Original Article

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Microbiology Section

Comparison of Tissue Culture Plate and Modified Tissue Culture Plate Method for Biofilm Detection in Members of Family Enterobacteriaceae

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ABSTRACT

Introduction: Microorganisms associated with biofilm formation have tendency to delay healing and show increased resistance to antimicrobial drugs resulting in chronic infection. This increases morbidity of patient as well as cost of treatment. Among the several methods of biofilm detection, Tissue Culture Plate (TCP) method and Modified Tissue Culture Plate (MTCP) method were studied.

Aim: 1) To detect biofilm production in pus isolates by TCP method and MTCP method; 2) To compare Antimicrobial Susceptibility Testing (AST) of biofilm producing and biofilm non-producing isolates.

Materials and Methods: In the present study, 240 pus samples obtained from patients attending Pt. B.D. Sharma PGIMS, Rohtak were studied. The organism was identified using standard microbiological procedures and AST was done by Kirby-Bauer disc diffusion method in accordance with CLSI guidelines 2016. Biofilm production was detected by TCP and MTCP methods.

Results: A total of 160 isolates were studied which included *Klebsiella* spp. (n=51), *Escherichia coli* (n=41), *Citrobacter* spp. (n=32), *Proteus* spp. (n=26), *Enterobacter* spp. (n=10). Out of these, 52.5% isolates showed biofilm production by TCP and 65.6% by MTCP method. The sensitivity of MTCP was found to be higher than TCP method. Also, it was observed that 79.7% biofilm producing isolates were multidrug resistant as compared to 29% non- biofilm producing strains. Overall 55.6% isolates were found to be multidrug resistant.

Conclusion: MTCP was found to be more accurate method for biofilm detection and quantification.

Keywords: Antimicrobial resistance, Biofilm detection, Wound infection

INTRODUCTION

A wound is a breach in the epidermis leading to the loss of skin integrity. This exposes the subcutaneous tissue and provides a nutritive and favourable environment promoting the microbial colonization and proliferation [1]. The invasion of microorganisms at wound site further adds to the tissue damage and may prolong wound healing by promoting inflammation [2]. Wide variety of microbes ranging from aerobic bacteria, anaerobic bacteria and fungi can infect wound [3]. The usual pathogens on skin and mucosal surfaces are Gram-positive cocci, which are derived from cutaneous commensals. Gram-negative aerobes and anaerobic bacteria which are part of respiratory, gastrointestinal, genitourinary flora are also important aetiological agents of wound infection [4].

There is substantial evidence that biofilms are one of the important causes of wound infection and therefore their management requires an understanding of mechanism of biofilm production by bacteria [2]. By definition, biofilms are matrix-enclosed aggregates of bacteria that are immobilized on surfaces or at interfaces in the ecosystems in which they are known to predominate [5]. They can form on environmental inert surfaces like clay particles, metal surface, air-water interfaces, dead and necrosed tissues. They can also form on living surfaces in the natural environment, such as plants, other microbes and animals. In human body, bacteria are present as biofilms in every niche that they colonize. These include both pathogenic and non-pathogenic flora of skin, oropharynx, nose and intestine [6].

Approximately 65% of all human microbial infections involve biofilms. These include native valve endocarditis, otitis media, chronic bacterial prostatitis, cystic fibrosis, periodontitis and chronic wounds. They are also found in indwelling biomedical devices like prosthetic heart valves, central venous catheters, orthopedic implants, contact lenses and intrauterine devices [7]. Biofilm formation is a multistep process involving initial bacterial adhesion (which is the most critical step), growth and Exopolysaccharide (EPS) production followed by gradual maturation and lastly cell dispersion [8]. The nature of biofilm structure and the physiological attributes of biofilm organisms confer an inherent resistance to antimicrobial agents, whether these antimicrobial agents are antibiotics, antiseptics, disinfectants or germicides. These factors constitute a clinical problem in the form of non-healing chronic wounds, resulting in high mortality as well as economic problem due to prolonged hospital stay [9]. Therefore, determining the species present and their relative contributions to biofilms is of great clinical importance.

