Comparative Evaluation of Methods Used For Detection of Biofilm Production In Multidrug Resistant Bacteria

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Abstract: Background: Multidrug resistant organisms (MDROs) are defined as bacteria resistant to at least one agent in three or more antimicrobial classes. Infections due to these bacteria lead to prolonged antimicrobial therapy and increase morbidity and mortality in the patients admitted in G.B.Pant Hospital, New Delhi. These bacteria tend to attach to various medical devices and form biofilms. Biofilm production of MDR strains has not been evaluated so far in this set up. Objective: The study was conducted to detect and compare biofilm production of MDR bacteria isolated by three different methods of biofilm detection, Congo Red Agar (CRA) method, Tube Method (TM) and Tissue Culture Method (TCP). Methods: A total of 200 bacterial isolates (MDROs) from various clinical samples of patients suffering from hospital acquired infections were subjected to biofilm detection along with positive and negative controls by all three methods (TCP, TM, and CRA). Result: Of the 200 isolates (MDROs), 152 (76%) were biofilm producers i.e. strong 28 (14%), moderate 57(28.5%), weak 67 (33.5%) by TCP Method. By TM 166 (83%) were biofilm producers i.e strong 34 (17%), moderate 86 (43%) and weak 46 (23%). By CRA Method 126 (63%) isolates were biofilm producers and 74 were non-biofilm producers. False positive rate was 15% and 16.5% and false negative rate was 8% and 29.5% respectively by TM and CRA methods, taking in to consideration TCP as a Gold Standard method of biofilm detection.[11] Conclusion: Tube Method has detected the highest number of biofilm producers followed by TCP and CRA. But Tube Method (TM) cannot be considered as most reliable method as the interpretation of the result depends upon the individual's observations. False negativity was high in case of CRA method. So it is concluded that TCP is the most reliable quantitative method of screening of biofilm production of bacterial isolates in Laboratories. [Gyaneshwar T NJIRM 2017; 8(5):1-8]

Key Words: Biofilm, Multidrug resistant bacteria, Congo red agar.

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Introduction: Emergence of bacterial resistance to antimicrobial agents in hospitals and community poses a public health problem[1]. Bacteria resistant to at least one agent in three or more antimicrobial categories are defined as Multi drug resistant organism (MDROs).[2] Infections with MDROs can lead to inadequate or delay in antimicrobial therapy and poor patient outcomes like increased morbidity, mortality and increased health care costs associated with treatment.[3, 4]

Bacteria contaminate medical devices and form biofilms.[5] Numerous studies to date indicate that human infections are, in large part, caused by the ability of bacteria to develop surface attached polymicrobial communities known as biofilms. They are a group of micro-organisms encased in an exopolysaccharide matrix. Biofilms have been considered to be an important virulence factor in device related infections. According to National Institute of Health (NIH) and Center for Disease Control and Prevention (CDC) more than 60% of all infections are caused by biofilm.[6, 7] Both Gram-positive and Gram-negative bacteria such as E. faecalis, S. aureus, S. epidermidis, Streptococcus viridans, E. coli, K. pneumoniae, Proteus mirabilis and P. aeruginosa produce biofilm.[8]

Various methods for detection of bacterial biofilm production are Congo Red Agar Method (CRA),[9] Tube Method (TM),[10] Tissue Culture Plate Method (TCP),[10,11] Bio- luminescent assay,[12] Piezoelectric sensors,[13] and Fluorescent microscopic examination.[14]

A large number of patients are admitted in G.B. Pant Hospital with indwelling catheters and other devices. Hospital acquired infections (HAI) are very common in these patients, that too with MDROs. No study has been made to date in this set up to determine the production of biofilm by these organisms. This study has been planned to determine the biofilm production in bacterial isolates.

Objective of the study: The study was conducted to detect and compare biofilm production of bacteria isolated from different clinical samples by three different methods of biofilm detection.

