ISSN 2515-8260 Volume 09, Issue 03, 2022

ORIGINAL RESEARCH

Biofilm formation of Enterococcus species among Diabetic foot ulcer Patients

[1]Sapre Rohit Rajendra, [2]Dr. Rohit Kumar

[1]PhD Research Scholar, [2]Associate Professor, Department of Microbiology, Index Medical College, Hospital & Research Centre, Malwanchal University, Indore, MP, India

Correspondence:
Dr. Rohit Kumar
Associate Professor, Department of Microbiology, Index Medical College, Hospital & Research Centre, Malwanchal University, Indore, MP, India

ABSTRACT

Introduction: Biofilm protects *Enterococci* from host immune response and antibiotics. Biofilm-producing *Enterococci* cause recurrent, chronic, and antibiotic-resistant infections. According to the National Institute of Health, 80% of infections are related to biofilm-forming microbes. Apart from biofilm-forming ability, *Enterococcus* spp. are known to produce various virulence factors. Biofilm formation is a major mechanism of adaptation that protects bacteria from antibiotics, due to several characteristics.

Materials and Methods: This is a prospective, descriptive and observational study conducted in the Department of Microbiology, Index Medical College, Hospital and Research center Indore from January 2019 December 2021. All isolates of Enterococcus species during the study period will be included. Enterococci isolated from clinical specimens like pus, wound swab and aspirates etc. received in Microbiology Department. All samples were processed by standard bacteriological procedures. Gram staining was done for pus, wound swab and aspirates and findings were recorded. Culture was done on 5% sheep blood agar and MacConkey agar. Inoculated plates were incubated at 37°C for 18- 24 hours.

Results: Of total 122 Enterococcus species isolated from diabetic foot ulcers, in that 66 (54.09%) isolates were biofilm producers and 56 (45.90%) were biofilm non-producers. In our study, Biofilm assay of High Congo red agar method 7.5%, tube method 24.24%, Tissue culture plate method 24.24%. On the other hand, Weak Congo red agar method 59.09%, tube method 16.66%, Tissue culture plate method 24.24%. In our study, among sensitive, few strains were sensitive to Clindamycin, Amikacin and Lenizolid, 1, 15, 17 respectively. Rate of resistance to Penicillin G: 6, Tetracycline 6, Gentamycin 3, Clindamycin 6, Amoxy-clav 6, Cefoxitin 6 and Ciprofloxacin 2.

Conclusion: In the present study, 48.33% of drug resistant bacteria were biofilm formers. Infections with bacteria forming biofilms are difficult to eradicate. These biofilms are not only less susceptible to host cell immune responses but also have a high tolerance to antibiotics than the planktonic cells. The resistance of biofilm forming bacteria towards antibiotics is due to obstruction in the permeability of the drug by the polysaccharide matrix and alteration of the drug efficacy in the biofilm environment.

Keywords: Biofilm formation, Antibiogram, Enterococcus species.

INTRODUCTION

Biofilm protects *Enterococci* from host immune response and antibiotics. Biofilm-producing *Enterococci* cause recurrent, chronic, and antibiotic-resistant infections. [1]

According to the National Institute of Health, 80% of infections are related to biofilm-forming microbes. Apart from biofilm-forming ability, *Enterococcus* spp. are known to produce various virulence factors. [2]

Biofilm formation is a major mechanism of adaptation that protects bacteria from antibiotics, due to several characteristics. Biofilm structure provides a protective layer against antimicrobial compounds. [3] Wounds biofilms are polymicrobial, formed by complex and order combinations of microorganisms. Hence, compounds produced by different bacterial strains might impair the contact between the bacterial cell wall and the antibiotic by changing the composition of the EPS. [4]

Moreover, clinical isolates have been reported to harbor gene coding for *esp* virulence factor rather than the commensal strains. [5] Hence, the study was done to know the prevalence of drug resistance in clinical isolates of *Enterococcus* spp. and to find the association of drug resistance with biofilm formation and *esp* genes in this part of the country. [6]