Biofilm associated diseases are associated with considerable diagnostic challenges for the clinical microbiological laboratory. These include false negative cultures, low colony count and decreased antimicrobial susceptibility. Various phenotypic and genotypic methods are available for assessing biofilm forming ability of microorganisms, but none of the method is universally applicable because of inherent analytical limitations associated with measurements of bacterial adhesion. Some of these methods include Tissue Culture Plate method (TCP), tube method, congo red agar method, flow cell method, confocal laser scanning microscopy, Calgary biofilm device, ATP bioluminescent assay and molecular methods for identifying genes responsible for EPS synthesis and bacterial adhesion [6,10]. Out of the available methods, TCP is considered the gold standard phenotypic method of biofilm detection. In this method, bacterial adherence is measured spectrophotometrically [11]. As the optical density is measured after complete drying of tissue culture plate, the method

does not measure bacteria adhered on the bottom as well as on the inner walls of the wells. This results in underestimation of slime production.

The present study was carried out to investigate the capacity of various members of family Enterobacteriaceae isolated from wound infections to produce biofilms along with their antibiotic susceptibility pattern. Also, TCP method was modified and a comparison of MTCP method with standard TCP method for detection of biofilm formation was done.

MATERIALS AND METHODS

This prospective study was conducted from February 2015-August 2016 in the Department of Microbiology, Pt. BD Sharma PGIMS, Rohtak, Haryana, India. During the study period, 240 pus samples submitted in the laboratory were processed. The study was approved by the institutional ethical committee.

Sample Collection and Processing

Skin was cleaned by 2% chlorhexidine and 70% alcohol. Pus was either aspirated in syringe or collected on sterile swab and sent to laboratory.

Non-repetitive pus samples obtained from various types of wounds and ulcers were included in the study. Isolates other than the members of Enterobacteriaceae were excluded from the study. Samples were subjected to microscopy and culture. Cultures were performed on blood agar and MacConkey agar plates. The colonies grown on the blood agar and MacConkey agar plates were processed further for the identification of the organisms by colony morphology, Gram staining and biochemical reactions as per standard microbiological protocol [12-14].

AST was done by Kirby-Bauer disc diffusion method in accordance with CLSI guidelines 2016 [15]. The antimicrobial drugs tested were gentamicin (10μg), amikacin (30μg), amoxicillin/clavulanic acid (20μg/10μg), piperacillin/tazobactam (100μg/10μg), ciprofloxacin (5μg), imipenem (10μg), meropenem (10μg), trimethoprim/sulfamethoxazole (1.25μg/23.75μg), ceftazidime (30μg), doxycycline (30μg). An isolate was considered as Multi-Drug Resistant (MDR) if it was resistant to atleast three classes of antimicrobial agents [16].

Biofilm Production was Detected by TCP and MTCP methods.

Tissue Culture Plate (TCP) method: This method was described by Christensen GD et al., [11].

Test organism was inoculated in Brain Heart Infusion (BHI) broth (Hi media laboratories, Mumbai) supplemented with 2% sucrose dispensed in test tubes and incubated overnight at 37°C. This broth was diluted in the ratio of 1:100 with fresh broth. A 200μL of this diluted culture broth was added to 96 well- flat bottom, non-adherent, non-treated polystyrene tissue culture plates (Hi media laboratories, Mumbai). These tissue culture plates were further incubated for 24 hours at 37°C. After incubation, the contents of the wells were removed and wells were washed four times with 0.2 mL of phosphate buffered saline. Adhered biofilms were fixed with 2% sodium acetate for 30 minutes and stained with crystal violet (0.1% w/v) for 30 minutes. Excess stain was rinsed off with distilled water. After drying, Optical Densities (OD) was determined by an automated micro ELISA reader at wavelength of 570nm.

Modified Tissue Culture Plate (MTCP) Method [17]

The standard TCP method was modified slightly by adding one step in the end. After staining with 0.1% crystal violet and drying, 160μL of 33% glacial acetic acid was added into the microwells. After 15 minutes, OD was taken by an automated micro ELISA reader at wavelength of 570nm. These OD values were considered as an index of bacterial adhesion and biofilm formation. The classification of bacterial adherance by TCP and MTCP method is shown in [Table/Fig-1].

Biofilm formation	Adherence	Mean OD value (TCP)	Mean OD value (MTCP)
None/ weak	None	< 0.120	< 2.66
Moderate	Moderate	0.120-0.240	2.66-5.32
Strong	High	>0.240	> 5.32

[Table/Fig-1]: Classification of bacterial adherence by TCP and MTCP method.