Method: Various clinical samples (blood, pus, fluids, respiratory samples, tips and urine) received from patients (admitted in ICUs and wards) of G.B. Pant Hospital having hospital acquired infection were processed as per standard microbiological methods over a period of 9 months from April to December 2015. Permission for the study was granted by Institutional Research Board (IRB). Approval for the study was taken from Ethical Review Board of the Institution. Informed consent was also taken from the patients.

Organisms isolated were identified by standard microbiological procedures and Antibiotic susceptibility test of isolated strains was performed by Kirby-Bauer disc diffusion technique as per CLSI guidelines.[15] on Mueller Hinton agar. The following groups of antibiotics: Cephalosporins, Aminoglycosides, Fluoroquinolones, Penicillins, Macrolides, Glycopeptides, Oxazolidones, Oxacillins and Carbapenams were used for drug susceptibility testing. All antibiotic discs were obtained from Hi-Media. The control strains used were Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853 and Staphylococcus aureus ATCC 29213. Identification and drug susceptibility testing of the bacterial isolates was confirmed by the automated Vitek II Compact system.

Isolates showing resistant to at least one agent in three or more antimicrobial categories was considered as MDROs.[2] Accordingly isolates were classified as shown in table (1.) A Total of 200 bacterial isolates (MDRO) were subjected to biofilm detection methods. Reference strains of positive biofilm producers were Staphylococcus epidermidis ATCC 35984, Staphylococcus aureus ATCC 35556, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 35218 and Staphylococcus epidermidis ATCC 12228 (non-slime producer) which were used as controls. Detection of biofilm production was performed by the following methods:

Tissue culture plate method: This quantitative test was done as described by Christensen et al.[10] and is considered as the gold-standard method for biofilm detection.[11] Freshly isolated colonies from Mueller Hinton Agar plates were inoculated in 10 ml of Trypticase Soy Broth (TSB)with 1% glucose and incubated at 37^{o}C for 24 hours. The cultures were then diluted to 1:100 dilutions with fresh TSB medium and 200 µl of the diluted cultures were added to wells of a sterile 96 well flat bottom polystyrene tissue culture plate. The control organisms were also processed in same manner. In wells with Negative control only 200 µl of sterile broth was added. The plate was incubated at 37^{o}C for 24 hours. After incubation, contents of each well were removed by gentle tapping and wells were washed with 0.2 ml of phosphate buffer saline (pH 7.2) four times in order to remove free floating bacteria. Biofilm formed by bacteria adherent to the wells was fixed with 2% sodium acetate and stained with crystal violet (0.1%). Excess stain from wells was removed by washing with deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm obtained was done by using ELISA micro plate reader at wavelength of 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.[16] as mentioned below:

Bacteria producing biofilms or not were classified as: non-biofilm producing, weak, moderate, and strong-biofilm producing, based on the following optical density (OD) average values:

- OD (isolate) ≤ OD (neg. control) = non-biofilm-producing;
- OD (neg. control) ≤ OD (isolate) ≤ 2OD (neg. control) = weak producing;
- 2OD (neg. control) ≤ OD (isolate) ≤ 4OD (neg. control) = moderate-producing;
- 4OD (neg. control) ≤ OD (isolate) = strong-producing.

Tube method: It is a qualitative method for biofilm detection and performed as described by Christensen et al.[10] A loopful of test organisms as well as control strains were inoculated in 10 ml of trypticase soy broth with 1% glucose in test tubes and incubated at 37^{o}C for 24 hours. After incubation, tubes were decanted, washed with phosphate buffer saline, PBS (pH 7.3) and dried. These tubes were stained with 0.1% crystal violet stain. Excess stain was removed by washing with deionized water. Tubes were dried in inverted position. The scoring for tube method was done in accordance to the results of the control strains. Biofilm formation was considered positive when visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-

none, 2-weak, 3-moderate and 4-high/strong. The experiment was performed in triplicate and repeated three times.

