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IP International Journal of Medical Microbiology and Tropical Diseases

Journal homepage: https://www.ijmmtd.org/

Original Research Article

An evaluation of three different biofilm detection methods in orthopedic implant associated infections and its implication in healthcare system

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ARTICLE INFO

Article history:
Received 15-10-2021
Accepted 23-12-2021
Available online 12-02-2022

Keywords:
Antibiogram
Biofilm
Implant
Tissue culture plate method

ABSTRACT

Background: Biofilms are defined as microbially derived sessile communities characterized by the cells that are irreversibly attached to a substratum. These organisms have been associated with implant associated infections**.**

Aims: The present study is therefore undertaken 1. To compare the three screening methods used for detection of biofilm formation (Tissue culture plate method, Tube method and Congo red agar method) and 2. To correlate the antibiotic resistance pattern of biofilm producers and non biofilm producers.

Materials and Methods: A prospective study was carried out in the department of microbiology, on all orthopedic implant associated infections from September 2015 to July 2016 on aspirated pus samples. A total of 120 non repetitive clinical isolates were taken and subjected to biofilm detection. All the bacterial isolates were identified by standard biochemical tests. Antibiotic susceptibility test of bacterial isolates was performed by Kirby Bauer disk diffusion method according to Clinical Laboratory Standard Institute (CLSI) guidelines on Muller Hinton Agar (MHA). A reference strain of Staphylococcus epidermidis ATCC 35984(positive biofilm producer) and Staphylococcus epidermidis ATCC 12228(non biofilm producer) were used as controls.

Results: Tissue culture plate method was considered as gold standard as it detected 25% biofilm producers which included 12% weak biofilm producers which was missed by congo red agar (CRA) and tube method (TM). Also, this correlated to methicillin resistant staphylococcus aureus (MRSA), ESBL, amp-c and MDR pattern of antibiogram.

Conclusion: TCP is considered as most reliable screening method for biofilm detection and should be routinely employed to prevent treatment failures as these organisms are intrinsically resistant to many antimicrobials leading to implant associated infections.

1. Introduction

Biofilms are a group of microbes along with their exopolysaccharide matrix which adhere on biotic and abiotic surfaces conferring antibiotic resistance especially in indwelling medical devices.[1] Infection is a major problem in orthopedic implant associated surgeries due to biofilm formation resulting in treatment failure.[2] Surgical skin incision exposes otherwise harmless bacteria to a change in environment leading to an opportunistic change in behavior. The process is dependent on local factors such as hydrophobicity, acidity, oxygen concentration, presence of inert material and ability of bacterium to initiate contact via pili/ flagella.[3–5] High antimicrobial concentrations are required to inactivate the biofilm organisms as they are as high as 1000 times more resistant than planktonic bacteria.[6]

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https://doi.org/10.18231/j.ijmmtd.2022.014

2. Materials and Methods

A prospective study was carried out in the department of microbiology, on all orthopedic implant associated infections from September 2015 to July 2016 on all aspirated pus samples. A total of 120 non repetitive clinical isolates were taken and subjected to biofilm detection. All the bacterial isolates were identified by standard biochemical tests. Antibiotic susceptibility test of bacterial isolates was performed by Kirby Bauer disk diffusion method according to Clinical Laboratory Standard Institute (CLSI) guidelines on Muller Hinton Agar(MHA). A reference strain of Staphylococcus epidermidis ATCC 35984(positive biofilm producer) and Staphylococcus epidermidis ATCC 12228(non biofilm producer) were used as positive and negative controls respectively. Biofilm detection was done by following methods:

Tube adherence method: Described by Christensen et al,[7] this is a qualitative method for biofilm detection. 10 ml of Trypticase soy broth with 1% glucose is inoculated with a loopful of test organisms, along with positive and negative controls. The broths are incubated at 37^0c for 24 – 48 hours. The culture supernatants are decanted and the tubes are washed with phosphate buffered saline. The tubes are dried and are stained with 0.1% crystal violet. The excess stain is washed away with deionised water. The tubes are dried in inverted position. The scoring for the tubes was done according to the control strains. Biofilm was considered positive when a visible film lined the wall and the bottom of the test tube. The amount of biofilm formed was scored as 1- weak/none, 2-moderate and 3- high/strong.

