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Antibiotic Susceptibility Pattern of Biofilm Forming and Biofilm Non Forming Enterococci Species

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Abstract
Background and objectives: *Enterococci, once considered as normal commensal flora in oral cavity, intestines and genital tract of humans and animals have emerged as major opportunistic pathogens. Biofilm formation is an important virulence factor of Enterococci species. Bacteria within biofilm are more resistant than their free living counterpart.Hencethis study was conducted to detect the biofilm formation by Enterococci isolates from our hospital and to compare the antibiotic resistance pattern of biofilm forming and non- biofilm forming species.*
Materials and methods:*Speciation and antimicrobial susceptibility testing was done for isolated Enterococci according to standard microbiological techniques. Biofilm formation was tested by Congo Red agar method, Tube method and Tissue culture plate method.*
Results*: A total of 78 Enterococci isolates [64 were from urine, 10 from pus and 4 from blood] were collected. E.faecalis [n=58] and E. faecium [n=20] were the two species isolated in our study. Biofilm forming Enterococci was 53 strains [68%] and 25 [32%] were biofilm non producers. E. faecalis[81%] has the highest rate of biofilm formation than E.faecium [30%].*
Conclusion:*Biofilm producing isolates were less sensitive to antibiotics than biofilm non formers.*
Keywords:*Antibiotics, Biofilm, Congo red agar, Enterococci, Enterococci faecalis, resistance*

I. Introduction

Enterococci are Gram positive aerobic and facultative anaerobic oval cocci arranged in pairs or short chains.[1] Being part of intestinal commensal flora of humans and animals, Enterococci are relatively low virulence organisms but over the past two decades they have emerged as important nosocomial pathogens.[1, 2] Enterococci are second most common cause of Nosocomial UTI next to E. coli and third most common cause of bacteremia.[1, 3] E. faecalis [80 to 90%] and E. faecium [5 to 10%] are the common species causing human infections.[4] E. gallinarum, E. casseliflavus, E durans, E. avium, and E. hirae, are the other species accounting for less than 5% of human infections.[5] Both intrinsic and acquired resistance are seen in Enterococci.[6] E.faecium is more resistant to antibiotics than E. faecalis.[7] The structured microbial community known as biofilm is formed by irreversible attachment of planktonic organisms to a surface or interface in which cells are embedded in extracellular polysaccharide slime matrix secreted by them.[8] Significant difference is seen between the behavior of bacteria in biofilm and their free living counterpart.[9] Biofilm formation is considered as one of the important virulence factor in Enterococci.[10] Biofilm offers its members protection from host immune responses, phagocytosis and antibiotics.[11] National institute of health stated that 80% of infections are related to biofilm forming microbes.[12] Biofilm associated infections are recurrent, chronic and highly resistant to antibiotics.[11] 10 to 1000 folds of more antibiotic concentrations required to kill the bacteria in biofilm than the free living forms.[12] Because drug resistance is a major concern in Enterococci, this study was conducted to detect the biofilm formation by Enterococci isolates from our hospital and to co relate them with their antibiotic resistant patterns.

II. Materials and Methods

All the Enterococci species which were isolated in our hospital from clinical samples like pus, urine, blood, body fluids, wound swabs and catheter tips during January to August 2015 were included in this study.

Speciation of Enterococci:

Identification of genus Enterococcus was done based on Gram staining, cultural characteristics, and physiological and biochemical tests, namely, bile esculin hydrolysis, PYR hydrolysis, and growth in 6.5% sodium chloride and at pH 9.6. Further speciation was done by standard set of biochemical tests including arginine dihydrolase test, mannitol, sorbitol, sorbose, arabinose, raffinose, lactose, sucrose, and pyruvate fermentation tests, according to Falckam Collins classification. [13]

Tests for Detection of Biofilm Formation:

1. Congo red agar method
2. Tube method
3. Tissue culture plate method

Congo Red Agar Method:

Freeman et al. have described a simple qualitative method to detect biofilm production by using Congo Red Agar (CRA) medium. CRA medium was prepared with brain heart infusion broth (Himedia, Mumbai) 37 g/L, sucrose 50 g/L, agar No. 1 (Himedia, Mumbai) 10 g/L and Congo red indicator (Himedia, Mumbai) 8 g/L. First Congo red stain was prepared as a concentrated aqueous solution and autoclaved (121°C for 15 minutes) separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose at 55°C. CRA plates were inoculated with test organisms and incubated at 37°C for 24 h aerobically. Black colonies with a dry crystalline consistency indicated biofilm production.[14]

Tube Method:

Trypticase soy broth with 0.25% glucose was prepared, poured in test tubes and inoculated with loopful of microorganism 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. [15]

Tissue Culture Plate Method:

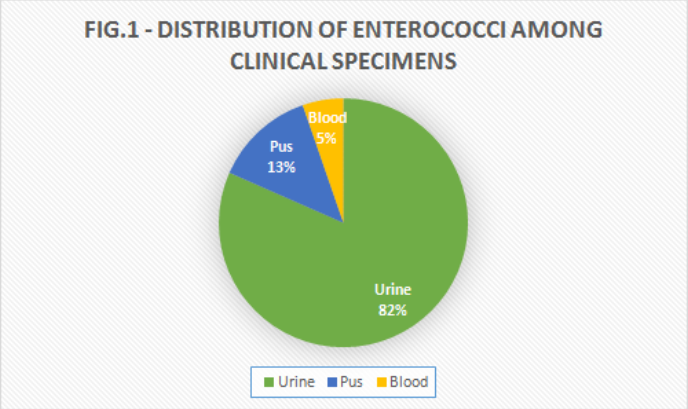
This quantitative test described by Christensen et al. is considered the gold-standard method for biofilm detection. Organisms isolated from fresh agar plates were inoculated in 10ml of trypticase soy broth with 1% glucose. Broths were incubated at 37°C for 24h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture treated plates were filled with 200µl of the diluted cultures. The plates were incubated at 37°C for 24 h. After incubation, contents of each well were removed by gentle tapping [14] and microtiter plate will be kept inverted for drying in room temperature for 1 hour. Dried wells will be stained with 0.1% safranin for 20 minutes at room temperature. Absorbance of the biofilm on the bottom surface of each well of the dried plates will be determined in an ELISA reader at 490nm. Optical density of strains was assessed for biofilm formation as: Weak <0.10, Moderate 0.10-0.20, High >0.20. [15]

Antibiotic Sensitivity Testing:

Antibiotic sensitivity testing was done in Muller Hinton agar according to standard procedures using the following antibiotic discs: penicillin, ampicillin, amoxyclav, chloramphenicol, erythromycin, gentamicin, amikacin, doxycycline, tetracycline, ciprofloxacin, levofloxacin, vancomycin and linezolid. Norfloxacin and nitrofurantoin were used only for urinary isolates. All the antibiotic discs were obtained from Himedia Laboratories, Mumbai, India.

Statistical calculation was done by using QuickCals from GraphPad software.

III. Results



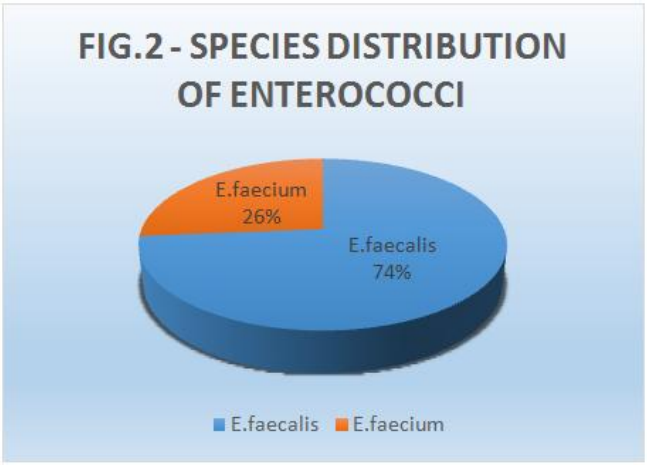


Table 1: Prevalence of Biofilm Forming Enterococci

Total no. of Enterococci isolates [%]	Biofilm producers	Biofilm Non formers
78 [100%]	53 [68%]	25 [32%]