Congo red agar method: Freeman et al.[9] have described this simple qualitative method of bacterial biofilm detection. Congo Red Agar (CRA) medium was prepared by adding brain heart infusion broth (37 grams), sucrose (50 grams) and Agar Agar powder (10 grams) in one liter distilled water and mixed well. Congo red stain was prepared as a concentrated aqueous solution by adding 8 grams of Congo red in 10-15 ml distilled water and autoclaved at 121^{o}C for 15 minutes separately from the other medium constituents. It was then added to the autoclaved brain heart infusion agar with sucrose at 55^{o}C.[17] CRA plates were inoculated with test organisms and incubated at 37^{o}C for 24 hours aerobically. Black colonies with a dry crystalline consistency indicated biofilm production.[17] the experiment was performed in triplicate and repeated thrice.

Results: Identification and classification of MDROs: During study period 215 strains associated with hospital acquired infection (HAI) were isolated from various clinical samples. 15 strains which were sensitive to various groups of antibiotics were excluded from the study. 200 isolates designated as multi drug resistant bacteria (MDRO) were included in the study. The pattern of MDRO is shown below (Table 1 &2):

Table 1: Resistance pattern of MDROs (Grams negative bacteria)

Name of the organisms	Number of organisms	Resistant to Cephalosporins, Fluoroquinolones & Aminoglycosides	Resistant to Cephalosporins, Fluoroquinolones & Carbapenams	Resistant to Aminoglycosides,Fluoroquinolones & Carbapenams	Resistant to all groups
Klebsiella pneumoniae	78	15	04	03	56
Esch. coli	50	14	08	08	20
Enterobacter cloacae	01	0	0	0	01
Proteus mirabilis	03	0	01	0	02
Providentia sp	01	0	0	0	01
Pseudomonas aeruginosa	28	11	05	02	10
Acinetobacter baumanii	23	02	03	02	16
Burkholderia cepacia	01	0	0	0	01

Table 2: Resistance patterns of Staphylococcus aureus (Methicillin Resistant) - MRSA

Name of organism	Number of Organis-ms	Resistant to Methicillin, Cephalosporines &Fluoroquinolones	Resistant to Methicillins, Fluoroquinolones & Marcolides	Resistant to Methicillin, Cephalosporines & Marcolides	Resistant to Methicillin, Fluoroquinolones & Oxazolidones	Resistant to Methicillins, Marcolides & Oxazolidones
Staph. aureus	15	5	4	4	1	1

Distribution of bacterial isolates from various clinical samples is as follows: 73 isolates were obtained from respiratory tract (sputum, bronchial wash, tracheal secretion), 47 isolates from various fluids (bile, drain fluid and CSF), 34 isolates from pus samples, 28 isolates from blood, 13 were from devices (implants, tips, stents) and 5 from urine.

Result of biofilm production by various methods: Please find it in below table**.** Of the 200 isolates (MDROs), 28(14%) isolates were strong biofilm producers, 57(28.5%) isolates were moderate biofilm producers and 67 (33.5%) isolates were weak biofilm producers as detected by Tissue Culture Plate Method (TCP), the gold standard method of biofilm detection[11]. By Tube method (TM), the number of strong biofilm producers were 34 (17%), moderate 86 (43%) and weak biofilm producers 46(23%). One hundred and twenty six (63%) isolates showed black colonies with crystalline appearance by the Congo Red Agar Method, indicating biofilm production (Table-3).

Table 3: Comparative results by three methods of biofilm detection:-

Name of isolates	No. of isolates	Result by TCP method					Result by Tube method					Result by CRA method	
		S	M	W	Total	N	S	M	W	Total	N	P	N
Staphylococcus aureus	15	1	8	5	14	1	6	8	0	14	1	15	0
Klebsiella pneumoniae	78	9	26	29	64	14	11	37	19	67	11	62	16
Esch coli	50	3	5	14	22	28	8	14	13	35	15	36	14
Proteus mirabilis	3	0	1	2	3	0	0	2	1	3	0	1	2
Provedencia sp.	1	0	0	1	1	0	1	0	0	1	0	0	1
Enterobacter cloacae	1	1	0	0	1	0	1	0	0	1	0	1	0
Pseudomonas aeruginosa	28	3	8	12	23	5	4	11	9	24	4	9	19
Acinetobacter baumanii	23	11	8	4	23	0	3	13	4	20	3	1	22
Burkholderia cepacia	1	0	1	0	1	0	0	1	0	1	0	1	0
Total	200	28	57	67	152	48	34	86	46	166	34	126	74
%		14	28.5	33.5	76	24	17	43	23	83	17	63	37