The Congo red agar (CRA) method – Freeman et al,[8] have described it as a simple qualitative method to detect biofilm production. The Congo red stain is prepared as a concentrated aqueous solution and is autoclaved at 121^0C for 15 minutes. This is added to autoclaved Brain heart infusion agar with sucrose at 55^0C. The plates are inoculated with the test organisms along with positive and negative controls and are incubated at 37^0C for 24 to 48 hours aerobically. Black colonies with a dry crystalline consistency indicate biofilm production.

The tissue culture plate (TCP) method – This is a quantitative test described by Christensen et al.,[7] is considered as gold standard method for biofilm detection.[9] The organisms isolated from fresh agar plates were inoculated in 10 ml of tryptic soy broth with 1% glucose and incubated at 37^0C for 24hrs. The cultures are then diluted 1:100 with fresh medium. Individual wells of the tissue culture plates are inoculated with a bacterial suspension (200μl) of diluted cultures, along with positive and negative controls and these are incubated at 37^0C for 24 to 48 hours. After incubation, contents of each well were removed by gentle tapping. Planktonic cells are removed by washing with phosphate buffered saline, four times. Biofilms are fixed with 2% sodium acetate and are stained with 0.1% crystal violet for 15 min. The excess dye is washed away with deionised water. The plates are dried properly and the optical densities of the stained biofilms are obtained spectrophotometrically using micro ELISA autoreader at wavelength 570nm. The experiment was performed in triplicate and repeated three times. The interpretation of biofilm was done according to the criteria of Stepanovic et al.[9]

2.1. Selection and description of participants

Observational study with.

2.2. Inclusion criteria

All orthopedic surgeries with implants, done in which are associated with implant failure.

2.3. Exclusion criteria

Surgeries done outside

3. Results

Table 1: Various organisms isolated in total positive cultures:

Organisms Isolated	Biofilm Producers	Non Biofilm Producers
Escherichia coli	02	01
Staphylococcus aureus	40	16
CoNS	06	06
Klebsiella species	11	03
Pseudomonas species	05	00
NFGNB	03	02
Citrobacter species	04	01
Providencia species	01	01

Observation: Biofilm producing organisms were 72 isolates (which were positive by any one of the method)Highest number of isolates were of Staphylococcus aureus(56%) of which 40 are biofilm producers, followed by Klebsiella species(14%) of which 11 are biofilm producers.
(CoNS)-Coagulase negative staphylococcus, Non fermenting gram negative bacilli(NFGNB)

Table 2: Screening of the isolates for biofilm formation by Tissue culture plate, Tube method and Congo red agar method

Biofilm Formation	TCP	TM	CRA
High	05	10	10
Moderate	07	27	25
Weak	18	00	00
Total number of positive biofilm forming organisms	30	37	35

Observation: Tissue culture plate method, has shown that 25% of Implant associated infection was due to biofilm producers, amongst which weak producers accounted to 12%.

.

Table 3: Resistance pattern (%) of biofilm producing Gram positive bacteria

Antimicrobial agent (mcg)	Biofilm producing Gram positive organisms(48)	Non biofilm producing Gram positive organisms(22)
Cefoxitin(30)	57%	45.45%
Ampicillin (10)	87%	77%
Clindamycin (2)	40.42%	36%
Erythromycin (15)	63.82%	54%
Teichoplanin(30)	19.14%	4.50%
Linezolid(30)	12.76%	8(36)%
Vancomycin(30)	6.38%	3%(9)
Gentamycin (10)	19.14%	9%
Amikacin(30)	10.63%	05%(31)
Amoxicillin clavulanic acid(30)	63.82%	42%
Ciprofloxacin(30)	32%	31%
Levofloxacin(5)	4.25%	0%
Cefepime (30)	34.04%	31%

Observation: High number of gram positive cocci were noted methicillin resistant in biofilm producers (60%) when compared to non biofilm producing Gram positive cocci. Also high resistance was noted to macrolides.

