Original Article

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Biofilm formation and slime production as virulence determinants among isolates from indwelling devices

Shrestha R,[1] Nayak N,[2] Bhatta DR,[3] Hamal D,[4] Gokhale S[2]

[1]Rajani Shrestha, Assistant Professor; [2]Niranjan Nayak, Professor; [3]Dharma Raj Bhatta, Associate Professor; [4]Deependra Hamal, Lecturer; [2]Shishir Gokhale, Professor, Department of Microbiology, Manipal College of Medical Sciences, Pokhara, Kaski, Nepal.

Abstract

Background: Biofilm is defined as an assembly of microorganisms which enclosed in a self-produced extracellular matrix principally of polysaccharide material and found in association with indwelling medical devices. This study was designed to determine the biofilm forming ability of isolates from devices associated infection.

Objectives: To compare and evaluate biofilm production with the virulence markers like multidrug resistance and slime production.

Methods: An analytical observational study was conducted in Manipal Teaching Hospital from 2020 June-2021 May after ethical clearance. A total of 106 clinical isolates were obtained from patients with indwelling medical devices. All bacteria were identified by conventional techniques. Antimicrobial sensitivity testing was performed on Mueller-Hinton agar plates with commercially available antibiotic discs using Kirby Bauer disc diffusion techniques and interpreted as per the guidelines of Clinical and Laboratory Standards Institute (CLSI). Biofilm and slime production were detected by two methods: Tissue culture plate method and Congo Red Agar method.

Results: Out of 106 total isolates, 79 (74.5%) isolates were detected in endotracheal tubes (ETTs). Besides, it was observed that 54 (68.3%) of the 79 ETT isolates were biofilm producers. Amongst the isolates, 90.4% (19/21) were *Klebsiella* species, 64.1% (25/39) *Acinetobacter* spp., 47.6% (10/21) *Pseudomonas* spp., and 54.5% (6/11) *Staphylococcus aureus* were biofilm producers.

Conclusion: Biofilm mediated persistence of infection in the nosocomial setting through indwelling devices. Significantly, higher number of the biofilm producers as well as slime producers were multidrug resistant (p-value <0.05).

Key words: Biofilm; Congo red; Multidrug resistance; Virulence.

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Address for correspondence

Dr. Rajani Shrestha
Assistant Professor, Department of Microbiology,
Manipal College of Medical Sciences,
Pokhara, Kaski, Nepal.
E-mail: rajani_sth7@hotmail.com

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INTRODUCTION

Biofilm is defined as an assembly of microbial cells with a surface either inert or living. Availability of key nutrients, chemotaxis towards surface, motility of bacteria, surface adhesions, and presence of surfactants are the factors that influence biofilm formation.[1] The multilayered cell clusters of biofilms facilitates the adherence of these microorganisms to biomedical surfaces. This protects them from the adverse effects of host immunity and antimicrobial agents. Microorganisms growing in a biofilm are more resistant to antimicrobial agents than planktonic cells. Both gram-positive and gram-negative bacteria have the capability to form biofilms. Commonly reported biofilm forming bacteria include *Enterococcus faecalis*, *Staphylococcus aureus, Staphylococcus epidermidis,* Viridians *Streptococcus, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis,* and *Pseudomonas aeruginosa.* Biofilms are estimated to be associated with 17.9-100% of nosocomial infections worldwide.[2] This study, therefore was designed to

determine the biofilm forming ability of each isolate and to compare biofilm production with the virulence markers like multidrug resistance and slime production.

METHODOLOGY

This was an analytical observational study conducted between 2020 June to 2021 May in the Department of Microbiology, Teaching Hospital of Manipal College of Medical Sciences (MCOMS), Fulbari, Pokhara, Kaski, Nepal after obtaining ethical approval from the Institutional Research Committee (IRC) of MCOMS (Ref. MEMG/IRC/GA).

