Section: Microbiology

Original Research Article

A STUDY ON BIOFILM FORMATION OF ENTEROCOCCAL SPECIES IN A TERTIARY CARE HOSPITAL

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ABSTRACT

Background: Bacteria in biofilms are extremely difficult to eradicate because they resist phagocytosis and are a cause of chronic infections (26). The biofilm matrix makes bacteria more resilient to antimicrobial agents and the immune system, leading to persistent infections that are difficult to treat (27). A wide variety of medical devices such as urinary and central venous catheters, cardiac pacemakers, prosthetic heart valves, joint prosthesis, contact lenses and hemodialysis equipment are colonized by biofilm producing bacteria (4). They are associated with human diseases such as urinary tract infections, burns wound infections, native valve endocarditis, gingivitis and cystic fibrosis (26).Enterococci have emerged as an important nosocomial pathogen and possessed many virulence factors including the ability to form biofilms on various biotic and abiotics surfaces(19)..Our study was designed to detect biofilm producing strains of Enterococcus species obtained from clinical isolates in a tertiary care hospital and also to assess the best method of detecting biofilm forming ability. **Materials and Methods:** The present study was conducted in KAPV Government Medical College, Trichy. The study period was from May 2024 to April 2025. Ethical committee clearance from the institution was obtained and informed written consent was received from the patients before collecting the specimens. Urine samples, blood samples, pus and wound swab samples were collected from 482 patients. All age groups and both sexes were included. Patients admitted to various wards (ICU, CCU, Surgery, Medicine, Pediatrics, Urology) with signs and symptoms suggestive of impending infections such as post operative wound infection, wound infection following burns, pyrexia of unknown origin, urinary tract infection, meningitis, endocarditis, intra-abdominal abscesses and septicemia were included in this study. The biofilm production in Enterococcal species was evaluated by three phenotypic methods such as Tissue culture plate (TCP) method, Tube method and Congo red agar (CRA) method. **Result:** A total of 482 clinical samples were collected from the patients admitted in various wards out of which 142 enterococcal isolates were taken up for the study. Among the three methods employed to detect biofilm formation, the Tissue culture plate method detected more biofilm produces in 52 samples (36.62%) followed by Tube method in 40 samples (28.17%) and Congo red agar method in 31 samples (21.83%). Tissue culture plate method was found to be more sensitive (100%) and specific (97.82%) than Tube method and Congo red agar method for the detection of biofilm formation in Enterococcal isolates. **Conclusion:** Tissue culture plate method was proved to be a simple and cost-effective method for the early diagnosis of biofilm formation in the Enterococcus species. Since the Tissue culture plate method was proved to be a simple and cost-effective method, it can be recommended for the early diagnosis of biofilm formation.

INTRODUCTION

Biofilm constitutes a complex community of microorganisms encased in hydrated matrix of exopolymer substances, proteins, polysaccharides and nucleic acids and attached irreversibly on various biotic (plants, animals, other microbes) and abiotic (minerals, carapaces of dead animals or air water interfaces) surfaces.[1-10] Complex developmental processes involved in biofilm formation are

irreversible attachment on a surface, interaction between cell to cell, formation of microcolony, biofilm formation and a three-dimensional biofilm structure development.[11-13]

Factors influencing formation of biofilm:

i) Nutrient contents like glucose, serum, availability of iron and CO2, osmalarity, pH and temperature of growth medium alter biofilm production.[6]

ii) The association of enterococcal surface protein (Esp) at high glucose concentration in biofilm formation has been reported.[14-16]

iii) Persistence and colonization of infection within the urinary tract is contributed by enterococcal surface protein of E. Faecalis. 93.5% enterococcal surface protein producing E. Faecalis produce biofilms on nonliving surfaces and E. faecalis isolates lacking enterococcal surface protein never produced biofilms.[1]

iv) Enhancement of biofilm production in E. Faecalis is more in 1% glucose supplemented tryptic soy broth medium than without glucose.[6]