Biofilm Isolates were sensitive to vancomycin, amikacin and levofloxacin.

Statistical significance calculated using Chi square test with p-value <0.05 was considered as significant test.

The resistance of ampicillin and amoxiclav was statistically significant among biofilm and non biofilm producers

Table 4: Resistance pattern (%) of biofilm producing Gram negative bacteria

Antimicrobial agent(mcg)	Biofilm producing Gram negative organisms(25)	Non biofilm producing Gram negative organisms(9)
Ampicillin(10)	90%	65%
Gentamycin(10)	64%	55%
Ciprofloxacin(30)	48%	22.22%
Levofloxacin(5)	8%	00%
Cefoxitin(30)	96%	00%
Amikacin(30)	32%	22%
Ceftriaxone(30)	92%	77%
Ceftazidime(30)	92%	55%
Cotrimoxazole (25)	96%	55%
Amoxicillin clavulanic acid(30)	92%	65%
Imepenem(10)	0%	0%
Piperacillin tazobactum(100/10)	30%	0%

Observation: Biofilm producing gram negative bacteria show maximum resistance to β-lactams when compared to non biofilm producers. The biofilm isolates were sensitive to levofloxacin, amikacin, imepenem and piperacillin tazobactem. 100% multi drug resistance was noted in all GNB biofilm isolates.

Table 5: Statistical evaluation of TM, CRA methods of biofilm detection in orthopedic implants with TCP as gold standard method

Method	Sensitivity	specificity	Positive predictive vaue	Negative predictive vaue	Accuracy
Tube	60%	45%	27%	62%	40%
Congo red agar	40%	35%	21%	60%	36%

Observation: Above table shows that comparison of sensitivity and specificity for biofilm detection between CRA and TM by Mc nemer test is not significant and TCP method is gold standard showing 30% biofilm producers which is alarming. Hence necessitating the need to intervene.

Fig. 1:

Fig. 2:

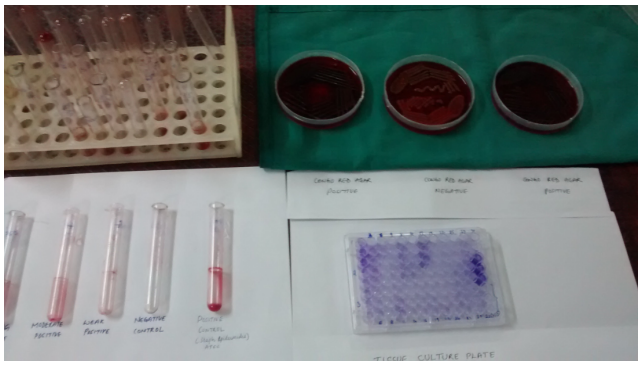

Fig. 3:

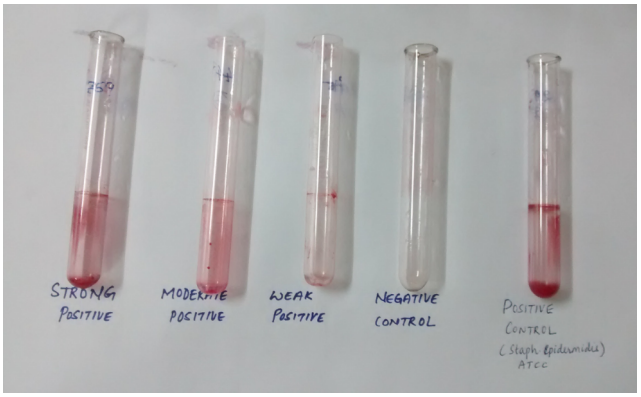

Fig. 4:

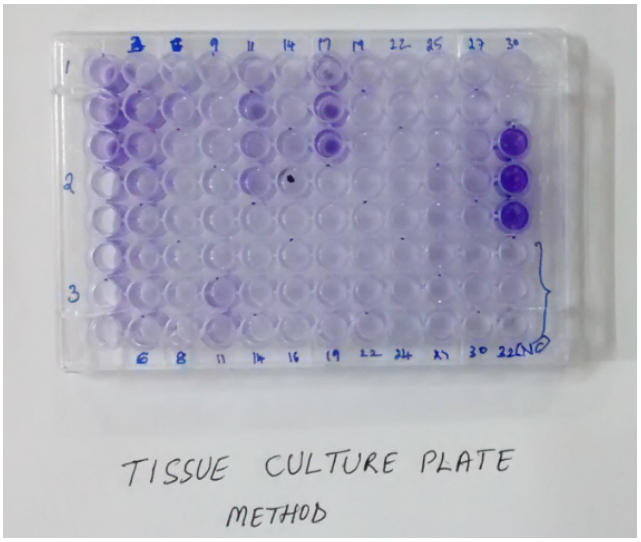

Fig. 5:

4. Discussion

Implant related infections continue to pose a problem for the orthopaedicians.[1] Research has established the properties of an inert material which influences the formation of biofilm.[10]

The use of prosthetic implants in orthopedics provides an ideal environment for biofilm formation as they are highly susceptible to infection. This is due to preoperative and post operative infection, local host immune response or device rejection leading to device failure.[11] The diagnosis and the treatment of these infections are complicated by the formation of a bacterial biofilm and an increase in the number of multidrug resistant bacteria.[12] This stresses the value of an adequate diagnosis, leading to aappropriate therapy of these patients.

However, the organisms which have adhered to the implant are occasionally impossible to detect by the common bacterial cultures. In the present study, aerobic gram positive cocci accounted for 68 in number and aerobic

gram negatives organisms accounted for 34 in number in accordance with Khosravi et al[13] and Anisha F et al[14] which also reported staphylococcus as most frequent isolate

The antimicrobial susceptibility testing revealed high rate of antimicrobial resistance in Staphylococcus aureus isolates to most of the routinely used antibiotics with 60% Methicillin resistant staphylococcus aureus(MRSA). Most of these organisms showed sensitivity to Vancomycin.(biofilm producing organisms) in accordance with Afreenish Hassan et al[15] and Nixon M et al[16] were vancomycin was the most effective antibiotic In gram negative biofilm producing organisms high prevalence of ESBL (20%), 06% AmpC producing Klebsiella species was found(14%) and 44% were AmpC co producers, probably the prolonged hospital stay along with other co morbid conditions in the patient contributes to such high drug resistance apart from biofilm production.[17] Various methods have been described to detect the biofilm production which showed

Tissue culture Plate method values of 30% positive, Tube method values of 37% positive, Congo red agar values of 35% positives. In this study TM, congo red agar method had almost similar detection rates but TCP method showed a relatively lower detection rates. Considering the accuracy of positive control and negative controls in this test we would suggest TCP method as a gold standard method in agreement to previous reports. Also conclude that the tube test due to observer variability cannot be considered as a diagnostic tool for biofilm detection and CRA methodis at par with TM method unlike Ruzicka et al[18] which showed TM is better than CRA.

WE suggest antibiotics aminoglycosides, flouroquinolones, linezolide, vancomycin based on antibiotic susceptibility profile for Gram positive cocci and piperacillin tazobactam, quinolones, aminoglycosides and imipenem for Gram negative bacilli.

5. Conclusion

The TCP method is found to be more reliable in biofilm detection as it could detect even the weakly positive biofilm producers. The results of this study which shows 25% biofilm producing organisms in orthopedic implants, emphasizes the need to account for the local factors while assessing the risk for orthopedic implants infections. The appropriate pre and post operative wound care for dirty wounds, especially when external fixators are used. Also insist appropriate antibiotic policies to eradicate the infections.

An improved understanding of biofilm within the orthopedic community will lead to a more streamlined approach to improve pre and post operative patient care.

As orthopedic implants lay lot of strain on the health services and the economy of the society it necessitates further studies to determine the causative organisms and their susceptibility pattern to treat the patient and also to be cost effective. However, larger studies with bigger sample sizes are required to attain these goals.

Finally to enhance the final results obtained in this study, it would be efficient to carry out other experiments, PCR for detection of icaADBC genes for confirming the pathogenicity.