A total of 106 samples were collected by convenience sampling technique. The sample size was derived using formula sample size: , where n = sample size, z = 1.96 at level of confidence 95%, P = expected prevalence, here in this study P = 0.95 (95%)[3,4] d= precision (0.05) and minimum sample size obtained was 72.9 ≈ 73. A total of 106 clinical isolates from patients with indwelling medical devices placed for more than 48 hours were included. The indwelling devices included Endotracheal tubes (ETTs), Central Venous Pressure Lines (CVPs), and Foleys catheters. The devices were sent to the laboratories under sterile precautions. The intraluminal surfaces were flushed with the sterile normal saline. The flushed-out fluid was inoculated onto Trypticase Soy Broth (TSB) which was incubated at 37°C overnight.[5] Those showing growth was sub-cultured onto Blood agar, MacConkey agar, and Chocolate agar plates. Out of 106 isolates, 79 were from ETTs, eight from CV Lines, and 19 from Foleys catheters. All bacteria were identified by conventional techniques.[6,7] Antimicrobial sensitivity testing was performed on Mueller-Hinton agar plates with commercially available antibiotic discs using Kirby Bauer disc diffusion techniques and interpreted as per the guidelines of Clinical and Laboratory Standards Institute (CLSI).[8] The antibiotic (Himedia, Mumbai, India) and their Conc/disc (mcg) were: Ampicillin (10), Piperacillin/tazobactam (100/10), Ciprofloxacin (5), Amikacin (30), Imipenem (10), Gentamicin (10), Tigecycline (15), ceftriaxone (30), ceftazidime (30) for gram-negative bacteria and Erythromycin (15), Amikacin (30), Gentamicin (10), Ciprofloxacin (5), and Clindamycin (2), vancomycin (30), teicoplanin (30) for gram-positive bacteria. The European Centre for Disease Prevention and Control (ECDC) criteria define multidrug resistant (MDR) as acquired non-susceptibility to at least one agent in three or more antimicrobial categories.[9]

Biofilm detection was performed by the following two methods: Tissue culture plate method and Congo red agar (CRA) method.[10,11] For the tissue culture plate method, organisms isolated from fresh agar plates were inoculated in 10 mL of TSB with 1% glucose. Broths were incubated at 37°C for 24 hour. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well-flat bottom polystyrene tissue culture plates (Sigma-Aldrich, Costar, USA) were filled with 200 µL of the diluted cultures. Negative control wells containing sterile broth were included in each batch. 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 four times with 0.2 mL of phosphate buffer saline (pH 7.2) to remove 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 deionised water and plates were kept for drying. Optical density (OD) of stained adherent biofilm were obtained by using micro-enzyme-linked immunosorbent assay (ELISA) autoreader (model 680, Biorad, UK) at a 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.[12]

The CRA test was performed as previously described by Freeman et al.[13] The CRA medium were 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. Congo Red stains were separately prepared as a concentrated aqueous solution and autoclaved (121°C for 15 minutes). Then it was added to the autoclaved brain heart infusion agar with sucrose at 55°C. CRA plates were inoculated with test, organism along with positive and negative controls (known slime producers and slime producers and incubated at 37°C for 24 h aerobically. Black colonies with a dry crystalline consistency indicated slime production.[14] The data were collected, entered and analysed using SPSS Statistics for Windows, version 21.0 (SPSS Inc., Chicago, Ill., USA). Categorical variables were calculated as frequency and percent. Chi-square test was used to compare two groups. All p-values <0.05 were considered as statistically significant.

RESULTS

Out of the total 106 isolates, 89 (83.9%) were gram-negative organism and 17 (16.0%) were gram-positive organism. *Acinetobacter* spp. were 39 (36.7%) the commonest gram-negative organism followed by *Klebsiella pneumoniae* 21 (19.8%), *Pseudomonas aeruginosa* 21 (19.8%), *Escherichia coli* seven (6.6%), and *Enterobacter* spp. one (0.9%). Among gram-positive

organism, *Staphylococcus aureus* 11 (10.3%) was the commonest one followed by *Enterococcus faecalis* four (3.77%), and coagulase-negative *Staphylococcus* two (1.8%) (Table 1).

