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GENOTYPIC DETECTION OF BIOFILM FORMATION AND ITS ASSOCIATION WITH ANTIBACTERIAL RESISTANCE IN CLINICAL ENTEROCOCCI ISOLATES FROM URINE SAMPLES

Medical Microbiology

Pooja M Gavade Post Graduate, JSS medical college, Mysuru, India.
Dr. Rashmi P Mahale Associate Professor, JSS medical college, Mysuru, India.

ABSTRACT

Enterococci are commonly found in the human gastrointestinal and female genital tract. Urinary tract infections are the most common infections caused by uropathogenic Enterococci. These bacteria are among the leading causes of multidrug-resistant, hospital-acquired infections. They produce various virulence factors, including biofilm formation. Biofilms allow bacteria to adapt and survive under many adverse environmental conditions, such as resistance to antibiotics. Enterococci acquire many resistance genes through biofilm. Many virulence factors influence biofilm formation in Enterococci, such as the Enterococcal Surface Protein (ESP). **Materials and methods:** In this 6-month hospital based retrospective study, a total of 100 urinary samples were collected in the department of microbiology and were processed according to the standard operative protocol. **Results:** A total of 50 clinical isolates of Enterococci were subjected to real-time PCR, out of which 35 (70%) were found to be positive for Esp gene and 15 (30%) were negative. Out of 35 isolates who harbored Esp gene, 33 (66%) were biofilm producers by phenotypic method. **Conclusion:** There was a strong correlation between biofilm formation and increased antibiotic resistance among biofilm forming Enterococci.

KEYWORDS

UTI, antimicrobial resistance, biofilm and Enterococci.

INTRODUCTION

The most common infection caused by Enterococci is urinary tract infection, followed by bacteremia, endocarditis and hospital acquired infections like CAUTI. Many infections are associated with biofilm. The biofilm formation is also responsible for developing antibiotic resistance at very higher rates.

Highly complicated Enterococcal infections are mainly due to the development of resistance towards antimicrobial agents like β-lactams, macrolides, glycopeptides and other clinically used antibiotics. The mechanism of developing resistance in Enterococci is either due to intrinsic or acquired through horizontal transfer of antibiotic-resistant genes. The three major mechanisms involved in intrinsic resistance includes low cellwall permeability, efflux of antibiotics or altering the membrane bound protein. Enterococci exhibit intrinsic resistance to many antibiotics, such as cephalosporins, aminoglycosides and cotrimoxazole. Several antibiotic-resistant genes are identified in Enterococci; blaZ, vanA, vanB, vanC-1, tetK, tetO, tetM, ermB, ermC, and aac (6') le- aph (2") la (7). (1). The mechanism of acquired resistance in Enterococci is due to mutations in genetic determinants and horizontal gene transfer, including the exchange of plasmids and transposons between bacteria within in biofilms. These factors interact with bacterial chromosomes, leading to the formation of new genetic components. The cells in the biofilm communicate with each other by secreting peptide pheromones and auto- inducing quorum signaling system. (2)

Esp (Enterococcal surface protein) is a large surface protein consisting of 1873 amino acids, with an N- terminal domain, a central core region and a C- terminal domain. Esp aids in the initial attachment of bacteria to surfaces. Some studies have shown that biofilm formation in *E. faecalis* is independent of Esp. Among the Enterococcal proteins, Esp is the only one whose role in virulence has not been defined well. (3)
In this study, the Esp gene was detected genotypically by real-time PCR and the isolates were then compared with their antibiotic resistance patterns and biofilm properties.

CASE STUDY

This is a prospective based hospital study carried over a period of 6 months in the department of microbiology, JSS medical college, Mysuru. All the urine samples from suspected patients of UTI were processed according to the standard operative protocol.

Antibiotic susceptibility test: A total of 100 Enterococcal isolates were subjected to antibiotic susceptibility testing by Kirby-Bauer disk diffusion method and results were interpreted according to CLSI guidelines. The antibiotics studied in this study included penicillin, ampicillin, tetracycline, high level gentamycin, linezolid, nitrofurantoin, norfloxacin, teicoplanin and vancomycin.

Biofilm detection by tissue culture plate method: A total of 100 Enterococcal isolates were subjected to biofilm detection by tissue culture plate method. The test was done in triplicates and average was taken for interpretation.

Table 1: O D readings interpretation.

OD readings	Interpretation
O D < O D C	Non biofilm formers
O D. C < O D ≤ (2x O D. C)	Weak biofilm formers
(2x O D. C) < O D ≤ (4x O D. C)	Moderate biofilm formers
(4x O D. C) < O D	Strong biofilm formers

Screening of Esp gene by real-time PCR.

DNA extraction was performed using by Helini Purefast Bacterial DNA Mini Spin Preparation Kit from pure cultures according to the kit protocol. The purified DNA was screened for the presence of ESP gene by real-time PCR. A total volume of 25µL reaction mixture was made by adding 12.5µL master mix solution, 1µL of extracted bacterial DNA, 1µL of forward TTGCTAATGCTAGTCCACGACC and reverse GCGTCAACACTTGCATTGCCGAA primer each and 9.5µL of distilled water. DNA amplification steps included denaturation step at 95°C for 10 seconds and 40 amplification cycles each with. Annealing step at 49°C for 15 seconds and extension step at 60°C for 45 seconds followed by an additional step of extension for 10 minutes at 60°C. Sterile water was used as negative control. The presence or absence of Esp gene was interpreted as positive and negative respectively, according to the graph and data obtained.