6. Conflict of Interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

7. Source of Funding

None.

References

1. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev*. 2002;15(2):167–93. doi:10.1128/CMR.15.2.167-193.2002.
2. Ercan B, Kummer KM, Tarquinio KM, Webster TJ. Decreased staphylococcus aureus biofilm growth on anodized nanotubular titanium and the effect of electrical stimulation. *Acta Biomater*. 2011;7(7):3003–12. doi:10.1016/j.actbio.2011.04.002.
3. Ribeiro Met al Infection of Orthopaedic implants with emphasis on bacterial adhesion process and techniques used in studying bacterial material interactions. *Biomatter*. 2012;2(4):176–94. doi:10.4161/biom.22905.
4. Arciola CR, Campoccia D, Speziale P, Montanaro L, Costerton JW. Biofilm formation in Staphylococcus implant infections. A review of molecular mechanisms and implications for biofilm resistant materials. *Biomaterials*. 2012;33(26):5967–82. doi:10.1016/j.biomaterials.2012.05.031.
5. Romano CL. Antibiofilm agents and implant related infections in orthopaedics: where are we. *J Chemother*. 2013;25(2):67–80.
6. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet*. 2001;358(9276):135–8.
7. Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime producing strains of Staphylococci epidermidis to smooth surfaces. *Infect*. 1982;37(1):318–26.
8. Freeman J, Falkinerfr, Keane CT. New method for detecting slime production by coagulase negative staphylococci. *J Clin Pathol*. 1989;42(8):872–4. doi:10.1136/jcp.42.8.872.
9. Stepanovic S, Vukovi D, Hola V. Quantification of biofilm in microtitre plates: overview of testing conditions and practical recommendations for assessment of biofilm production by Staphylococci. *APMIS*. 2007;115(8):891–9.
10. Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of Staphylococci: an evaluation of three different screening methods. *Indian J Med Microbiol*. 2006;24(1):25–9.
11. Zimmerli W. Clinical presentation and treatment of orthopedic implant associated infection. *J intern Med*. 2014;276(2):111–9. doi:10.1111/joim.12233.
12. Banerjee P, Singh M, Sharma V. Biofilm formation: A Comprehensive Review. *Int J Pharm Res Health Sci*. 2015;3(2):550–60.
13. Khosravi AD, Ahmadi F, Salmanzadeh S, Dashtbozorg A, Montazei EA. Study of bacteria isolated from orthoctions and their antimicrobial susceptibility pattern. *Microbial Research J*. 2009;4(4):158–63. doi:10.3923/jm.2009.158.163.
14. Anisha F, Meenad. the microbiological profiles of infected prosthetic implants with emphasis on the organisms which form biofilm. *J Clin Diagn Res*. 2013;7(2):219–23.

15. Hassan A, Usman J, Kaleem F, Omair M, Khalid A, Iqbal M. Detection and antibiotic susceptibility pattern of biofilm producing gram positive and gram negative bacteria isolated from a tertiary care hospital of Pakistan. *Malaysian J Microbiol*. 2011;7(1):57–60.
16. Nixon M, Jackson B, Varghese P, Jenkins D, Taylor G. Methicillin resistant staphylococcus on orthopedic wards. *J Bone Joimt Surgery(br)*. 2006;88(6):812–7. doi:10.1302/0301-620X.88B6.17544.
17. Sinha P, Sharma R, Rishi S, Sood S, Pathak D. prevalence of extended spectrum betalactamase producers among Escherichia coli in a tertiary care hospital in Jaipur. *Indian J Pathol Microbiol*. 2008;51(3):367–9.
18. Ruzicka F, Holá V, Votava M, Tejkalová R, Horvát R, Heroldová M, et al. Biofilm detection and clinical significance of Staphylococcus epidermidis isolates. *Folia Microbiol*. 2004;49(5):596–600. doi:10.1007/BF02931540.

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Cite this article: Mansabdar P, Medegar S, Patil AB. An evaluation of three different biofilm detection methods in orthopedic implant associated infections and its implication in healthcare system. *IP Int J Med Microbiol Trop Dis* 2022;8(1):63-68.