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Assessment of biofilm formation by *Enterococcus faecalis* causing nosocomial infections and their statistical analysis

KEYWORDS: Enterococci, Biofilm, Pathogencity, Virulence, transcriptional regulator, Intrinsic resistance.

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ABSTRACT *The aim of the study is to evaluate three method for detection of biofilm formation in Enterococcus faecalis. For detection of biofilm formation 198 clinical isolates of Enterococcus faecalis were observed by Tissue culture plate (TCP), Tube method (TM) and Congo Red agar (CRA) method. Out of the 198 Enterococcus faecalis 149(75%) displayed a biofilm positive phenotype under the optimized condition.TCP method were further classified as high 160(80.8%), moderate 28(14.1%) and weak 10(5.05%) isolates or non biofilm producer. During observation it was seen that TM and CRA did not correlated with TCP method for detection of biofilm formation in Enterococcus faecalis. The TCP method was found to be most 94% sensitive, 83% accurate and reproducible screening method for detection of biofilm production by Enterococcus faecalis.*

Introduction:

A biofilm is composed of living, reproducing microorganisms such as bacteria, that exist as a colony or community. Bacteria stick to any surface and produce a slimy polysaccharide matrix. This state of microorganism activity is now universally known as biofilm. These communities of cells are organized through an exopolysaccharide matrix made of complex carbohydrate rich polymers and other macromolecules such as DNA, RNA and proteins (Sutherland, 2001; Branda *et al.*, 2005). The complex architecture of biofilm-associated matrix provides enhanced resistance to multiple stress factors and allows the influx of nutrients, water and small signalling molecules that in turn provide effective communication between the cells (Watnik and Kolter, 2000; Tarver, 2009). Regarded as nosocomial pathogen of the 1990's Enterococci have become increasingly important not only because of their ability to cause serious infections but also due to their increasing resistance to many antimicrobial agents. Enterococci, recognized as opportunistic pathogens, are natural inhabitants of the oral cavity, normal intestinal microflora, and female genital tract of both humans and animals. They are common nosocomial agents that infect the urinary tract, bloodstream, intra-abdominal and pelvic regions, surgical sites and central nervous system (Murray and Weinstock, 1999; Richards, 2000). *Enterococcus faecalis* is the most common *Enterococcus* species, and it is responsible for 80-90% of human Enterococcal infections (Jett *et al.*, 1994; Jones *et al.*, 2004). *Enterococcus faecium* accounts for the remaining infections caused by Enterococci spp. (Jett *et al.*, 1994). The adherence (Joyanes *et al.*, 1999; 2000) and production of a biofilm by *E. faecalis* and *E. faecium* on different biomaterials has been demonstrated. (Baldassarri *et al.*, 2001; Toledo-Arana *et al.*, 2001; Distel *et al.*, 2002; Mohamed *et al.*, 2003, 2004). Enterococci in biofilms are known to be more resistant to antibiotics than planktonically growing Enterococci, thus the potential impact of biofilm formation could be significant. The capacity of Enterococci to bind to various medical devices, such as biliary stents (Dowidar *et al.*, 1991), ureteral stents (Keane *et al.*, 1994), intravascular catheters (Sandoe *et al.*, 2003) and silicone gastrostomy devices (Dautle *et al.*, 2003) has been associated with their ability to produce biofilms. *E. faecalis* is reported to form biofilm on ocular lens materials, such as polymethymethacrylate, silicone and acrylic, has also been documented (Kobayakawa *et al.*, 2005).

Materials and Methods: -

Bacterial strains:- The clinical isolates of *Enterococcus faecalis*, isolated from blood, urine, pus, ear swabs, catheter tips, wounds from SMS Medical college. Initially, standard microbiological techniques including Gram staining, catalase were used to identify the isolates. All cultures were maintained on Brain heart infusion medium.

Methods For Detecting Biofilm Formation:

Tissue Culture Plate (TCP) Method:- E.faecalis isolates from each sample were grown overnight in TSB with 0.5% glucose at 37 oC. This culture was diluted 1:40 in fresh TSB-0.5% glucose. From this cell suspension 200 µl was used to inoculate sterile 96-well flat bottomed polystyrene microtitre plates at 37 oC for 48 hrs. The wells were gently washed three times with distilled water and the microtitre plates were then dried in air in an inverted position for 1 hr at room temperature. The adherent biofilms were stained with 0.1% saffranin and allowed to stand for 20 min. at room temperature. The absorbance of the biofilm on the bottom surface of each well of the dried plates was determined at 490 nm using an enzyme linkedimmunosorbent assay microplate reader. Culture medium without any bacteria was used as blank. Each experiment was carried out in three wells and was repeated three times. All values were expressed in OD 490 as average with standard deviation. The biofilm formation was assessed as: Weak <0.10, Moderate 0.10-0.20, High >0.20 (Jayenthi et al., 2008).

