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ORIGINAL ARTICLE

Assessment of diverse biofilm discovery techniques in the Dental experimental separates

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ABSTRACT
Bio-films are a group of micro-organisms, surface associated, covered by an exo-polysaccharide matrix contribute too many infections e.g. dental plague, cariesetc. Refractory to antibiotic therapy. The study was conducted to analyze and evaluate biofilm detection assay (TCP, TM and CRA) in dental samples to choose a sensitive method with good reproducibility that can be employed in routine microbiology laboratory. A total of 100dental specimens were analyzed. And isolates were identified by Gram Staining and standard biochemical tests. Biofilm formation was detected by Congo Red Agar Method (CRA), Tube Method (TM) and Tissue Culture Plate Method (TCPM). Out of 100,A total of 91(91%) isolated dental samples showed positive biofilm formation by TCPM which was considered the gold standard for biofilm detection. When compared with the TCPM, TM truly identified 60(60%) biofilm producers and 40(40%) non-biofilm producers Very different results were observed by the CRA method, with which only (18) isolates showed black colonies with crystalline appearance. In TCP Highest 100 % SBP pattern was observed for S. pneumonia &E. bacterium sp. and lowest 30 % for S. aureusin gram positive while in gram negative highest 83.33 % SBP pattern was detected by P.aeruginosa and lowest 50 % by N. gonorrhoaea and Y. pestis. In TM method among gram positive strains 100 %SBP was observed for S. pyogen and lowest 30 % for S. aereus, in gram negative highest 91.66 % SBP pattern was observed by P.aeruginosa and lowest 58.33 % byE.aerogen.In CRA method gram positive bacteria SBP bacteria are M. luteus 66.66 %, Corynebacterium sp.50 %, S. aureus40 %, and S. epidermidis25 %. In case of 48 strains, 4 (8.33 %) SBP, 0 (0.00 %) MBP and 44 (91.66 %) were NBP, in which only one strain E. aerogen showed SBP pattern 33.33 %. Furthermore, most of the gram negative bacterial strains showed 100 % NBP pattern following the CRA method. Detection of biofilms can be recommended for persistent and unmanageable infections before administration of empirical antibiotics. It is concluded that TCPM is a method with good reproducibility and specificity which can be used for detection of biofilms in resource limited settings.
Keywords: *TCP, TM, CRA, Biofilm detection Assay*

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INTRODUCTION

A biofilm is extracellular polymeric substances (EPS) and have complex social structure that composed of reproducing and communicating microorganism for example bacteria that actually live as a colony, or you may define Bio-films are defined as microbial derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other and show an altered phenotype with respect to growth rate and gene transcription [1-2]. Biofilm confers a number of advantages such as protection from

antimicrobial agents, exchange of genetic material and exchange of nutrients [3-4].Within a biofilm, bacteria communicate with each other by quorum sensing in which they produce of chemo-tactic particles or pheromones, Biofilm production is affected by the number of key factors like availability of key nutrients, chemo-taxis towards surface, motility of bacteria, surface adhesins and presence of surfactants [5].

According to a publication by the National Institutes of Health, more than 80% of all infectious pathogen involve in bio-films formation like pathogen involve inupper respiratory tract infections (uRTI), dental plaque, urogenital and peritonitis infections. Both Gram-positive and Gram-negative bacteria have the ability to produce bio-films. Bacteria commonly involved include *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans, Escherichia coli, K. pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa*. There are a number of methods to detect biofilm including the Transmission electron microscope (TEM), Scanning electron microscope (SEM), Tissue Culture Plate (TCP), Tube method (TM), Congo Red Agar method (CRA), bioluminescent assay, piezoelectric sensors, and fluorescent microscopic examination [6-9].

In current study we screened isolated organisms for biofilm production by three different simple methods, (TCP, TM and CRA) and to evaluate, which could be used in a routine clinical laboratory.

