isolates (n = 17)	%
Amoxicillin[a]	14 (n = 14)	100.0	15 (n = 15)	100.0
Cefixime	19	95.0	16	94.1
Ceftriaxone	19	95.0	16	94.1
Amikacin	7	35.0	5	29.4
Co-trimoxazole	11	55.0	9	52.9
Ciprofloxacin	13	65.0	9	52.9
Levofloxacin	13	65.0	9	52.9
Meropenem	12	60.0	5	29.4
Amoxyclav	17	85.0	13	76.5
Piperacillin–Tazobactam	7	35.0	5	29.4
Cefoperazone–Sulbactam	8	40.0	6	35.3
Tigecycline	2	10.0	0	0.0
Polymyxin-B[b]	0 (n = 14)	0.0	0 (n = 15)	0.0
Colistin Sulphate[b]	0 (n = 14)	0.0	0 (n = 15)	0.0

[a] Except for *Klebsiella pneumoniae*

[b] Except for *Burkholderia cepacia* complex

Table 5 Antibiotic resistance pattern in Gram-positive isolates

Antibiotics	Biofilm positive isolates (n = 11)	%	Biofilm negative isolates (n = 19)	%
Amoxicillin	9	81.8	13	68.4
Cloxacillin[a]	4 (n = 5)	80.0	4 (n = 11)	36.4
Amikacin[a]	4 (n = 5)	80.0	3 (n = 11)	27.3
Ciprofloxacin	7	63.6	9	47.4
Levofloxacin	7	63.6	8	42.1
Nitrofurantoin	5	45.5	4	21.1
Meropenem	8	72.7	8	42.1
Amoxyclav	8	72.7	10	52.6
Piperacillin–Tazobactam	6	54.5	6	31.6
Tigecycline	0	0.0	0	0.0
Vancomycin	0	0.0	0	0.0

[a] Except for *Enterococcus feacalis*

be due to restricted penetration of antibiotics into biofilms, decreased growth rate of bacteria in biofilm, elevated expression of efflux pump and expression and exchange of resistance genes among bacteria within a biofilm [16]. Empirical use of antimicrobial agents and the superimposed complex nature of bacteria in biofilms colonising IMDs have resulted in resistant and chronic DRIs. It is a challenge to treat and also to eradicate such MDR organism because bacteria in biofilm are reported to require much greater concentration of antibiotics that is not indicated [3]. This adds up to the overall healthcare costs for the treatment of biofilm associated infections.

Conclusion

Nearly half of the isolates from IMDs were found to be biofilm producers. Tigecycline and Vancomycin for Gram-positive isolates and Polymixin B, Colistin Sulphate and Tigecycline for Gram-negative isolates, were found to be more effective antibiotics for most of the biofilm producers. It is necessary to carry out continuous surveillance of antimicrobial susceptibility of biofilm-forming isolates to generate data useful for optimising empirical antimicrobial therapy for such drug-resistant bugs. We recommend TA method for routine dectection of biofilm production in country like ours where molecular methods and sophisticated microscopy techniques are constrained, realising the fact that this method is easy to adopt, cost-effective and above all it confers reliable results with better sensitivity and specificity.

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