v) The gelatinase, which is an extracellular zinc metalloprotease is important for biofilm production.[17-19]

vi) E.faecalis regulator (fsr) the two-component quorum – sensing signal transduction system regulates the expression of gelatinase and serine protease and helps in biofilm formation.[10]

vii) Other genes associated with biofilm formation in E. faecalis are atn (Autolysin), bec (biofilm enhancer in enterococcus), bop (biofilm on plastic surface), dltA (D-alanine lipoteichoic acid) ebpA, B, C (endocarditis and biofilm associated pili), epa (enterococcal polysaccharide antigen), Sal A, B (Secretory antigen like A & B).[7]

Detection of Biofilm formation: Biofilm formation in enterococcus can be detected by qualitative methods such as Congo red agar (CRA) method and Tube method (TM) and quantitative method such as Tissue culture plate (TCP) method.[17] In this study, comparison is done between all the three methods and to find out the most reliable and sensitive method for the detection of biofilm formation. The present study was undertaken to detect the biofilm producing Enterococci which were isolated from various clinical materials by three different phenotypic methods such as Tube method, Congo red agar method and Tissue culture plate method.

MATERIALS AND METHODS

Source and Sample size: Urine samples, blood samples, pus and wound swab samples were collected from 482 patients. Out of 482 samples collected 142 enterococcal isolates were obtained which were subjected to species identification and biofilm formation by phenotypic methods.

Detection of Biofilm production

1. **Congo red agar method:** It is a qualitative method used for the detection of biofilm formation. The medium used was Congo red agar (CRA) medium.

Procedure: The test organisms were inoculated in CRA medium and kept for incubation at 37°C for 24 hr aerobically ().

Interpretation:

Biofilm producer	Colony Morphology
High	Colonies with black color and a dry crystalline consistency
Moderate	Darkening of the colonies without dry crystalline consistency
Weak / Non-biofilm producers	Pink colored colonies

2. **Tube method:** A qualitative method for detection of biofilm production

Procedure:

1. The test organisms were inoculated in 10ml of trypticase soy broth taken in the sterile test tubes. The tubes were kept for overnight incubation at 37°C.
2. Then the tubes were decanted and by using phosphate buffer saline (pH 7.3), the tubes were washed and then allowed to dry.
3. By using 0.1% safranin, the tubes were stained and deionized water was used to remove excess stain.
4. Tubes were kept in inverted position and allowed to dry. The control strains were included in the test and according to the results the scoring was done.[3]

Interpretation

Biofilm production: The wall and the bottom of the tube were lined by a visible film.

The amount of biofilm formed was scored as 1 – weak / none, 2 – moderate, 3 – strong.

3. **Tissue culture plate method:** This is a quantitative method for biofilm detection.

Procedure

1. The test organisms were inoculated in 10ml of trypticase soy broth and kept for overnight incubation.
2. A dilution of 1: 100 was done for the cultures by using fresh broth. 200µl of the diluted cultures was added into individual wells of sterile 96 well flat bottom polystyrene tissue culture plate and then incubated along with positive and negative control. (Biofilm producer was Enterococcus faecalis ATCC 29212 and biofilm nonproducer was Staphylococcus aureus ATCC 25923).
3. Gentle tapping was done to remove the contents of the well.
4. Washing of the wells was done with 0.2ml of phosphate buffer saline (pH 7.2) and then wells were washed four times to remove the free-floating bacteria.
5. After washing, 2% sodium acetate was used to fix adherent bacteria in the wells and by using 0.1% crystal violet, the wells were stained and deionized water was used to remove excess and then allowed to dry.

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6. Reading was taken at wavelength 490nm by micro-ELISA auto reader. As the bacteria forms biofilm and adheres to the wells, these optical density values were taken as an index of bacterial adherence to the wells.[5]

Interpretation

Mean OD values	Biofilm production
< 0.1	Non / weak
0.1 – 0.2	Moderate
> 0.2	High

OD cut off value = average of negative control + 3 x Standard deviation (SD) of negative control.