MATERIAL AND METHODS

Place and duration of the study:

Sampling was collected from the POF Hospital Wahcantt Taxila Punjab Pakistan and the experimental procedure was conducted at the laboratory of Biosciences Department, University of Wah**.**

Sample collection, Isolation and identification

100 Dental samples were collected randomly (healthy and non-healthy)from dental Department of the hospital in the sterile eppendrof containing 1ml of nutrient broth with the help of sterilized cotton swabs and then transferred to laboratory of Biosciences Department, University of Wah, where they were incubated overnight at 37˚C in the shaking incubator. Collected dental samples were initially examined by standard microbiological techniques. Samples were inoculated on CLED agar plates that were then incubated at 37°C for 24-48 hours. The oral pathogens were identified by colonial morphology, Gram staining and biochemical Test such as Mannitol Salt Agar, Starch Test, Simon Citrate, Oxidase, Catalase, MacConkey, VogesProskauer, Urease, indole, Methyl Red and Coagulase Test etc. (General Microbiology Laboratory Manual by Rachel Watson)

Detection of Biofilm Production

Biofilm production by isolated dental pathogen were process for the biofilm by three phenotypic methods which included TCPM, TM and CRA. Reference strains of. Biofilm production was graded into strong, moderate and non/weak. Strong and moderate results were interpreted as positive biofilm production, while, non/weak results were interpreted as negative biofilm production.

Tissue Culture Plate Method

Organisms isolated from fresh agar plates were inoculated in 10 mL of trypticase soy broth with 1% glucose. Broths along with isolated organism were incubated at 37 ^{o}C for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well-flat bottom polystyrene tissue culture treated plates were filled with 100 cultures. After that the control organisms were also incubated, *Staphylococcus epidermidis* ATCC 12228 as negative control and *Staphylococcus epidermidis* ATCC 31484 as positive control strains, diluted and added to tissue culture plate. After incubation, at 37 ^{o}C for 24 h, a content of each well was removed by gentle tapping. The wells were washed four times with 0.2 mL of phosphate buffer saline (pH 7.2). The free floating bacteria were removed by this step. Biofilm formed by bacteria adherent to the well were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using de-ionized water and plates were kept for drying. Optical densities (OD) of stained adherent biofilm were obtained by using micro ELISA auto reader (model 680,) at wavelength 570 nm. The experiment was performed two times for accuracy. From the calculated OD values biofilm production was graded into strong, moderate and non/weak (Table 1) as described in previous studies [7-11].

Table 1: Biofilm Grading.

Optical densities values	Adherence	Biofilm formation
< 0.120	Non	Non/weak
0.120- 0.240	Moderate	Moderate
> 0.240	Strong	Strong

Tube Method

A qualitative method for biofilm detection in which a loop-full of test organisms were inoculated in 10mL of trypticase soy broth with 1% glucose in test tubes. After incubation of the tubes at 37 °C for 24 h, were decanted and washed with phosphate buffer saline (pH 7.3) then dried in air and were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored 1 for weak/none, while 2 for moderate and 3 for high/strong visible biofilm. The experiment was repeatedly performed two times [7].

Congo red Method

CRA media was prepared with brain heart infusion broth (Oxoid, UK) 37 g/L, sucrose 50 g/L, agar No. 1 10 g/L and Congo Red indicator 8 g/L. CRA plates were inoculated with test organisms and incubated at 37 °C for 24 h aerobically. Biofilm production was observed by appearance of black crystalline colonies. The experiment was performed two times.

RESULT

After Gram staining and biochemical test the identified bacteria were from both of the groups gram positive and gram negative in which 48 % were gram negative and 52% were gram positive(table 2 shows all the identified bacterial strains).

