Original Article

E. faecalis are common for biofilm production than E. faecium and tissue culture plate method is gold standard for biofilm detection in Enterococci.

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Abstract :

Objective: *The ability of Enterococci to produce biofilms is fundamental in causing endodontic and urinary tract infections, as well as endocarditis. The aim of the study were to investigate that E. faecalis are common for biofilm production than E. faecium and tissue culture plate method (TCP) is gold standard for biofilm production in Enterococci.* ***Materials &Method:*** *Biofilm of Enterococci was detected by tissue culture plate method, tube method and congo red agar method.* ***Results:*** *Biofilm formation were more in E. faecalis than E. faecium (P = 0.005) and the TCP method was considered the gold standard method for detection of biofilm in Enterococci(sensitivity and specificity 100%).* ***Conclusion:*** *The purpose of the study was to show biofilm producing Enterococci and the gold standard method of biofilm detection in Enterococci.*

Key words: Biofilm, tissue culture plate method

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Introduction:

Biofilm are defined as microbial derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. They are embedded in a matrix of extracellular polymeric substance they are produced, and exhibit an altered phenotype with respect to growth rate and gene transcription[1]. With a biofilm bacteria communicate with each other by production of chemotactic particles or pheromones, a phenomenon called quorum sensing[2]. Availability of key nutrients, chemotaxis towards surface, motility of bacteria, surface adhesins and presence of surfactants are some factors which influence biofilm formation[2]. According to a publication by the National Institutes of Health, more than 80% of all infections involve biofilms. Biofilm are associated with many medical conditions including indwelling medical devices, dental plaque, upper respiratory tract infection, peritonitis and urogenital infections[3]. Both gram positive and gram negative bacteria have the capability to form biofilms. Bacteria commonly involved include Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Viridans streptococci, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis and Pseudomonas aeruginosa[4]. The two most common Enterococci species are Enterococcus faecalis and Enterococcus faecium, both are capable of producing biofilms, which consists of a population of cells attached irreversible on various biotic and abiotic surfaces, encased in a hydrated matrix of exopolymeric substances[5].

Materiais and Method

This cross-sectional study carried out at the department of microbiology, Dhaka Medical College from January 2015 to December 2015. Among 350 urine samples, 30 Enterococcus faecalis and 10 Enterococcus faecium were

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detected by culture and PCR method. Among the Enterococci, biofilm detection was done by tube method, congo red agar method and tissue culture plate method.

Tube method

This is a qualitative method for biofilm detection. A loopful of test organisms was inoculated in 10 ml of brain heart infusion broth (BHIB) with 1% glucose in test tubes. The tubes were incubated at 370C for 24 h. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.2) and dried. Tubes 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 as 1-weak/none, 2-moderate and 3-high/strong. The experiment was performed in triplicate and repeated three times.[6]

Congo Red Agar method

A simple qualitative method to detect biofilm production by using Congo Red Agar (CRA) media. CRA medium was 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. First Congo Red stain was prepared as a concentrated aqueous solution and autoclaved (121ºC for 15 minutes) separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose at 550C. CRA plates were inoculated with test organisms and incubated at 370C for 24 h aerobically. Black colonies with a dry crystalline consistency indicated biofilm production. The experiment was performed in triplicate and repeated three times.

Tissue Culture Plate Method (TCP)

The microorganisms are grown in polystyrene tissue culture plates for 24 hours then after washing, fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). Biofilm formation is detected by measuring optical density with ELISA reader[6].

The organisms were grown overnight in brain heart infusion broth (BHIB) with 0.25% glucose at 37°C. The culture was diluted 1:40 in TSB 0.25% glucose, and 200 µl of this cell suspension was used to inoculate sterile 96 well polystyrene microtiter plates. After 24h at 370C, wells were gently washed three times with 200 µl of phosphate buffered saline (PBS), dried in an inverted position, and stained with 0.1 % crystal violet for 15 min. The wells were rinsed again, and the crystal violet was solubilized in 200 µl of acetone (80:20, vol/vol). The optical density at 595nm (OD595) was determined using a microplate reader. Each assay was performed in triplicate and repeated three times[7].

Calculation of OD values

OD value was calculated by using the following method.The average OD values were calculated for all tested strains and negative controls, since all tests were performed in triplicate and repeated three times. Second, the cut off value (ODc) was established. It was defined as three standards (SD) above the mean OD of the control: ODc = average OD of negative controls + (3 X SD of negative control). Final OD value of a tested strain was expressed as average OD value of the strain reduced by ODc value (OD = average OD of a strain – ODc). ODc value was calculated for each microtiter plate separately. If a negative value is obtained, it should be present as zero, while any positive value indicates biofilm.