Abbreviations used for: S= Strong, M= Moderate, W= Weak, P= Positive, N= Negative

Table 4: Biofilm producing bacterial strains by different methods:

Name of isolates	No. of isolates	Biofilm producer isolates by TCP method N (%)	Biofilm producer isolates by Tube method N (%)	Biofilm producer isolates by CRA method N (%)
Staphylococcus aureus	15	14(93.3)	14(93.3)	15 (100)
Klebsiella pneumoniae	78	64(82.1)	67(85.9)	62(79.5)
Esch coli	50	22(44)	35(70)	36(72)
Proteus mirabilis	03	03(100)	03(100)	01(33.3)
Provedencia sp.	01	01(100)	01(100)	0
Enterobacter cloacae	01	01(100)	01(100)	01(100)
Pseudomonas aeruginosa	28	23(82.1)	24(85.7)	09(32.1)
Acinetobacter baumanii	23	23(100)	20(86.9)	01(4.3)
Burkholderia cepacia	01	01(100)	01(100)	01(100)
Total	200	152 (76)	166(83)	126(63)

The majority of the organisms associated with biofilm production by TCP method were Acinetobacter baumanni (100%) followed by Staphylococcus aureus (93.3%), Pseudomonas aeruginosa (82.1%), Klebsiella pneumoniae (82.1%) and Esch. coli (44%). The biofilm producers as detected by Tube method were Staphylococcus aureus (93.3%), followed by Acinetobacter baumanni (86.9%), Pseudomonas

aeruginosa (85.7%), Klebsiella pneumoniae (85.9%) and Esch. coli (70%). Biofilm producing strains detected by CRA method were Staphylococcus aureus (100%) followed by Klebsiella pneumoniae (79.5%), Esch. coli (72%), Pseudomonas aeruginosa (32.1%) and Acinetobacter baumanni (4.3%) (Table-4). Biofilm production by different methods from various clinical samples is shown in table: 5.

Table 5: Biofilm producing bacterial strain by different methods Vs clinical samples

Method	Respiratory (n=73)	Fluids (n=47)	Pus (n=34)	Blood (n=28)	Tips (n=13)	Urine (n=5)
TCP	56(76.7%)	30(63.8%)	29(85.3%)	24(85.7%)	10(76.9%)	3(60%)
TM	62(84.9%)	29(61.7%)	33(97.1%)	27(96.4%)	10(76.9%)	5(100%)
CRA	43(58.9%)	32(68.1%)	21(61.8%)	21(75%)	7(53.8%)	2(40%)

Abbreviation 'n' used for total number.

Table 6: Comparative analysis of the Positive Vs Negative results of three methods of detection of biofilm formation:

Test	TCM n (%)	CRA n(%)	TM n (%)
Positive	152(76)	126(63)	166(83)
Negative	48(24)	74(37)	34(17)
Total	200 (100)	200 (100)	200 (100)

Table 7: Comparison between Tissue Culture Method (gold standard) and CRA

		Tissue culture method		Total
		Positive	Negative	
CRA	Positive	93	33	126
	Negative	59	15	74
Total		152	48	200

Table 8: Comparison between Tissue Culture Method (gold standard) and Tube method

		Tissue culture method		Total
		Positive	Negative	
Tube method	Positive	136	30	166
	Negative	16	18	34
Total		152	48	200

Table 9: Diagnostic parameters of CRA and TM for Biofilm detection

Test	Sensitivity %	Specificity %	PPV %	NPV %	Accuracy %
CRA	61.18	31.25	73.8	20.27	54
Tube Method	89.47	37.5	81.92	52.94	77