Table.2. TCP for gram positive and negative bacterial strains

Gram positive Bacterial isolates	Frequency (%)	Gram Negative Bacterial isolates	Frequency (%)
Streptococcus pneumonia	4(7.69)	*Acinetobacterradioresistens*	6(12.5)
Streptococcus pyogens	1(1.92)	*Klebsiella pneumonia*	5(10.41)
Corynebacterium	12(23.07)	*Escherschia coli*	1(2.08)
Actinomyces	10(19.23)	*Y. pestis* 4	12(25)
Micrococcus luteus	3(5.76)	*Neisseria spp*	8(16.66)
Clostridium difficile	6(11.53)	Veillonella	12(25)
S. sobrinus	2(3.84)	Haemophilus	4(8.33)
Corynebacteriumspp	2(3.84)	Total	48
Exiguobacteriumspp	3(5.76)		
Lactobacillus	5(9.61)		
Bacillus cereus	4(7.69)		
Total	52		

Out of total 52 gram positive strains, strong biofilm producers (SBP) 31 (59.61 %), moderate (MBP) 15 (28.84 %) and non- biofilm producers (NBP) 6 (11.53 %) were obtained. Highest 100 % SBP pattern was observed for *S. pneumonia* &*E. bacterium sp.* and lowest 30 % for *S. aureus*. While *S. pyogen* had highest 100 % and *C. difficile* with lowest 16.66 % MBP pattern. Furthermore, *Corynebacterium sp.* were recorded for 50 % and *S. epidermidis* for 8.33 % NBP patterns.

In 48 gram negative strains, 32 (66.66 %) SBP, 13 (27.08 %) MBP and 3 (6.25 %) were NBP. Out of total bacteria, highest 83.33 % SBP pattern was detected by *P.aeruginosa* and lowest 50 % by *N. gonorrhoaea* and *Y. pestis.* While 100 % MBP pattern was demonstrated by *E.coli* and lowest 8.33 % by *P.aeruginosa.* Only 3 strains showed NBP pattern including *Y. pestis, K. pneumonia* & *P.aeruginosa* 25 %, 20 % and 8.33 %, respectively.

TM for gram positive and negative bacterial strains

Table.2. revealed that total 52 strains, 10 (19.23 %), 16 (30.76 %), and 26 (50 %) were strong, moderate and non- biofilm producers, respectively. Out of total gram positive bacteria, 100 %SBPwas observed for *S. pyogen* and lowest 30 % for *S. aereus*. While 100% MBP was detected for *Coryne bacteriumsp* and lowest 16.66 % for *C. difficile.* Furthermore *S. pneumoniae, E. bacteriumsp.*Showed100% and *S. epidermidis* exhibited the lowest 16.66 % NBP pattern.

Out of total 48 gram negative strains, 25 (52.08 %) strong, 9 (18.75 %) moderate and 14 (29.16 %) were NBP were found. Highest 91.66 % SBP pattern was observed by*P.aeruginosa* and lowest 58.33 % by*E.aerogen.* While 100 % MBP pattern was demonstrated by *E.coli* andlowest 16.66 % by *A. radioresistens.* Furthermore highest NBP pattern was showed for *A. radioresistens* 83.33 % and lowest 8.33 % for *P.aeruginosa.*

CRA for gram positive and negative bacterial strains

In this method 100 % non- biofilm formation pattern was exhibited by most of the bacterial strains.

Figure.1. shows that out of total 52 gram positive strains following the CRA method, strong, moderate and non- biofilm producing are 10 (19.23 %),4(7.69%) and 38(73.07 %) respectively in which SBP bacteria are*M. luteus*66.66 %, *Corynebacterium sp.*50 %, *S. aureus*40 %, and *S. epidermidis*25 %. In addition only 3 strains showed the MBP pattern including *L. monocytogen*100%, *Corynebacterium sp.*50 %, *W. confusa*20 % and 100% NBP pattern was observed by *S. pneumoniae, S. pyogen, C. difficile, E. bacteriumsp, B. cereus* and lowest by *M. luteus*33.33 %.

In case of 48 strains, 4 (8.33 %) SBP, 0 (0.00 %) MBP and 44 (91.66 %) were NBP, in which only one strain *E. aerogen* showed SBP pattern 33.33 %. Furthermore, most of the gram negative bacterial strains showed 100 % NBP pattern following the CRA method.

