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Assessment of Biofilm Formation by *Enterococcus* spp. from Clinical Isolates

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The purpose of the present study is to evaluate three method for detection of biofilm formation in *Enterococcus* spp. for detection of biofilm formation, 150 clinical isolates of *Enterococcus* spp. were observed by Tissue culture plate (TCP), Tube method (TM) and Congo Red agar (CRA) method. Out of the 150 *Enterococcus* spp. 98 (65%) displayed a biofilm positive phenotype under the optimized condition. TCP method were further classified as high 28(18.66%), moderate 50(33.33%) and weak 72(48%) 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 spp*. The TCP method was found to be most sensitive, accurate and reproducible screening method for detection of biofilm production by *Enterococci*.

Key Words: *Enterococci*, Biofilm, Pathogenecity, Virulence, Transcriptional regulator, Intrinsic resistance.

Enterococci are known to produce slime that is an amorphous extracellular substance made of polysaccharides, and is one of the major components of bacterial biofilm[1] . Biofilm protects the microorganisms from host defense and some antimicrobial drugs. Biofilm formation is an increasing problem in medicine, due to the intrinsic resistance of microorganisms in the biofilm mode of growth against the host immune system and antimicrobial therapy. Adhesion is an important step in biofilm formation, influenced, among other factors, by the surface hydrophobicities and charges of both the substratum and the adhering microorganisms[2] and is an important factor in the attachment of *Enterococci* to surfaces and other cells .It is also one of the virulence factors in many pathogenic *Enterococci* strains. The composition of the growth media has an influence on the production of biofilm by the *Enterococci*. Many bacterial species are able to colonize the surfaces of biomedical devices and form biofilms. Biofilm growth protects the bacteria against host defenses and the action of antimicrobial agents, and therefore biofilms can be a source of persistent infections [3-5]. The human commensal *Enterococcus faecalis* is a major cause of nosocomial infections and is able to form biofilms on biomedical devices such as urinary catheters and central venous catheters[6]. *E. faecalis* is also one of the most predominant strains involved in the formation of biofilms in biliary stents, used to palliate obstructions in the common bile duct[7]. Biofilm formation takes place in a complicated series of events that commences with the formation of a conditioning film on a surface and the subsequent adhesion of bacteria, followed by the formation of micro colonies and the production of a matrix of extracellular polymeric substances that define the biofilm .The presence of additional carbohydrates

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or depletion of iron favor the biofilm production[8] .Other factors that favor the production of biofilm are the transcriptional regulator BopD[9], the gene products of the quorum sensing locus *fsr* and gelatinase (GelE)[10] .The *Enterococcus faecalis* for two-component system controls biofilm development through production of gelatinase[11]. In clinical strains of *E. faecium* slime production is more frequent than in environmental strains and isolates from healthy individuals, therefore biofilm may be considered to be one of the virulence factors of *Enterococci*[12]. A number of studies have identified different virulence factor, most important among them being [13-19] haemolysin, gelatinase ,enterococcal surface protein [E_{sp}], aggregation substance [As],MSCRAMM Ace (Microbial surface component recognizing adhesive matrix molecule adhesion of collagen from Enterococci) ,serine protease, capsule, cellwall polysaccharide and superoxide. These factors have been associated with the virulence of E.*faecalis* in animal models[20-23]. Clogging of biliary stents caused by microbial biofilms is a common complication, necessitating removal of the device, severely affecting the quality of life of the patient, and raising the cost of health care. The majority of clinical isolates of *E. faecalis* have the ability to form a single-species biofilm in vitro [24, 25]. Biofilm forming colony morphology was detected for microorganism isolates on *CRA* plates containing 21 g Mueller–Hinton broth, 15 granulated agar; 36 g sucrose and 0.8 g Congo red per liter of distilled water[26]. A number of tests are available to detect slime production by *Enterococcus*; viz Tissue culture plate[27] (TCP) ,Tube Method[28] (TM) ,Congo Red Agar (CRA) , Bioluminent Assay and Light or Fluorescence microscopic examination . In the present study clinical isolates of *Enterococcus* spp. by TCP, TM, and CRA methods were screened for determining their ability to form biofilm and also the reliability of these methods was evaluated in order to determine most suitable screening method.

MATERIALS AND METHODS

Bacterial strains

The clinical isolates of *Enterococcus spp*, isolated from blood, urine, infected devices from SMS medical college. Initially, standard microbiological techniques including Gram staining, catalase, coagulase and biochemical test were used to identify the isolates. All cultures were maintained on Brain heart infusion medium.