Statistical analysis of Tissue Culture Plate, Tube and Congo Red Agar methods: In this study data of TM and CRA method was compared with TCP method (the gold standard method) [11] of detection of bacterial biofilm. Sensitivity, specificity, negative predictive value, positive predictive value and accuracy like parameters were also calculated. Sensitivity and specificity of TM was 89.47% and 37.5%, respectively whereas for CRA method, sensitivity and specificity were 61.18% and 31.25%, respectively (Table 9). Bacterial strains showing positive result by all three methods (TCP, TM, and CRA) are considered as True positives or true biofilm producers. Isolates showing positive results by TM and CRA methods and negative results by TCP method are false positive or false biofilm producers. Bacterial strains, which were non-biofilm producers by TM and CRA but producing biofilm by TCP method, were considered as false negative biofilm producers. Those isolates which were non biofilm producers by all the three methods are considered as True negatives. By TM 136 isolates were found to be true positive, 30 isolates were false positive, 18 were true negative and 16 were false negative (Table 8). Whereas by CRA method 93 isolates were found to be true positive, 33 were false positive and 15 were true negative while 59 were false negative (Table 7).

Discussion: Biofilm producing bacteria are responsible for many chronic infections and are very 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.[18] There are various methods of biofilm detection.[9-14] In this study 200 MDROs were evaluated by three screening methods for their ability to form biofilms. It was found that the majority of biofilm producing bacteria were isolated from blood (85.7%) followed by pus (85.3%), tips (76.9%), respiratory sample (76.7%), fluids (63.8%) and urine (60%). By TCP method, the number of isolates showing biofilm production was 152(76%), whereas non biofilm producers were 48(24%). Regional data from India also showed that out of the 152 isolates tested, the number of bio- film producers identified by TCP method was 53.9 %, and non-biofilm

producers were 46%.[11] The majority of the organisms associated with biofilm production in this study were Acinetobacter baumanni (100%) followed by Staphylococcus aureus (93.3%), Pseudomonas aeruginosa (82.1%), Klebsiella pneumoniae (82.1%), and Esch. coli (44%). Study of Sabina Fatima et al. showed biofilm production among the Gram negative isolates was 66.6% for Acinetobacter spp, 58.3% for Pseudomonas spp, 50% for Citrobacter spp, followed by 45 % for Klebsiella spp, 40 % Enterobacter spp, 38.4% Proteus spp. and 29.4% Esch.coli.[19] According to a study conducted by Jeetendra Gurung et al out of 109 isolates 42.2% showed biofilm production which included 33% Pseudomonas aeruginosa and 50% Acinetobacter baumanii.[20] Biofilm production in Acinetobacter baumanii and Pseudomonas aeruginosa promote increased colonization and persistence leading to higher rate of device related infections.[21,22] Afreenish Hassan et al. reported that out of the 110 isolates 22.7% were strong, 41% were moderate and 36.3% were weak or non- biofilm producers as detected by TCP method.[23] S. Nagaveni et al. in their study found with isolates of Pseudomonas aeruginosa -41.6% strong, 33.3% moderate and 25% weak biofilm producers.[24]

83% isolates were detected as biofilm producers and 17% as non-biofilm producers in this study by Tube Method. Similar studies by Rao RS et al, Rodriguez – Bano J et al and Marti S et al on biofilm detection by TM showed 62-63% biofilm producers among isolates of Acinetobacter baumanii.[25,26,27] Another study by S. Nagaveni et al. showed similar findings with 50% strong, 25% moderate and 25% weak biofilm producers amongst the isolates of Pseudomonas aeruginosa.[24] Afreenish et al found 49% biofilm producers and 51% non- biofilm producers by Tube method.[23] In a study by Ruzicka et al.[28] 53.7% of Staphylococcus epidermidis were found to be biofilm producers by TM. Baqai et al in their study found 75% of isolates showed biofilm production by TM among uropathogens.[29] Tube method correlated well with TCP for identifying biofilm producers. This method is 89.47% sensitive, 37.5% specific and 77% accurate for biofilm detection. By this method, 30 isolates were found to be false positive and 16 were false negative. In accordance with the preceding studies by Mathur T et al.[11] and Christensen et al.[30] TM cannot be considered as general screening test to identify biofilm producing isolates. In another study, Ruzicka et al. noted that out of 147 isolates of S. epidermis, TM detected biofilm formation in 79 (53.7%) isolates. They showed that TM is better for biofilm detection than CRA.[28] Baqai et al. tested TM to detect biofilm formation among uropathogens. According to their results, 75% of the isolates exhibited biofilm formation.[29] Though Tube method is easy and simple to perform but reading of the results may be difficult. The interpretation of results is often difficult for the observers particularly in case of weak reactions. However as adherence alone may not complete the cycle of process of biofilm formation, there might be many other mechanisms that could explain adherence.