Table 2: Prevalence of Biofilm Forming Enterococci Species

Enterococci species	Biofilm formers	Biofilm Non formers
E. faecalis [58]	47 [81%]	11 [19%]
E.faecium [20]	6 [30%]	14 [70%]

Table 3: Biofilm Assay

No.Of Isolates	Congo Red Agar Method			Tube Method			Tissue Culture Plate Method		
	High	Moderate	Weak	High	Moderate	Weak	High	Moderate	Weak
53	4 [8%]	16 [3o%]	33 [62%]	15 [28%]	30 [57%]	8 [15%]	18 [34%]	26 [49%]	9 [17%]

Table 4: Antibiotic Susceptibility Pattern of Enterococcal Isolates

Antibiotics	E.faecalis(n=58)		E.faecium(n=20)		Total no. Of sensitive isolates	Total no. Of resistant isolates
	Sensitive isolates	Resistant isolates	Sensitive isolates	Resistant isolates		
Penicillin	16 (28%)	42 (72%)	4 (20%)	16 (80%)	20 (25.6%)	58 (74.4%)
Amoxycillin clavulanic acid	35 (60%)	23 (40%)	12 (60%)	8 (40%)	47 (60.3%)	31 (39.7%)
Gentamicin	33 (57%)	25 (43%)	12 (60%)	8 (40%)	45 (57.7%)	33 (42.3%)
Amikacin	25 (43%)	33 (57%)	10 (50%)	10 (50%)	35 (44.7%)	43 (55.3%)
Tetracycline	10 (17%)	48 (83%)	8 (40%)	12 (60%)	18 (23.1%)	60 (76.9%)
Erythromycin	9 (16%)	49 (84%)	2 (10%)	18 (90%)	11 (14%)	67 (86%)
Ciprofloxacin	22 (38%)	36 (62%)	6 (30%)	14 (70%)	28 (36%)	50 (64%)
Nitrofurantoin	47 (81%)	11(19%)	16 (80%)	4 (20%)	63 (81%)	15 (19%)
Norfloxacin	12 (21%)	46 (79%)	6 (30%)	14 (70%)	18 (23.1%)	60 (76.9%)
Vancomycin	56 (97%)	2 (3%)	20 (100%)	0 (0%)	76 (97.4%)	2 (2.6%)

Table 5: Antibiotic Susceptibility According to Biofilm Formation

Antibiotics	Sensitive isolates		Resistant isolates		P [fisher's exact test]
	Biofilm formers	Biofilm non formers	Biofilm formers	Biofilm non formers	
Penicillin	4	16	38	20	Significant
Amoxycillin clavulanic acid	4	42	18	14	Significant
Gentamicin	28	17	12	21	Significant
Amikacin	24	11	26	17	In significant
Tetracycline	4	14	36	24	Significant
Erythromycin	0	11	50	17	Significant
Ciprofloxacin	6	22	30	20	Significant
Nitrofurantoin	23	40	10	5	Significant
Norfloxacin	4	14	38	22	Significant
Vancomycin	16	60	2	0	In significant

IV. Discussion

In our study maximum number of Enterococci species were isolated from urine samples [82%] followed by pus [13%] and blood [5%] [Fig.1], this similar observation is seen in the studies conducted by Bose et.al and Jada et.al.[16, 17] Bose et al reported in their study that 62.13% of the isolates were from urine sample.[16]Regarding species prevalence of Enterococci only E. faecalis and E. faecium were isolated in our study based on biochemical tests. E.faecalis [74%] was the predominant species followed by E.faecium [26%] [Fig. 2]. This finding can be compared with the studies conducted by Preeti Srivastava et al[18] and Bose et al. [16] In the study conducted by Preeti Srivastava et al, 92% of the isolates were E. faecalis and 8% were E. faecium. Bose et al also found 82% E.faecalis and 12% E.faecium in their study. Higher incidence of E.faecalis infection might be due to its greater intrinsic virulence.