STATISTICAL ANALYSIS

Biofilm production by both the methods was graded as weak/none, moderate and strong. High and moderate biofilm production by each method was considered positive and weak/none biofilm production was considered negative. Based on the literature, TCP method was considered as the gold standard method of biofilm detection and parameters like sensitivity, specificity, negative predictive value, positive predictive value and accuracy of MTCP method were calculated. Association of two or more set of variables was analysed using Chi-square test. A p-value <0.05 was considered as statistically significant. SPSS version 20.0 was used for data analysis.

RESULTS

A total of 160 isolates were obtained out of 240 pus samples. Bacteriological profile includes *Klebsiella* spp. (n=51), *Escherichia coli* (n=41), *Citrobacter* spp. (n=32), *Proteus* spp. (n=26), *Enterobacter* spp. (n=10). Out of these, 52.5% isolates showed biofilm production by TCP and 65.6% by MTCP method. The rate of biofilm detection by two methods was found to be statistically significant (p-value =0.004). Quantification of biofilm production has been shown in [Table/Fig-2]. In our study, biofilm production as per standard TCP method was more prevalent in *Klebsiella* spp. (32, 62.7%) followed by *Proteus* spp. (15, 57.7%), *Enterobacter* spp. (5, 50%), *E. coli* (19, 46.3%) and *Citrobacter* spp. (13, 40.6%). The magnitude of biofilm production by individual bacterial spp. is depicted in [Table/Fig-3].

Biofilm formation	TCP		MTCP	
	N	%	N	%
Strong	25	15.6	44	27.5
Moderate	59	36.9	61	38.1
Weak/None	76	47.5	55	34.4
Total	160	100	160	100

[Table/Fig-2]: Grading of biofilm formation by TCP and MTCP method.

Organism	Total number of isolates (n)	Number of biofilm forming isolates by TCP method (%)	Number of biofilm forming isolates by MTCP method (%)
Klebsiella spp.	51	32 (62.7)	38 (74.5)
E. coli	41	19 (46.3)	25 (60.9)
Citrobacter spp.	32	13 (40.6)	19 (59.4)
Proteus spp.	26	15 (57.7)	17 (65.4)
Enterobacter spp.	10	05 (50)	06 (60)
Total	160	84 (52.5)	105 (65.6)

[Table/Fig-3]: Detection of biofilm formation by TCP and MTCP.

Antibiogram of the isolates revealed high resistance to routinely administered antibiotics like ciprofloxacin, co-trimoxazole, gentamicin, ceftazidime and doxycycline while carbepenems were found to be the most effective class of antimicrobials. High resistance by biofilm forming isolates was observed against ceftazidime (73, 87%) followed by doxycycline (70, 83.3%), co-trimoxazole (64, 76.1%), gentamicin (60, 71.5%), amoxyclav (56, 66.7%), ciprofloxacin (54, 64.3%), amikacin (47, 55.9%), piperacillin+tazobactum (36, 42.8%), meropenem (31, 37%) and imipenem (7, 8.3%). The antimicrobial resistance pattern of biofilm

producing and non-biofilm producing isolates is shown in [Table/Fig-4]. MDR was seen in 55.6% (n= 89) isolates. Out of which 79.7% (n=67) biofilm producing isolates and 29% (n=22) of non- biofilm producing isolates were MDR [Table/Fig-5]. Sensitivity of MTCP method was found to be 95.45% while specificity was 78.26%. Positive Predictive Value (PPV) was 84.84%, Negative Predictive Value (NPV) was 93.1% and accuracy was found to be 87.89%.

Antibiotics	Resistance in BF isolates	Resistance in NBF isolates
	%	%
Gentamicin	71.5	34.9
Amikacin	55.9	32.5
Amoxicillin-clavulanate	66.7	22.9
Piperacillin-tazobactam	42.3	12.5
Ciprofloxacin	64.3	31.3
Meropenem	37	2.4
Imipenem	8.3	00
Co-trimoxazole	76.1	42.2
Ceftazidime	87	62.6
Doxycycline	83.3	43.4

[Table/Fig-4]: Antibiotic resistance pattern of biofilm forming (BF) and non-biofilm forming (NBF) isolates.