Finally, the production of degradative enzymes by different pathogens can act in synergy against antibiotics. These biofilm aspects are responsible for a reduced diffusion of the antibiotic within the biofilm matrix leading to an inefficient activity of the antibiotic treatment. [7] In addition to this feature, the ability to form a biofilm is an effective strategy to enhance survival and persistence of microorganisms by increasing their antimicrobial resistance. [8]

The antimicrobial resistance in organisms producing biofilms acts by delayed penetration of the antimicrobial agents through the biofilm matrix, altered growth rate of biofilm organisms, and other physiological changes due to the biofilm mode of growth. [9]

Bacterial biofilms are complex surface attached communities of bacteria held together by self-produced polymer matrixs mainly composed of polysaccharides, secreted proteins, and extracellular DNAs. It is now understood that about 40–80% of bacterial cells on earth can form biofilms. It is now understood that about 40–80% of bacterial cells on earth can form biofilms. The formation of biofilms was detrimental in several situations. For example, in food industries, pathogenic bacteria are able to form biofilms inside of processing facilities, leading to food spoilage, and endangering consumer's health. [10]

MATERIALS AND METHODS

This is a prospective, descriptive and observational study conducted in the Department of Microbiology, Index Medical College, Hospital and Research center Indore from January 2019 December 2021. All isolates of Enterococcus species during the study period will be included.

INCLUSION CRITERIA

- All patients over 18 years of age having chronic diabetic foot ulcers where ulcer duration is greater than three months were included in the study
- Persons willing to give consent.

EXCLUSION CRITERIA

- Children (<18 years) was excluded.
- Pregnant women
- Patients with other comorbid conditions like chronic venous insufficiency, and osteomyelitis
- Persons not willing to give consent.

Enterococci isolated from clinical specimens like pus, wound swab and aspirates etc. received in Microbiology Department.

All samples were processed by standard bacteriological procedures. Gram staining was done for pus, wound swab and aspirates and findings were recorded. Culture was done on 5% sheep blood agar and MacConkey agar. Inoculated plates were incubated at 37°C for 18- 24 hours. Preliminary identification of Enterococci was done by standard bacteriological techniques including colony morphology, gram staining and catalase test. Farther specification was done by Bile Aesculine test, Pyrrolidonyl Arylamidase (PYR) test, growth in presence of 6.5% Sodium chloride, growth at 10°C and 60°C and heat resistance test.

RESULTS

Table 1: Gender wise distribution of the study subjects

Gender	E. faecalis		E. faecium	
	No. of isolates	%	No. of isolates	%
Male	42	58.33	34	68
Female	30	41.66	16	32
Total	72	100	50	100

A total number of 72 isolates of E. faecalis, 42 (58.33%) were isolated from males whereas 30 (41.66%) from female patients of diabetic foot ulcers. In case of E. faecium, 34 (68%) were isolated from males and 16 (32%) isolated from females. In present study, males [76 (62.29%)] predominance was noted over females [46 (37.70%)]. This revealed that diabetic foot ulcer was prevalent in the male population in our study. (Table no. 2)

Table 2: Frequency of Age distribution among the study subjects

Age	No. of individuals	Percentage
31-40 years	25	20.49
41-50 Years	28	22.95
51-60 Years	39	31.96
61-70 Years	30	24.59
Total	122	100.0

According to age wise distribution of the study subjects, the majority of the patients (39 patients) belonged to the age group 51-60 years. Followed by 30 patients in the age group of 61-70 years. The age and gender wise distribution of the study subjects is shown in the following table no.3.

Table 3: Prevalence of Biofilm Producer Enterococci

Total no. of Enterococci isolated	Biofilm producers	Non-biofilm producers
122(100%)	66 (54.09%)	56 (45.90%)

In table 5, Of total 122 Enterococcus species isolated from diabetic foot ulcers, in that 66 (54.09%) isolates were biofilm producers and 56 (45.90%) were biofilm non-producers.