Out of 106 isolates, 79 (74.5%) were from endotracheal tubes, eight (7.5%) were from central venous lines, and 19 (17.9%) were from Foley's catheter tips (Table 2). Out of 79 ETT isolates, 35 (44.3%) were *Acinetobacter* spp., 18 (22.7%) were *Pseudomonas aeruginosa,* 17 (21.5%), Klebsiella *pneumoniae,* three (3.8%) each of *Escherichia coli, and Staphylococcus aureus,* two (2.5%) coagulase negative *Staphylococci* and one (1.26%) *Enterobacter*. From among eight CVP line, *Staphylococcus aureus* were obtained from three (37.5%), *Acinetobacter* from three (37.5%) and *Klebsiella pneumoniae* from two (25%). Likewise, out of 19 Foley's catheter, four (21%) yielded *Enterococcus spp*., five (26.3%) yielded *Staphylococcus aureus*, four (21%) *Escherichia coli,* three (15%) *Pseudomonas,* two (10.5%) *Klebsiella*, and one (5.2%) yielded *Acinetobacter* spp**.**

It was observed that 38 (92.6%) of slime producing organism under study formed biofilm (Table 3). Contrary to that 32 (49.2%) among the 65 non-slime producers were capable of producing biofilm. These differences were found to be statistically significant meaning thereby that slime being an adhesion could help the organism to adhere to the devices first leading subsequently to the formation of biofilms.

It was observed that 71.4% (50/70) of the biofilm producing organisms were MDR, as compared to only 19.4% (7/36) of the non-biofilm producing organisms which were MDR (Table 4).

As 30 (73.1%) slime producers were MDR compared to only 27 (41.53%) MDR non slime producers (Table 5).

Table 1: Frequency of organisms isolated from indwelling devices (N = 106)

Gram-negative organisms	Organisms isolated, n (%)
Acinetobacter spp.	39 (36.7)
Pseudomonas aeruginosa	21 (19.8)
Klebsiella pneumoniae	21 (19.8)
Escherichia coli	7 (6.6)
Enterobacter spp.	1 (0.9)
Gram-positive organisms	
Staphylococcus aureus	11 (10.3)
Coagulase negative Staphylococcus	2 (1.8)
Enterococcus faecalis	4 (3.7)
Total	**106 (100)**

Table 2: Organisms isolated from various medical devices, n (%)

Organisms	ETT	CVP line	Foley's catheter	Total
Klebsiella pneumoniae	17 (21.5)	2 (9.5)	2 (9.5)	21
Staphylococcus aureus	3 (27.2)	3 (27.2)	5 (45.4)	11
Coagulase negative *Staphylococci*	2 (100)	-	-	2
Enterococcus	-	-	4 (100)	4
Pseudomonas aeruginosa	18 (22.7)	-	3 (14.2)	21
Acinetobacter spp.	35 (44.3)	3 (7.6)	1 (2.5)	39
Enterobacter spp.	1	-	-	1
Escherichia coli	3 (42.8)	-	4 (57.1)	7
Total	**79**	**8**	**19**	**106**

p <0.001

Table 3: Correlation of slime production with biofilm production, n (%)

		Tissue culture plate method for biofilm formation		
		Positive	Negative	
Congo red agar (For slime production)	Producer	38 (92.6)	3 (7.3)	p <0.001
	Non-producer	32 (49.2)	33 (50.7)	
Total		**70 (66.0)**	**36 (33.9)**	

Table 4: Multidrug resistance among biofilm producing and non-biofilm producing organisms

Biofilm production (N = 106)	Multidrug resistance detected (%)	No multidrug resistance not detected (%)
Positive (70)	50 (71.4)	20 (28.5)
Negative (36)	7 (19.4)	29 (80.5)
Total	**57**	**49**

p <0.001

Table 5: Multidrug resistance among slime producers and non-slime producers

Slime production N = 106	Multidrug resistance Detected (%)	Multidrug resistance not detected (%)
Positive (41)	30 (73.1)	11(26.8)
Negative (65)	27 (41.5)	38 (58.4)
Total	**41**	**65**

(p = 0.001)

DISCUSSION

During the last three decades, deep seated infections due to bacterial agents were ascribed to their pathogenic potential related to the inherent property of slime production. Slime as a virulence factor of many bacterial species in implant/device related infections were documented in the past.[15-17] Thus it was planned to study the virulence characteristics of these organisms in context to their biofilm forming, slime producing abilities and drug resistance, in order to derive information on the prevalence of these virulence traits among the isolates causing various device infections in MCOMS Teaching Hospital set up.