Methods for detection biofilm formation

Tube method

A qualitative assessment of biofilm formation was done by Tube method as described Christensen *et al*. (1982). Trypticase soya broth (TSB) with 0.25% glucose was prepared and inoculated with loopful of microorganism from overnight cultured blood agar plates and incubated for 24 hours at 37°C. The tubes were 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 (Fig. 1). The biofilm formation was assessed as: 0-Weak , 1- Moderate, 2- Strong.

Tissue culture plate method(TCP)

Biofilm formation by Microtitre Plate Method was carried out as reported by Kristich *et al* (2004). Briefly , *Enterococcus* isolates were grown overnight in trypticase soy broth with 0.5% glucose at 37°C . The culture was diluted 1:40 in

Table 1. Classification of bacterial adherence by TCP Method

Mean OD_{490} value	Adherence	Biofilm Formation
> 0.20	High	High
<0.10	Moderate	Moderate
>0.10	Weak	Non/ Weak

Table 2. Screening of 150 *Enterococcus* isolates for detection of biofilm formation by TCP, TM and CRA method

Clinical Isolates	Biofilm Formation	Screening Method		
		TCP	TM	CRA
	High	28	15	9
	Moderate	50	43	12
	Weak/ Non	72	92	129

fresh TSB- 0.5% glucose , and 200 µl of this cell suspension was used to inoculate sterile 96- well flat bottomed polystyrene microtitre plates. These were prepared at 37°c for 48 hours . The wells were gently washed three times by rinsing in distilled water and the microtitre plates were then dried in air in an inverted position for an hour at room temperature, the adherent biofilms were stained with 0.1% safranin and allowed to stand for 20 minutes at room temperature (Fig. 2). The absorbance of the biofilm on the bottom surface of each well of the dried plates was determined at 490 nm using an enzyme linked immunosorbent 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 by *Elisa* Auto Reader.

Congo Red Agar

Qualitative detection of Biofilm production by *Enterococcus* strains was also assessed by cultivation on Congo Red Agar (CRA) as described by Freeman et al 1989. The medium composed of brain heart infusion (BHI) broth (37gms/L), sucrose (36gms/L), agar (30gms/L) and Congo red dye (0.8gms/L). It was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 min. Inoculated CRA plates were incubated at 37°C for 24 hours followed by subsequent storage at room temperature. Colonies on CRA were observed at 48 and 72 h. Strains producing black colonies with a rough, dry and crystalline consistency were considered biofilm producers. Strains producing red or pink colonies were classified as non- biofilm producers (Fig. 3).

Fig. 1. Showing Detecting Biofilm Formation

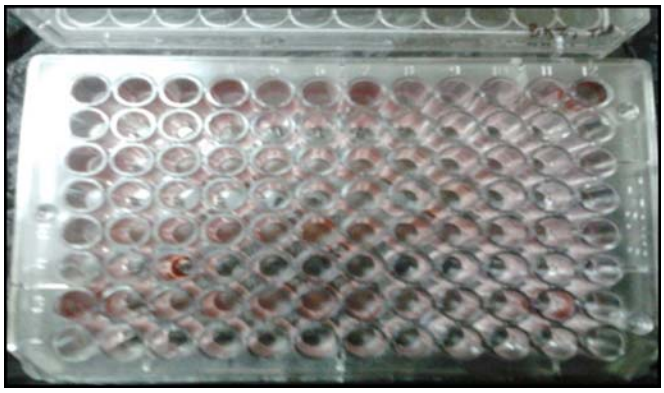

Fig. 2. Tissue Culture Plate Method

Fig. 3. Biofilm production by *Enterococcus* strains on Congo Red Agar

RESULTS AND DISCUSSION

Enterococcus is one of the major etiologic of nosocomial infections .Tissue culture plates made polystyrene were found to be most sensitive, accurate and reproducible screening method for detection of biofilm formation in clinical isolates. In this study 96 well polystyrene microtitre plates were used .The detection of high biofilm former showed a threat for nosocomial infection.

In this study 150 clinical isolates of *Enterococci* were tested by three *in vitro* screening procedures for their ability to form biofilm. Out of 150 *Enterococcus spp.* 98 (65%) displayed a biofilm positive phenotype under the optimized condition .TCP method were further classified as high 28(18.66%), moderate 50(33.33%) and weak 72(48%) isolates or non biofilm producer. Here *E.faecalis* ATCC 19433, *E.faecalis* ATCC 29212 and *E.faecalis* ATCC 51299 were used as quality control strains.

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