Table 4: Biofilm assay

No. of isolates	Congo red agar method			Tube method			Tissue culture plate method		
	High	Moderate	Weak	High	Moderate	Weak	High	Moderate	Weak
66	5(7.5 %)	22(33.33 %)	39(59.09 %)	16(24.24 %)	39(59.09 %)	11(16.66 %)	16(24.24 %)	34(51.51 %)	16(24.24 %)

In our study, Biofilm assay of High Congo red agar method 7.5%, tube method 24.24%, Tissue culture plate method 24.24%. On the other hand, Weak Congo red agar method 59.09%, tube method 16.66%, Tissue culture plate method 24.24% in table 4.

Table 5: Antibiotic susceptibility pattern according to Biofilm formation of Enterococcus species

Antibiotics	Sensitive		Resistant	
	Biofilm former	Non biofilm former	Biofilm former	Non biofilm former
Cefoxitin	12	35	35	40
Clindamycin	3	33	40	46
Penicillin G	17	10	38	46
Amikacin	60	47	14	11
Ciprofloxacin	16	34	50	45
Lenizolid	65	42	06	06
Gentamycin	36	14	36	36
Amoxy-clav	14	36	41	26
Tecoplanin	83	20	12	7
Vancomycin	80	37	04	1
Tetracycline	12	35	32	49

In our study, among sensitive, few strains were sensitive to Clindamycin, Amikacin and Lenizolid, 1, 15, 17 respectively. Rate of resistance to Penicillin G: 6, Tetracycline 6, Gentamycin 3, Clindamycin 6, Amoxy-clav 6, Cefoxitin 6 and Ciprofloxacin 2. Moreover, among resistant few strains were sensitive to Clindamycin, Amikacin and Lenizolid 9, 2, 2 respectively. Rate of resistance to Penicillin G: 5, Tetracycline 6, Gentamycin 7, Clindamycin 7, Amoxy-clav 7, Cefoxitin 5 and Ciprofloxacin 1 in Table 5.

DISCUSSION

In our study, of the 190 samples collected from patients with diabetic foot ulcers, it is found in the present study that the male to female ratio is 2.6:1 (121 males as compared to 69 females. [11] Male incidence is higher and the possible reasons may be males are exposed more to trauma during heavy manual work. [12] Smoking habits are higher in males, may cause peripheral arterial disease that may coexist with diabetes which flare up the lesions. This was almost similar to study conducted by M Madan et al where 70% of males were affected as compared to 30% of females. [13] Even study conducted by Vinod kumar et al showed males were affected more than females (M:F=1.6:1) and also male to female ratio was similar to our study in a tertiary hospital in Nigeria which was 2.3:1. [14]

In our study, the maximum number of infections was found in patients aged 51-60 years. In the literature, the maximum number of infections was reported in patients aged 51-60 years by Ibrahim et al. [15] and in patients aged 60-65 years by Shanmugam et al . [16] This may be attributed to the high prevalence of comorbid conditions in this age group. When compared with the recent study of M Madan et al, age difference was almost similar with the present study conducted on hundred patients. [17]

In our study, eighty-nine (48.33%) of the isolates showed biofilm formation. *Staphylococcus aureus* was the predominant biofilm former, with 24 (26.9%) of the isolates testing positive for biofilm formation. The second highest biofilm formation was by *Pseudomonas aeruginosa* was 17 (19.1%) followed by *Citrobacter sp.* was 13 (14.6%), *Enterococcus sp.* was 5 (13.4 %) and *Proteus sp.* (4.4 %). A study by Vinod kumar et al showed Pseudomonas as an emerging pathogen in diabetic foot infections which were detected in 54 out of 310 patients on pus culture specimens (17%). [18] This present study also showed pseudomonas in 10% of pus culture specimens. Also in a study conducted by Tiwari S et al, it was found that E. Coli was the most common organism followed by Staphylococcus aureus and other pathogens. [19]

In our study, Biofilm assay of High Congo red agar method 7%, tube method 25%, Tissue culture plate method 25%. On the other hand, Weak Congo red agar method 58.33%, tube method 16.66%, Tissue culture plate method 25%.