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of each test were calculated by using true positive, true negative, false positive and false negative values obtained in all the three methods.

RESULTS

The number of Enterococcal isolates obtained from various clinical samples were as follows. (n=142).

Table 1: No of Enterococcal isolates from various clinical samples

Specimen	No of Entrococci isolates	Percentage
URINE	58	40.84%
BLOOD	32	22.53%
PUS	27	19.01%
WOUND SWAB	25	17.60%

Table 2: No of Enterococcal species isolated from various clinical samples

Speciman	E.Faecalis	E.Faecium	E.Raffinosus	E.Sulfurous	Total
URINE	34	19	4	1	58
BLOOD	18	12	2	0	32
PUS	16	8	2	1	27
WOUND SWAB	18	6	0	1	25
TOTAL	86	45	8	3	142

Table 3: No of Biofilm Producing Enterococcal isolates percentage by different phenotypic methods

Bio film production		TCP	TM	CRA
No of bio film producers	High	30(21.31%)	26(18.31%)	19(13.38%)
	Moderate	22(15.49%)	14(9.86%)	12(8.45%)
	Total	52(36.62%)	40(28.17%)	31(21.83%)
No of nonbio film producers	Weak/none	90(63.38%)	102(71.83%)	111(78.17%)

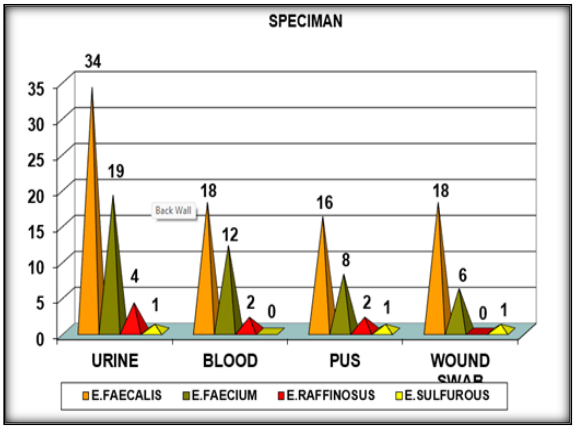

Chart 1: No of Enterococcal isolates from various clinical samples

From the above table, it is observed that Tissue culture plate method detected more biofilm producer in 52 samples (36.62%) followed by Tube method in 40 samples (28.17%) and Congo red agar method in 31 samples (21.83%).

Species and Specimen wise distribution of Biofilm producing Enterococci (n=52)

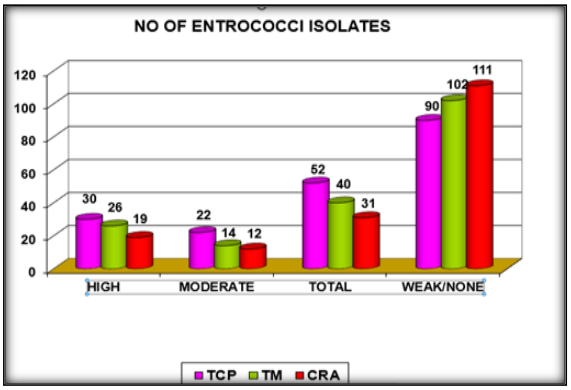

Chart 2: Biofilm Producing Enterococcal isolates percentage by different phenotypic methods

Table 4: No of Biofilm Producing species of Enterococcus from various clinical samples

Specimen	E.faecalis	E.faecium	TOTAL
URINE	21(40.38%)	4(7.7%)	25
BLOOD	8(15.38%)		8
PUS	7(13.46%)	2(3.8%)	9
WOUND SWAB	10(19.23%)		10
TOTAL	46(88.46%)	6(11.54%)	52

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Out of 52 biofilm producing enterococci, 46 (88.46%) constituted E. faecalis and only 6 (11.54%) constituted E. faecium. E.raffinosus and E.sulfureus did not produce any biofilms. Biofilm producers were more isolated from urine samples followed by wound swab samples.