Tube Method (TM) :-TSB with 0.25% glucose was prepared, poured in test tubes and inoculated with loopful of microorganims from overnight cultured blood agar plates of each clinical isolate and incubated for 24 hrs at 37°C. Each tube was decanted and washed with sterile phosphate buffer saline (PBS, pH 7.4) and dried. After drying the tubes were stained with 0.1 % crystal violet. Excess stain was removed and tubes were washed with sterile distilled water. Tubes were dried in an inverted position and observed for biofilm formation. When a visible stained film lined the wall and bottom of the tube then the biofilm formation was considered positive.

Congo Red Agar (CRA) :- The medium i.e. Congo Red Agar was prepared as a concentrated aqueous solution and poured in the petriplates. The culture of E. faecalis from each sample was streaked on these plates. Colonies were observed after incubation for 48 hrs at37oC. Black bacterial colonies with a rough, dry and crystalline consistency are biofilm producers. Red or pink bacterial colonies are classified as weak or non

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biofilm producers.

Statistical Analysis:-Biofilm assay was confirmed with three screening methods *i.e.* TCP method, TM and CRA method. The specificity, sensitivity, positive predictive value and negative predictive value of these tests were statistically analyzed by "Gold standard test. The TCP method was considered as standard for this study and was compared with TM and CRA.

Gold standard test is considered to be the most accurate test or the criteria by which scientific evidence is evaluated. A hypothetical ideal "Gold standard test" has a sensitivity of 100% with respect to the presence of the disease (it identifies all individuals with a well defined disease process; it does not have any false-negative results). A specificity of 100% means that it does not falsely identify someone with a condition that does not have the condition; it does not have any false-positive results.Sensitivity and specificity are statistical measures of the performance of a binary classification test. Sensitivity measures the proportion of actual positives which are correctly identified. Specificity measures the proportion of negatives which are correctly identified.

$$\text{Sensitivity} = \frac{\text{No. of true positive}}{\text{No. of true positive + No. of false negative}} = \frac{\text{No. of true positive}}{\text{No. of positives}}$$

$$\text{Specificity} = \frac{\text{No. of true negative}}{\text{No. of true negative + No. of false positive}} = \frac{\text{No. of true negative}}{\text{No. of negative}}$$

True positive: Sick people correctly identified as sick.

False positive: Healthy people incorrectly identified as sick.

True negative: Healthy people correctly identified as healthy.

False negative: Sick people incorrectly identified as healthy.

Results:-
Tissue Culture Plate (TCP) Method: 198 isolates of *E. faecalis* were tested for biofilm formation by this method. Amongst these 160 (80.8%) were found to be high biofilm formers, 28 (14.1%) were medium and 10 (5.05%) were weak.
On the basis of OD_{490}:High>0.20, Moderate 0.10-0.20 and Weak<0.10. (Figure 1,2; Table 1).

Tube Method (TM):-The same 198 isolates were tested for biofilm formation by TM. After incubation for 24 hrs 87 (43.9%) strong biofilm producers were obtained, 85 (42.9%) were moderate and 26 (13.1%) isolates were considered as weak or non biofilm producers (Figure 1,3; Table 1).

Congo Red Agar Plate (CRA) Method:- The same 198 isolates were then tested for biofilm formation by CRA method. After 24 hrs, strong biofilm producers obtained were 55 (27.7%), 53 (26.7%) were moderate and 90 (45.4%) isolates were found to be weak or non biofilm producers (Figure 1,4 ;Table 1).

Statistical Analysis
Gold Standard Test:-The TCP method was considered the gold-standard for this study and compared with data from TM and CRA methods. Parameters like sensitivity, specificity, negative predictive value, positive predictive value and accuracy were calculated. True positives were biofilm producers by TCP, TM and CRA method. False positives were biofilm producers by TM and CRA method and not by TCP method. False negatives were the isolates which were non-biofilm producers by TM and CRA methods but were producing biofilm by TCP method. True negatives are those which were non biofilm producers by all the three methods.Sensitivity and specificity of TM was 77% and 86%, respectively. For CRA method, sensitivity and specificity remained low and were 38% and 64% respectively (Table 3; Figure 5).

Discussion and Conclusion:-Biofilm producing bacteria are responsible for many recalcitrant infections and are notoriously difficult to eradicate. They exhibit resistance to antibiotics by various methods like restricted penetration of antibiotic into biofilm and expression of possible resistance genes(Kim,2001). Knobloch *et al.*,2002 also found TCP method to be more suitable for biofilm detection as compared to CRA method. Similarly, Hittinahalli *et al.,* 2012 and Ira *et al.*,2013 found TCP method to be superior to TM and CRA methods. Chandrakanth *et al.*,2012 too recommended TCP method to be more reliable as compared to the TM method. Christensen *et al.*,1982 and Mathur *et al.*,2006 reported that TM method cannot be recommended as a general screening test to identify biofilm producing isolates. Contrary to it, Ruzicka *et al.*,2004 found TM method better for biofilm detection. However, Freeman *et al.*,1989 suggested that CRA method is more reliable and has no correlation with the TCP and TM methods. But Hassan *et al.*,2011 did not recommend CRA method for detection of biofilm producing isolates when compared to TCP and TM methods. The results of the present study indicate that the TCP method is most reliable method for screening of biofilm forming microorganisms. The present data also showed that TCP was 94% sensitive and 83% accurate while TM was 77% sensitive and 81% accurate. The CRA method also show less sensitive (38%) and accurate (44%) compared to the TCP method. It can therefore, be concluded that the TCP method is the most sensitive, accurate and reliable screening method for detection of biofilm formation.