In our study, Among E. facalis organism, few strains were sensitive to Clindamycin, Amikacin and Lenizolid 33.33%, 100%, 100% respectively. Rate of resistance to Penicillin G 12 (80.00%), Tetracycline 13 (86.66%), Gentamycin 11 (73.33%), Clindamycin 10(66.66%), Amoxy-clav 10 (66.66%), Cefoxitin 9 (60.0%) and Ciprofloxacin 12 (80.00%). Moreover, Among E. faecium organism few strains were sensitive to Clindamycin, Amikacin and Lenizolid 25%, 50%, 75% respectively. Rate of resistance to Penicillin G: 6 (75%), Tetracycline 3 (37.5%), Gentamycin 3 (37.5%), Clindamycin 6(75.0%), Amoxy-clav 3 (37.5%), Cefoxitin 5 (62.5%) and Ciprofloxacin 6 (75%).

In our study, among sensitive, few strains were sensitive to Clindamycin, Amikacin and Lenizolid, 1, 15, 17 respectively. Rate of resistance to Penicillin G: 6, Tetracycline 6, Gentamycin 3, Clindamycin 6, Amoxy-clav 6, Cefoxitin 6 and Ciprofloxacin 2. Moreover, among resistant few strains were sensitive to Clindamycin, Amikacin and Lenizolid 9, 2, 2 respectively. Rate of resistance to Penicillin G: 5, Tetracycline 6, Gentamycin 7, Clindamycin 7, Amoxy-clav 7, Cefoxitin 5 and Ciprofloxacin 1.

The percent of biofilm formers in our study is significantly larger in comparison to a previous study and corresponds to studies by Swarna et al. and James et al. [20] The higher percentage of biofilm formers in diabetic wounds could be due to ineffective debridement procedure or longer duration of ulcer in patients. [21]

Studies have shown that biofilm-associated microorganisms can be up to 1,000 times more resistant to antibiotics than free-floating planktonic bacteria. [22] The biofilm structure has been analysed microscopically and biochemically to show multiple layers of bacteria encased in a biofilm matrix containing proteins, DNA, and polysaccharides. The mechanism of multidrug resistance in biofilm-forming organisms is believed to be a direct result of close cell-cell contact in the biofilm, which allows for easy transfer of plasmids containing MDR genes amongst one another. [23]

Organisms, which form biofilms, are also characterised by tolerance, which is a temporary, non-heritable characteristic. [24] The mechanisms for tolerance are: (1) Antibiotics whose mechanism of action depends on the division of cells are inactive against microbes in a biofilm, which are in a slow-growing, dormant state. [25] (2) Drug permeation is hindered the polysaccharide matrix of the biofilm. [26] (3) Drug efficacy is altered in the microenvironment of the biofilm (pH and osmotic variations). [27] In addition to their effect on antimicrobial agents, biofilms also block host defences. They have an anti-phagocytic property, which inactivates leukocytes in the polysaccharide matrix. There is also an element within the matrix that disables both complement and host antibodies. [28]

Wounds can become infected by bacteria that encapsulate themselves in biofilms over time or when the body's natural defense mechanisms are impaired. [29] A non-healing wound is an indicator of the presence of biofilm. [30] Biofilms cause a delay in healing by initiating an immune response leading to chronic inflammatory cycle and tissue damage due to high levels of proteases and reactive oxygen species. [31] Several virulence genes are implicated in biofilm formation, like icaA and icaD, responsible for the biosynthesis of polysaccharide intercellular adhesion (PIA) molecules, containing N-acetylglucosamine, the main constituent of the biofilm matrix in the accumulation phase. [32]

CONCLUSION

In the present study, 48.33% of drug resistant bacteria were biofilm formers. Infections with bacteria forming biofilms are difficult to eradicate. These biofilms are not only less susceptible to host cell immune responses but also have a high tolerance to antibiotics than

the planktonic cells. The resistance of biofilm forming bacteria towards antibiotics is due to obstruction in the permeability of the drug by the polysaccharide matrix and alteration of the drug efficacy in the biofilm environment.

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