Table 1 - Among the 78 isolates of Enterococci taken for our study 53 [68%] were found to be biofilm producers and 25 [32%] were non biofilm producers. This is consistent with the study conducted by Abdul-Razak SH. Hasan et al[2], in which they found 77.3% of the Enterococci isolates were producing biofilm and 22.7% were not forming biofilms. Table 2 - E.faecalis has highest rate of biofilm formation 47 [81%] than E. faecium 6 [30%] in our study. This observation is also seen in other study conducted by Asha Peter et al[19] in which Enterococcal isolates from various sources were tested for biofilm formation. In that study regardless of source of isolation, E.faecalis isolates showed higher rate of biofilm formation than E. faecium establishes its association with infection. Table 3 - Congo red agar method [CRA], Tube method [TM] and tissue culture plate method [TCP] are the three methods used for detection of biofilm formation in microorganisms. Many studies have statistically evaluated the sensitivity and specificity of these methods. Most of the studies recommend TCP method for general screening on biofilm formation. Tube method also correlates well with TCP but it is difficult to differentiate the moderate, weak and non-biofilm producers due to the changeability in the results detected by different observers. CRA method showed very little correlation with other method. Hence in our study we performed all 3 biofilm detection methods in Enterococcal isolates and we found that TM and TCP were able to detect strong and moderate biofilm producers than CRA method which is similar to study conducted by Afreenish Hassan et al. [14]

Table 4 – In our study maximum resistance of Enterococci was shown against Erythromycin [86%]. There were 2 Vancomycin resistant strains in our study, but this result is obtained by disk diffusion method. Other studies in India have reported 0 to 30% prevalence rate for Vancomycin Resistant Enterococci [VRE]. All the isolates tested in our study were showing 100% sensitivity to Linezolid. Chakraborty, et al [20] observed that all the isolates tested in their study were 100% sensitive to Vancomycin and Linezolid and also maximum resistance to Erythromycin [71.24%].

Decreased permeability and transfer of resistance genes between the bacteria present inside biofilm are factors contributing to more resistance of biofilm forming organisms than their free living counterpart. Table 5 – According to this table, biofilm forming isolates were significantly more resistant to antibiotics like, Erythromycin [n=50], Penicillin [n=38], Norfloxacin [n=38], Tetracycline [n=36], Ciprofloxacin [n=30], Amoxycillin clavulanic acid [n=18], Gentamicin [n=12], Nitrofurantoin [n=10] than biofilm non forming isolates. Resistant pattern of other antibiotics like Amikacin and Vancomycin were insignificantly higher for biofilm forming Enterococci species. Overall biofilm forming Enterococcal isolates in our study were showing higher resistance patterns than their counterpart. This observation is also noted in some other studies conducted by Chakraborty et al [20] and Abdul-Razak SH. Hasan et al. [2] Chakraborty et al [20] reported the resistance pattern of biofilm forming Gram positive organisms as 100% to Penicillin, 70% to Rifampicin, 40% to Ciprofloxacin, 40% to Erythromycin and 30% to Cotrimoxazole and biofilm non formers were more sensitive these antibiotics. Abdul-Razak SH. Hasan et al [2] also observed significant higher resistant of biofilm producing Enterococci species in their study.

V. Conclusion

We conclude that Enterococci can attach to surface of various medical devices like urinary catheters, intravascular catheters etc. and exhibit their ability to biofilm. Though Enterococci are intrinsically resistant to many antibiotics, biofilm formation will also decrease their susceptibility to antibiotics. They will also account for recurrent and recalcitrant infections. Prevalence of biofilm forming resistant Enterococci strains is increasing now a days as shown in our study. Hence timely detection and control of biofilm formation is necessary to increase patient outcome.

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