Organism	Number of BF isolates	BF MDR		Number of NBF isolates	NBF MDR		'p' value
		N	%		N	%	
Klebsiella spp.	32	25	78.1	19	06	31.6	0.001
Escherichia coli	19	14	73.7	22	06	27.2	0.003
Citrobacter spp.	13	11	84.6	19	05	26.3	0.001
Proteus spp.	15	12	80	11	03	27.2	0.007
Enterobacter spp.	05	05	100	05	02	40	0.03
Total	84	67	79.7	76	22	29	0.00

[Table/Fig-5]: Comparision of multidrug resistance among biofilm forming (BF) and non-biofilm forming (NBF) bacterial isolates.

DISCUSSION

The finding of biofilm in the wounds has great relevance in the wound management. Early identification of biofilm producing strains and appropriate antibiotic selection might help to prevent relapse of such infections. Bacteriological profile in the current study showed *Klebsiella* spp. to be most common bacterial isolate followed by *Escherichia coli*. In an Indian study by Subramanian P et al., it was found that the most commonly isolated microorganism included *Klebsiella pneumoniae* (40.2%) followed by *E. coli* (30.9%) [18]. This is similar to the present study.

Other studies conducted by Fatima S *et al.,* and Zubair M et al., showed *Escherichia coli* as the most common bacterial isolate [19,20].

Fatima S et al., found rate of biofilm production to be 33.3% in *E. coli* followed by 22.9% in *Proteus* spp., 12.5% in *Klebsiella* spp., 6.3% in *Citrobacter* spp. and 2.1% in *Enterobacter* spp. [19]. Zubair M et al., observed biofilm production in 59.4% isolates. The prevalence of biofilm production was highest in *Klebsiella* spp. (70%) [20]. Subramanian P et al., found 59.2% bacterial isolates were biofilm producers. The rate of biofilm production was maximum in *K. pneumoniae* (55.9%) [18]. This was similar to present study.

In the study by Subramanian P et al., resistance pattern of biofilm positive isolates showed 62%, 20%, 74%, 60%, 03%, and 03% resistance to gentamicin, amikacin, ceftriaxone, ciprofloxacin, piperacillin-tazobactum and imipenem respectively as compared to 29%, 11.6%, 37.7%, 24.6%, 1.4%, and 2.9% resistance shown by biofilm non-producers for the same antibiotics [18]. Fatima S et al., also compared rate of biofilm production and drug susceptibility pattern of gram negative isolates. They observed that 69% biofilm producing isolates were MDR while only 41.5% non-biofilm producers were MDR [19]. Similarly, Zubair M et al., also found biofilm producing isolates to show high degree of resistance for routinely administered antibiotics [20].

In the current study, two phenotypic methods for detecting biofilm formation were used and their results were compared to find out most appropriate method for demonstrating biofilm formation. Out of the 160 isolates, the TCP method could detect biofilm in 84 isolates (52.5%). The MTCP method detected biofilm in 105 isolates (65.6%). The present study showed MTCP method to be more sensitive than TCP method (p-value < 0.05). Stepanović et al., evaluated 30 clinical isolates of staphylococci for biofilm formation. In their study, TCP method identified 73.3% isolates as biofilm producer while MTCP method detected 83.3% isolates to be biofilm producer. The authors compared the results of both the methods and found the difference to be statistically significant [21]. Another investigator Babapour E et al., studied 156 clinical isolates of *Acinetobacter* spp. On comparison, the rate of biofilm formation was 66.7% and 73.7% by TCP and MTCP methods respectively. The authors concluded MTCP method to be more accurate than TCP method in evaluating biofilm formation [22]. The result of present study is in accordance with the above mentioned studies.

LIMITATION

This study is based only on phenotypic methods of biofilm detection. Molecular methods identifying gene responsible for biofilm production were not included due to lack of facility.

CONCLUSION

High rate of biofilm formation shown by the members of Enterobacteriaceae suggests it to be one of the important mechanisms of anti microbial resistance. MTCP method is better than TCP method for biofilm detection and quantification. This is a simple, reliable accurate method and can be utilized for biofilm screening.