Results

Among 42 isolates, TCP, detected 28 (66.67%) biofilm producers. By TM, the number of biofilm

producers were 22 (52.38%) and non-biofilm producers were 20. Very different results were observed by the CRA method, with which only 11 (26.19%) were biofilm producers. Among 30 E. faecalis, 25 (83.33%) were biofilm producers and among 10 E. faecium, 3 (30%) were biofilm producers. Biofilm formation were more in E. faecalis than E. faecium (P = 0.005).

Table I: Screening of the isolates for biofilm by Tissue culture plate (TCP), Tube method (TM) and Congo Red Agar (CRA) method (N=42)..

Method	E. faecalis (N=30) n (%)	E. faecium (N=10) n (%)	Unidentified (N=2) n (%)	Total n (%)
TCP	25(83.33)	3 (30.00)	0(0.00)	28(66.67)
TM	19 (63.33)	3 (30.00)	0(0.00)	22(52.38)
CRA	10 (30.00)	1 (10.00)	0(0.00)	11(26.19)

N = Total number of Enterococci.
n = Number of biofilm formation.

A Significant difference was observed in biofilm formation among E. faecalis and E. faecium (P = 0.005).Statistical analysis of Tissue Culture plate, Tube method and Congo Red Agar method. The TCP method was considered the gold-standard for this study and compared with data from TM and CRA methods. Sensitivity and specificity of TM was 78.57% and 100% respectively. For CRA methods, sensitivity and specificity were 25% and 71.42% respectively.

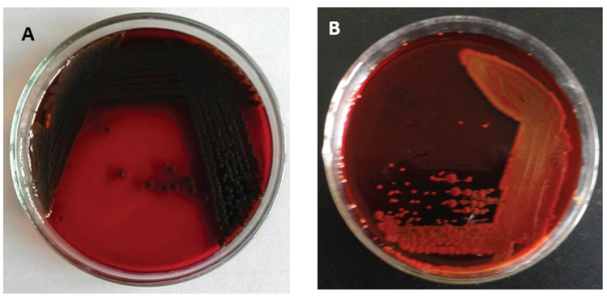
Table II: Statistical analysis of tissue culture plate, tube method and Congo Red Agar methods for biofilm detection of Enterococcus

Method	Sensitivity	Specificity	PPV	NPV	Accuracy
TCP	100%	100%	100%	100%	100%
TM	78.57%	100%	100%	100%	85.71%
CRA	25%	71.42%	63.63%	32.25%	40.47%

Discussion

In the present study, 83.33% E. faecalis were biofilm producers and 30% E. faecium were biofilm producers and these results are similar to the data reported by Baldassarriwhere 80% for E. faecalis and 48% for E. faecium isolated from infected patients which were able to form biofilm[8]. Seno reported that 100% E. faecalis isolates isolated from urinary tract infection were capable of producing biofilm[9]. In this study, E. faecalis produced biofilm more than E. faecium (P = 0.005). Other investigators have reported that E. faecalis (95%) isolates produce a biofilm more often than E.faecium (29%).

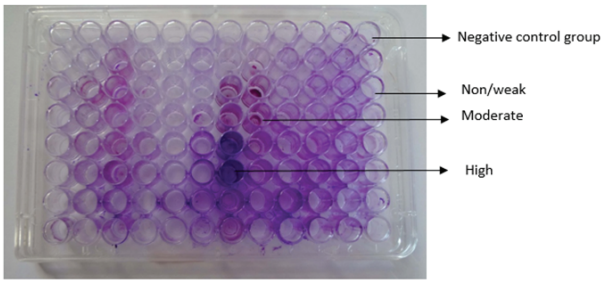
In the present study, the TCP method was considered the gold-standard and compared with data from TM and CRA methods. Hasan also considered the TCP method as a gold-standard for their study compared with TM and CRA methods. Parameters like sensitivity, specificity, positive predictive value, negative predictive value and accuracy were calculated[10]. In the present study, sensitivity, specificity, PPV, NPV and accuracy of TM was 78.57%, 100%, 100%, 70% and 85.71% respectively. Similar results were reported in other study where sensitivity, specificity, PPV, NPV and accuracy was 73%, 92.5%, 94%, 66% and 80% respectively[10]. In this study, for CRA method, sensitivity and specificity remained low which were 25% and 71.42% respectively. Hasan reported that sensitivity and specificity were 11% and 92% respectively for CRA method[10].



Enterococci in congo red agar media.

Left: (A) black crystalline colonies of biofilm positive strain.

Right: (B) pinkish-red colonies of biofilm negative strain.



Screening of biofilm producers of Enterococci by TCP method.

Conclusion

Results of the present study showed that the presence of biofilm is more common in E. faecalis than E. fauciumand among the three procedure of biofilm detection in Enterococci,tissue culture plate method is the gold standard method.

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