Out of a total of 106 isolates, a majority that is 79 were from ETTs which accounted for 74.5% of the total (Table 2). Other researchers, too, reported high rate of bacterial isolation (67.5%) from patients with ETTs.[18] Results from current study showed majority that is 54 (68.35%) of the 79 ETT isolates formed biofilm on ETT. The ETT being an inert substance, provided a nidus for conditioning film formed by host secretion. Following this event, biofilm forming bacteria like *Acinetobacter* spp. and *Pseudomonas* spp. were able to colonise on those surface of ETT forming a monolayer onto which subsequent adherence of other bacterial cells having the potential to form biofilms.[5]

The implication of biofilm formation of ETT has tremendous clinical relevance. First of all, once formed in vivo condition provides a protective architecture for the bacteria lying in its interior.

These bacterial communities are non-responsive to antibiotics therapy and also are protected from the host immunity. Over and above the biofilm upon ageing dislodges from its original site of attachment and the biofilm bacteria attach to another inert surfaces on ETT forming a fresh sessile architecture, leading thereby to chronicity.[19]

Gil-Peroton et al.[20] reported that 87% of patients were colonised based upon ETT culture and in 56% cases the same microorganism grew on ETT culture and on biofilm assay. Other authors have also shown high prevalence of biofilm producing bacteria on ETT from mechanically ventilated patients.[21] At the same time, it was noted that fourteen out of Seventy five (19%) patients on ETT progressed to develop Ventilator associated pneumonia (VAP). These observations from current study as well as from others, point towards the fact that airway colonisation in association with biofilm formation were necessary for development of VAP. In such a clinical scenario, ETT could act as a means of direct conduit for

the organisms to descend to the lower respiratory tract, producing pneumonia.

Amongst the clinical isolates in current study, 90.4% (10/21) of *Klebsiella* species, 64.1% (25/39) of the *Acinetobacter* spp., 47.6% (10/21) *Pseudomonas* spp., and 54.5% (6/11) *Staphylococcus aureus* were biofilm producers. Such high degree of biofilm detection among gram-negative bacilli is challenging, in consideration with the earlier observations that airway colonisation with *Acinetobacter* and *Pseudomonas* species were accounted for increased risk factors for VAP.[22,23]

Antibiotic resistance of bacteria embedded in biofilms were detailed in previous studies with regard to difficult to treat device related infections.[24] Current study findings that majority, and significantly higher number of the biofilm producers as well as slime producers were multidrug resistant was of concern (Tables 4, 5). As reported previously, bacterial survival in the core of the biofilm formed on the indwelling devices could account for recalcitrance to antibiotic therapy in many device infections, including those due to ETT, central venous lines, and Foley's catheter. As proposed elsewhere, such persistence and chronicity of infections due to biofilm forming bacteria could be due to slow and incomplete penetration of antibiotics into the biofilms, an altered chemical mechanism inside the biofilm microenvironment, and evolution of colony variants in the interior of biofilms.[25,26] Interestingly, in this study, it was noted that most isolates from infections associated with ETT, central venous lines, and Foley's catheter were biofilm producers, and slime producers as well.

Moreover, it was noteworthy to observe that majority (38, 92.6%) of the slime producing bacteria were capable of forming biofilm. This finding of current study reiterated the notion put forth earlier that slime being a true adhesin, could initiate the organism's adherence on to the devices, eventually triggering the formation of biofilm.[19] Therefore, slime, which is the extracellular polymeric matrix could lay the foundation for primary attachment with micro-colony formation, leading subsequently to the formation multicellular complex structural community of biofilms.[27] Thus, all findings in the present study indicated biofilm mediated persistence of infection in the nosocomial setting through indwelling devices.

The limitations of this study could be that the authors have estimated biofilm activity only phenotypically.

CONCLUSION

In conclusion, this study denoted the dynamic relation among colonisation in the airway and other body sites, formation of biofilms and development of deep-seated infections, including VAP. The intricate mechanism of biofilm persistence of bacteria and their unresponsiveness to antibiotic therapy highlights the importance of evolving novel strategies of eliminating the biofilm from the indwelling devices.

The authors recommend further studies with molecular characterisation of the isolates by looking at the biofilm determining gene for more precise result. Correlation of biofilm activity of the isolates with antimicrobial resistance pattern could be of much clinical relevance in patient management.

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