REFERENCES

[1] Cooper R, Okhiria O. Biofilm, wound infection and the issue of control. Wounds UK. 2006;2(3):48-57.
[2] James GA, Swogger E, Wolcott R, Pulcini E, Secor P, Sestrich J. Biofilms in chronic wounds. Wound Repair Regen. 2008;16(1):37-44.
[3] Shaw TJ, Martin P. Wound repair at a glance. J Cell Sci. 2009;122(Pt 18):3209-13.
[4] Singhal H, Kaur K. Wound infection clinical presentation. Medscape Medical News from Web MD. 2015
[5] Brook I, Frazier EH. Aerobic and anaerobic bacteriology of wounds and cutaneous abscesses. Arch Surg. 1990;125(11):1445-51.
[6] Sharma M, Yadav A. Biofilms: microbes and disease. Braz J Infect Dis. 2008;12(6):526-30.
[7] Wolcott R, Costerton JW, Raoult D, Cutler SJ. The polymicrobial nature of biofilm infection. Clin Microbiol Infect. 2013;19(2):107-12.
[8] Wolcott RD, Rhoads DD, Dowd SE. Biofilms and chronic wound inflammation. J Wound Care. 2008;17(8):333-41.
[9] Phillips PL, Wolcott RD, Fletcher J, Schultz GS. Biofilms made easy. Wounds International. 2010;1(3):1-6.
[10] Bose S, Ghosh AK. Biofilms: a challenge to medical science. J Clin Diag Res. 2011;5(1):127-30
[11] Christensen GD, Simpson WA, Younger JA, Baddour LM, Barret FF, Melton DM, et al. Adherence of coagulase negative Staphylococci to plastic tissue cultures: a quantitative model for the adherence of Staphylococci to medical devices. J Clin Microbiol. 1985;22(6):996-1006.
[12] Colle JG, Marr W. Specimen collection, culture containers and media. In: Colle JG, Fraser AG, Marmon BP, Simmons A, editors. Mackie and McCartney Practical Medical Microbiology. New York: Churchill Livingstone. 1996: 95-111.
[13] Colle JG, Miles RB, Watt B. Tests for identification of bacteria. In: Colle JG, Fraser AG, Marmon BP, Simmons A, editors. Mackie and McCartney Practical Medical Microbiology: Churchill Livingstone. 1996:131-49.
[14] Duguid JP. Staining Methods. In: Collee JG, Fraser AG, Marmon BP, Simmons A, editors. Mackie and McCartney Practical Medical Microbiology. Churchill Livingstone. 1996:793-812.
[15] CLSI. Performance standards for antimicrobial disc susceptibility testing. 26th ed. CLSI supplement M100-S. Wayne, PA: Clinical and Laboratory Standards Institute; 2016.
[16] Cdc.gov.in[Internet]. Atlanta (USA): CDC, Inc.; c2006 Management of MDRO's in healthcare settings,2006; [updated 2015 Feb; cited 24 Aug 2015]
[17] Saxena S, Banerjee G, Garg R, Singh M. Comparative study of biofilm formation in *Pseudomonas aeruginosa* isolates from patients of lower respiratory tract infection. J Clin Diagn Res. 2014;8(5):9-11

[18] Subramanian P, Umadevi S, Kumar S, Stephen S. Determination of correlation between biofilm and extended spectrum β lactamases producers of *Enterobacteriaceae*. Scho Res J. 2012;2(1):2-6.
[19] Fatima S, Prasanthi K, Nagamani K. Comparative evaluation of biofilm production in multidrug resistant and sensitive Gram negative clinical isolates. Int J Curr Microbiol App Sci. 2015;4(6):918-26.
[20] Zubair M, Malik A, Ahmad J, Rizvi M, Farooqui KJ, Rizvi MW. A study of biofilm production by gram-negative organisms isolated from diabetic foot ulcer patients. Biol Med. 2011;3(2):147-57.
[21] Stepanovic´ S, Vukovic´ D, Dakic´ I, Savic´ B, S̆vabic´-Vlahovic M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods. 2000;40(2):175–79.
[22] Babapour E, Haddadi A, Mirnejad R, Angaji SA, Amirmozafari N. Biofilm formation in clinical isolates of nosocomial *Acinetobacter baumannii* and its relationship with multidrug resistance. Asian Pac J Trop Biomed. 2016;6(6):528–33.

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