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Table - 1
Biofilm assay by TCP, TM and CRA methods

No of Isolates 198	Tissue culture Plate Method			Tube Method			Congo Red Plate Method		
	High	Moderate	Weak	High	Moderate	Weak	High	Moderate	Weak
	160 (80.8%)	28 (14.1%)	10 (5.05%)	87 (43.9%)	85 (42.9%)	26 (13.1%)	55 (27.7%)	53 (26.7%)	90 (45.4%)

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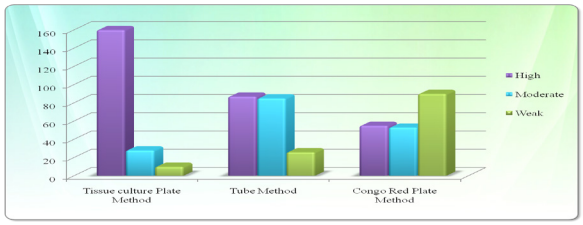

Figure 2 : Biofilm assay by TCP, TM and CRA methods

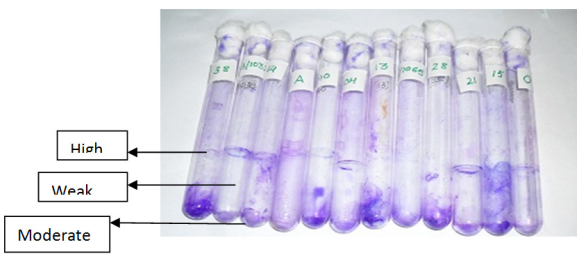

Figure 2: Biofilm assay by Tube Method

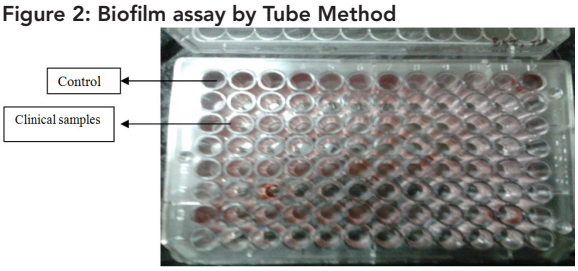

Figure 3: Biofilm assay by Tissue Culture Plate Method

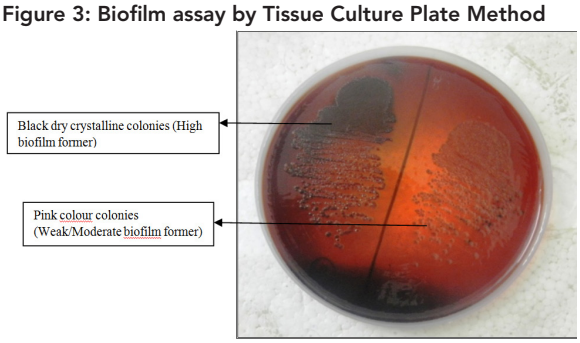

Figure 4: Biofilm assay by Congo Red Agar Plate Method

Table - 2
Screening for Gold standard test

Screening Method	True Positive (a)	False Positive (b)	False negative (c)	True Negative (d)
TCP	160	5	10	23
TM	87	12	26	73
CRA	55	19	90	34

a=True positive (Sick people correctly diagnosed as sick)

b= False positive (Healthy people incorrectly identified as sick)

c=True negative (Healthy people correctly identified as healthy)

d=False negative (sick people incorrectly identified as healthy)

Table - 3
Gold standard test

Screening Method	Sensitivity (%)	Specificity (%)	Positive predictive Value (%)	Negative predictive Value (%)	Accuracy (%)
TCP	94	82	97	70	83
TM	77	86	88	74	81
CRA	38	64	74	27	44

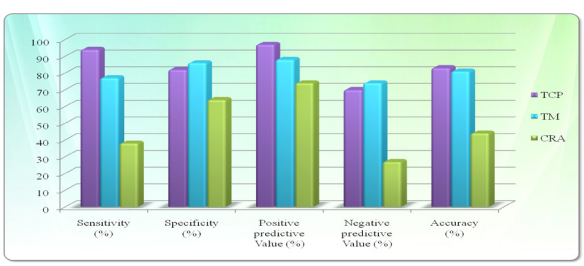

Figure 5: Gold standard test

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