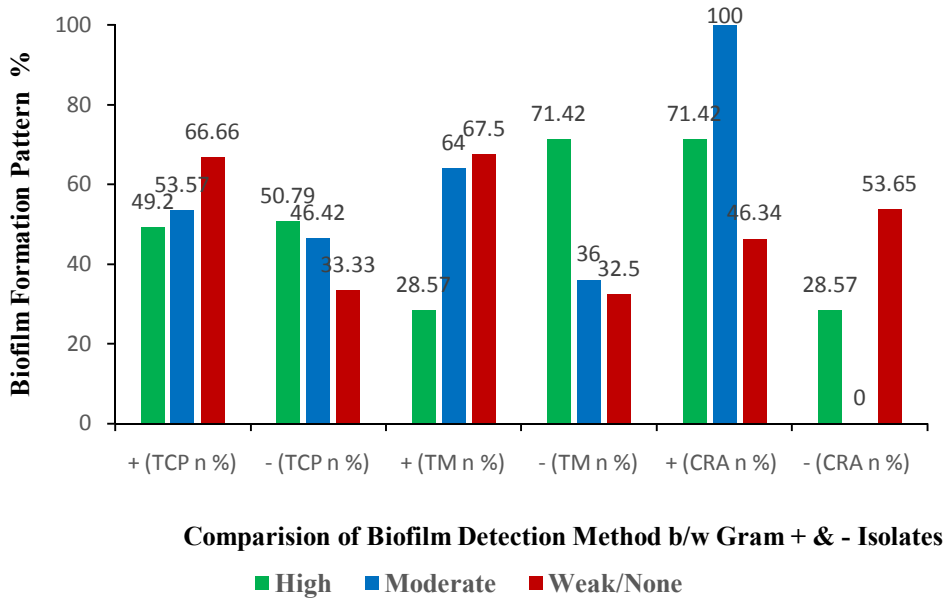

Figure.1. Overall comparison of gram positive and negative bacterial strains (n=100) for biofilm production pattern

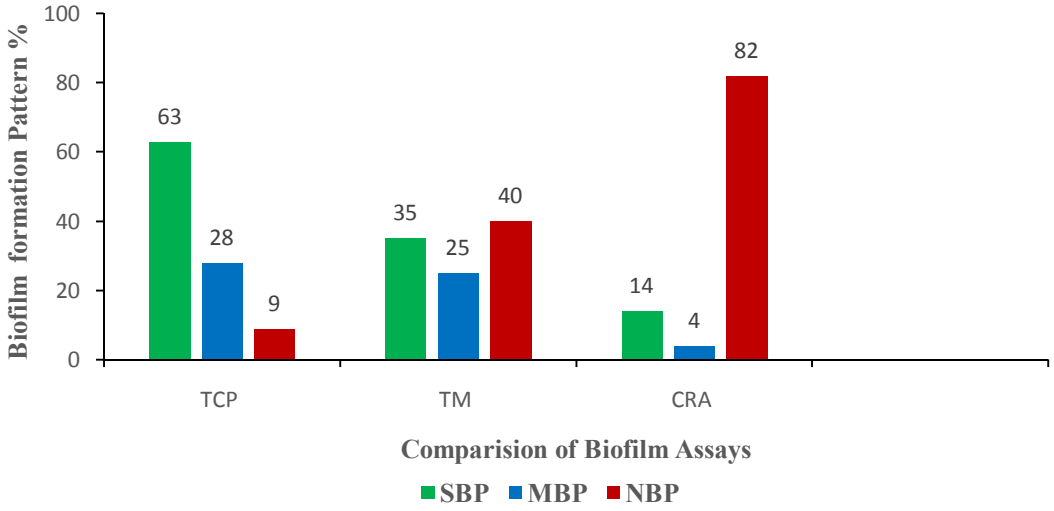

Figure.2.Comparision of Biofilm Assays.

In addition, comparison of different assays revealed that TCP detected, 63% SBP, 28 % MBP and 9 % were NBP. Percentages of biofilm producers as detected in TM were 35%, 25% and 40% for SBP, MBP and, NBP respectively. In contrast, CRA method only 18% bacterial strains showed black colonies with crystalline appearance.