Table 5: True Vs False positives of different phenotypic methods

METHOD	TRUE(+VE)	FALSE(-VE)	FALSE(+VE)
TCP	50	--	2
TM	29	6	5
CRA	10	14	7

From the above table, it was found that Tissue culture plate method showed only two false positives compared to other methods.

Table 6: Sensitivity and Specificity of various phenotypic methods

Method	Sensitivity	Specificity	PPV	NPV
TCP	100%	97.82%	96.15%	100%
TM	82.86%	95.32%	85.29%	94.44%
CRA	41.67%	94.07%	58.82%	88.80%

From the above table, it was observed that Tissue culture plate method had the highest sensitivity (100%) and specificity (97.82%) with PPV and NPV of 96.15% and 100% respectively. Tube Method showed 82.86% sensitivity and 95.32% specificity with 85.29% PPV and 94.44% NPV. Congo Red Agar method had the least sensitivity (41.67%) and specificity (94.07%) with PPV and NPV of 58.82% and 88.8% respectively.

DISCUSSION

Enterococci have emerged as an increasingly important cause of nosocomial infections. In the present study, out of 482 samples processed, 142 (29.46%) were Enterococcal species Bacteria in biofilms causes a more persistent infections which respond poorly to conventional antibiotic therapy.[18] Biofilm formation is commonly regulated by inter and intraspecies quorum sensing mechanisms.[19-21] The present study showed 52 (36.62%) out of 142 Enterococcal isolates were biofilm producers. Out of 52 biofilm producers, 30 (21.31%) were high biofilm producer and 22(15.49%) were moderate biofilm producer. In Giridhara Upadhyaya PM et al study they documented that out of 65 biofilm producer, 23 (11.5%) were high biofilm producer and 42(21%) were moderate biofilm producer. In the present study, out of 52 biofilm formers, 46 (88.46%) were E. faecalis and 6 (11.54%) were E. faecium. This result was in discordance with the study of Jonathan A.T. Sandoe et al and in their study, they documented 100% E. faecalis and 42% E. faecium formed biofilms.[22] In this study, the percentage of biofilm production detected by Tissue culture plate method (36.62%) was high followed by tube method (28.17%) and Congo red agar method (21.83%). This finding correlated with Mathur et al showed that the number of biofilm producers identified by TCP method was high (53.9%) and followed by TM (11.8%) and CRA method.[23] In another study conducted by Manpreet Kour et al, noted that 80.8% biofilm producer were detected by TCP method, 43.9% by TM and 27.7% by CRA method.[24] Knobloch et al, HiHinahalli et al, Ira et al found that TCP method to be more accurate and sensitive for biofilm detection as compared to TM and CRA method. The sensitivity and specificity of TCP method, TM and CRA method were 100%, 82.86%, 41.67% and 97.82%, 95.32% and 94.07% respectively. In Soni et al study, the sensitivity and specificity of TCP method, TM, CRA method were 94%, 77%, 38% and 83%, 81% and 44% respectively.[25-28]

CONCLUSION

1. A total of 142 Enterococcal isolates were collected to study biofilm production
2. In the present study, E. faecalis was the predominant species with an isolation rate of about 60.56% followed by E. faecium 31.69%. Other species isolated were E. raffinosus (5.63%) and E. sulfureus (2.11%). E. faecalis had the highest isolation in urine samples.
3. Phenotypic detection of biofilm production among Enterococcal isolates were high in Tissue culture plate method when compared to Tube method and Congo red agar method.
4. Predominant biofilm producer was E. faecalis 88.46% (46/52) followed by E. faecium 11.54% (6/52).
5. Among the phenotypic method, Tissue culture plate method had the highest sensitivity and specificity as compared with other phenotypic methods.
6. On evaluation of bioform forming ability of Enterococci by three phenotypic methods, Tissue culture plate method showed high sensitivity and specificity of 100% and 97.82% respectively.

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