Congo Red Agar method detected 126(63%) biofilm producing isolates and 37% non- biofilm producers in the study. Similar results were reported by Alicia Veleria Zaranza in their study, which showed biofilm production by 52% strains.[31] Ruzicka et al.[28] noted that out of 147 isolates of Staphylococcus epidermidis, CRA detected biofilm formation in 64 (43.5%) isolates. Afreenish Hassaan et al.[23] found only 11% strains as biofilm producers. Knobloch et al.[32] did not recommend the CRA method for biofilm detection in their study as out of 128 isolates of Staphylococcus aureus, CRA could detect only 3.8% biofilm producing bacteria. S. Nagaveni et al. findings by CRA method was as that 75% were weak/non biofilm producers while 16.6% were moderate producers of biofilm among 12 strains of Pseudomonas aeruginosa.[24] The CRA method could correlate well with the other methods with parameters of sensitivity (61.18%), specificity (31.25%) and accuracy (54%). By this method 33 isolates were false positive while 59 were false negative.

Conclusion: It can be concluded from the present study that TCP is a quantitative and reliable method to detect biofilm forming micro-organisms, when compared to TM and CRA methods. As false positive rate was 15% and 16.5% and false negative rate was 8% and 29.5%respectively by TM and CRA methods.

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References:

1. Moellering RC Jr, Graybill JR, McGowan JE Jr, Corey L. Antimicrobial resistance prevention

initiative-an update: proceedings of an expert panel on resistance. Am J Med. 2007;120 (7):S4-25.
2. Magiorakos A.P, Srinivasan A, Carey R.B, Carmeli Y, Falagas M.E, Giske C.G. et al. Multidrug resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance, Clinical Microbiol Infection 2012;18: 268-281.
3. Bergogne-Berezin E, Decre D, Joly-Guillou ML. Opportunistic nosocomial multiply resistant bacterial infections- their treatment and prevention. J Antimicrob Chemother. 1993;32(Suppl A): 39-47.
4. McGrath EJ, Asmar BI. Nosocomial infections and multidrug-resistant bacterial organisms in the pediatric intensive care unit. Indian J Pediatr. 2011;78: 176-184.
5. Revdiwala S, Rajdev BM, Mulla S. Characterization of bacterial etiologic agents of biofilm formation in medical devices in critical care set up. Crit. Care Res. Pract. 2012; 945-805.
6. Arciola C. R, Baldassarri L, Montanaro L. Presence of icaA and icaD genes and slime production in a collection of Staphylococcal strains from catheter associated infections. J. Clin. Microbiol.2001; 39: 2151–2156.
7. Research on microbial biofilms (PA-03-047). NIH, National Heart, Lung and Blood Institute. 2002; p12-20.
8. Donlan RM. Biofilms and device-associated infections. Emerg. Infect. Dis. 2001; 7(2): 277-818.
9. Freeman J, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative Staphylococci. J. Clin. Pathol.1989; 42:872-4.
10. Christensen GD, Simpson WA, Younger JJ 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; 22:996-1006.
11. 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.
12. Donlan RM, Murga R, Bell M et al. Protocol for detection of biofilms on needleless connectors attached to central venous catheters. J Clin Microbiol.2001; 39:750-3.
13. Aparna MS, Yadav S. Biofilms: microbes and disease. Braz. J Infect Dis. 2008; 12(6):526-30.