DISCUSSION

In oral cavity various species of the genus *Streptococcus, Lactobacillus, Staphylococcus, Corynebacterium, Veillonella, Bacteroids* and *Enterococcus* are commonly found. In oral cavity, some beneficial micro-flora shifts their lifestyle to destructive pathogens when they gain access into the oral tissue and blood stream. It has been reported in previous investigation that bacterial strains are responsible for the dental caries and dependent upon interactions of protective and pathologic factors that reside in saliva [12].A total of 100 bacterial isolates were recovered from the dental samples in our study. Gram-Positive bacteria found to be the predominant, constituting 52% of the total isolates (52/100). *Corynebacterium* (12/100) *Y. pestis* (12/100) and *Veillonella* (12/100) were the most prevalent isolates from the dental samples followed by *Actinomyces*10/100 and *Neisseria spp*8/100. Some other research group discover almost same flora from the oral cavity like One study from the explore lactobacillus 56 % which is higher from current studies 19.23%. While in case of Actinomyces spp. they isolates 6.7% and in our study we have found 19.23 % which is higher from their studies *P. aeruginosa* 13%. Many more worker discovered the oral microfolora and majority of the strains were similar [13-14].

Oral cavity contains a diverse variety of the microorganism which may associated with the multiple infection and biofilm production which is attributed with the protection of the microbes from the drugs and harsh environment, hence a source for the drug resistance, which is a major problem in the treatment of the infection. In current study all 100 bacterial isolates were further subjected to TCPM, TM and CRA methods for phenotypic detection of biofilm production. The TCPM, the gold standard method, detected biofilm formation in 89 out of 100 bacterial isolates (89%). The highest biofilm production was found among gram Positive Bacteria, in which SBP was 80% by the *Lactobacillus* and*M. luteus* followed by the *Bacillus cereus* 70% and in case of the gram negative the *A. radioresistens shows 70% the highest SBP followed by the Haemophilus 60%*. In the present study, Escherichia coli show no biofilm formation and *K. pneumonia* shows 50%. These results were considerably lower than those of who reported that Escherichia coli 60 % and *K. pneumonia* produced biofilm 63%.Further other studies were carried out for biofilm detection assays with different samples, like regional data from India also showed that out of 152 isolates tested, the number of biofilm producers identified by TCP method was 53.9 %, and non-biofilm producers were 46%[15]. This is lower than our studies. Tube method detected 60% isolates as biofilm producers and 40% as non-biofilm producers. TM is 93% sensitive, 81.4% specific and 80% accurate for biofilm detection. With PPV value 67.8 % and NPV value 96 %, this method is well correlated with TCP for identifying strong biofilm producers, but it cannot be used to differentiate between moderate, weak and non-biofilm producers due to the changeability in the results detected by different observers. In accordance with the former studies, TM cannot be suggested as general screening test to identify biofilm producing isolates but if compare with CRA, it is give better and accurate result than CRA [16-20]. In another study, Ruzicka et al. noted that out of 147 isolates of *S. epidermidis*, TM detected biofilm formation in 79 (53.7%) and CRA detected in 64 (43.5%) isolates. They showed that TM is better for biofilm detection than CRA did not recommend the CRA method for biofilm detection in their study. Out of 128 isolates of *S. aureus*, CRA detected only 3.8% as biofilm producers as compared to TCP which detected 57.1% as biofilm producing bacteria [21-24]. Baqai *et al* tested TM to detect biofilm formation among uropathogens. According to their results, 75% of the isolates exhibited biofilm formation.20 With the CRA method, we found only 20% were found to be biofilm producing bacteria and 80% as non-biofilm producers [25-27].

There is no clear explanation for such variations in these studies. Though, this might be attributed to the modified methods and different samples used in experiments.

CONCLUSION

We can conclude from our study that TCP is a quantitative and trustworthy method to detect biofilm forming microorganisms compared to other two methods TM and CRA and TCP can be suggested as a general screening method for detection of biofilm producing bacteria in Microbiology laboratories.

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