14. Zufferey J, Rime B, Francioli P, Bille J. Simple method for rapid diagnosis of catheter associated infection by direct Acridine orange staining of catheter tips. J Clin Microbiol.1988; 26:175-7.
15. Bauer AW, Kirby M M, Sherris JC, Jurek M. Antibiotic susceptibility testing by a standardized single method. Am J Clin Pathol.1966; 45:493-6.
16. Stepanovic S, Vukovi D, Hola V et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by Staphylococci. APMIS. 2007; 115:891-9.
17. Reid G. Biofilms in infectious disease and on medical devices. Int. J. Antimic. Ag. 1999; 11:223-6.
18. Kim L. Riddle of biofilm resistance. Antimic Ag chemother. 2001; 45(4):999-1007.
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): 918-926.
20. Gurung J, Khyriem A.B, Banik A, et al. Association of biofilm production with multidrug resistance among clinical isolates of Acinetobacter baumannii and Pseudomonas aeruginosa from intensive care unit. Indian J Crit Care Med. 2013 Jul-Aug; 17(4): 214–218.
21. Dijkshoorn L, Nemec A, Seifert H. An increasing threat in hospitals: Multidrug resistant Acinetobacter baumannii. Nat Rev Microbiol. 2007; 5: 939-51.
22. Navon-Venezia S, Ben-Ami R, Carmeli Y. Update on Pseudomonas aeruginosa and Acinetobacter baumannii infections in the health care setting. Curr Opin Infect Dis. 2005; 18:306-13.
23. Hassan A, Usman J, Kaleem F, et al. Evaluation of different detection methods of biofilm formation in the clinical isolates. Braz J Infect Dis. 2011;15(4):305-311.
24. Nagaveni S, Rajeshwari H, Oli A. K, et al. Evaluation of biofilm forming ability of the multi-drug resistant Pseudomonas aeruginosa. The Bioscan, 2010;5(4):563-566.
25. Rao RS, Karthika RU, Singh SP, Shashikala P, Kanungo R, Jaychandran S, et al, Correlation

between biofilm production and multi drug resistance in Imipenem resistant clinical isolates of Acinetobacter baumanii. Indian J Med Microbiol.2008; 26:333-7.
26. Rodriguez-Bano J, Marti S, Soto S, Fernandez-Cuenca F, Cisneros JM, Pachon J, et al. Spanish group for the study of Nosocomial Infections (GEIH). Biofilm formation in Acinetobacter baumanii, associated features and clinical implications. Clin Microbiol Infect. 2008; 14:276-8.
27. Marti S, Rodriguez-Bano J, Catel-Ferreira M, Jouenne T, Vila J, Seifert H, et al. Biofilm formation at the solid- liquid and air-liquid interfaces by Acinetobacter species. BMC Res Notes. 2011; 4:5.
28. Ruzika F, Hola V, Votava M, et al.Biofilm detection and clinical significance of Staphylococcus epidermidis isolates. Folia Microbiol (Prabha).2004;49(5):596-600.
29. Baqai R, Aziz M, Rasool G. Urinary tract infection in diabetic patients and biofilm formation of uropathogens. Infect Dis J Pakistan. 2008; 17(1):7-9.
30. Christensen GD, Simpson WA, Bisno AL, Bachey EH, Adherence of slime producing strains of Staphylococcus epidermidis to smooth surfaces. Infect Immun 1982; 37:318-26.
31. Zaranza A.V, Morais F.C, Carmo M.S, et al. Antimicrobial Susceptibility, Biofilm production and Adhesion to HEp-Cells of Pseudomonas aerigunosa strains isolated from Clinical Samples. Journal of Biomaterials and Nanobiotechnology, 2013; 4: 98-106.
32. Knobloch JK, Horsetkotte MA, Rohde H, Mack D. Evaluation of different detection methods of biolfilm formation in Staphylococcus aureus. Med Microbial Immunol 2002; 191(2):101-6.

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