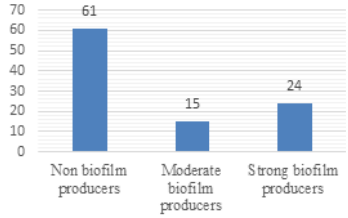
RESULTS

Phenotypic detection of biofilm formation by TCP method.

All the 100 isolates of Enterococci were subjected to biofilm detection by tissue culture plate method. Out of 100 isolates, 61(61%) were found to be non-biofilm producers and 39 (39%) were found to be biofilm producers. Among the 39 biofilm producers, 15 (15%) were moderate biofilm producers and 24 (24%) were strong biofilm producers.

Table 2: Biofilm test results.

Biofilm results	No. of isolates %
Non biofilm producers	61
Moderate biofilm producers	15
Strong biofilm producers	24
Total	100



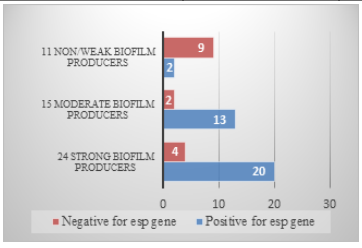
Graph 1: Biofilm test results.

Genotypic detection by real -time PCR.

Among 100 Enterococcal isolates, 50 were screened for the presence of Esp gene by real-time PCR, out of 50 isolates, 39 were biofilm producers and 11 were non/weak -biofilm producers by phenotypic method. Among the 50 isolates, 35 (70%) were found to be positive for Esp gene and 15 (30%) were found to be negative. Out of 35 (70%) isolates which harbored Esp gene, 33 (66%) were biofilm producers by phenotypic method and 2 (4%) were non/weak biofilm producers.

Table-3: - PCR results of the isolates.

No. of isolates	Positive for Esp gene	Negative for Esp gene
24 Strong biofilm producers	20 (40%)	4 (8%)
15 Moderate biofilm producers	13 (26%)	2 (4%)
11 Non/weak biofilm producers	2(4%)	9 (18%)
Total 50	35 (70%)	15 (30%)



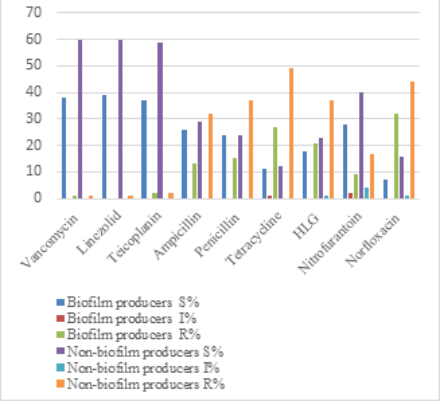
Graph 2: PCR results of the isolates.

Comparison of antibiotic sensitivity pattern between biofilm producers and non-biofilm producers.

Among 39 biofilm producers, 38 (97.4%) were sensitive to vancomycin and 1 (2.5%) was resistant to vancomycin, all were sensitive to linezolid, 37 (94.8%) were sensitive to teicoplanin and 2 (5.1%) were resistant, 24 (61.5%) were sensitive to penicillin and 15 (38.4%) were resistant, 26 (66.6%) were sensitive to ampicillin and 13 (33.33%) were resistant, 11 (28.2%) were sensitive to tetracycline, 1 (2.5%) was intermediate and 27 (69.2%) were resistant, 28 (71.79%) were sensitive to nitrofurantoin, 2 (5.1%) were intermediate and 9 (23.07%) were resistant, 18 (46.15%) were sensitive to HLG and 21 (53.8%) were resistant, 7 (17.94%) were sensitive to norfloxacin and 32 (82.05%) were resistant.

Table 4: Comparison of antibiotic sensitivity pattern between biofilm producers and non- biofilm producers.

Antibiotics	Biofilm producers			Non-biofilm producers			Total
	S%	I%	R%	S%	I%	R%	
Vancomycin	38	0	1	60	0	1	100
Linezolid	39	0	0	60	0	1	100
Teicoplanin	37	0	2	59	0	2	100
Ampicillin	26	0	13	29	0	32	100
Penicillin	24	0	15	24	0	37	100
Tetracycline	11	1	27	12	0	49	100
HLG	18	0	21	23	1	37	100
Nitrofurantoin	28	2	9	40	4	17	100
Norfloxacin	7	0	32	16	1	44	100



Graph 3: Comparison of antibiotic sensitivity pattern between biofilm producers and non-biofilm producers.

DISCUSSION

The most common infection caused by Enterococci is urinary tract infection. Enterococci cause up to 15 to 20% of hospital acquired, urinary tract infections. Antibiotic resistance in Enterococci is another major problem to treat severe infections as they are resistant to antibiotics like β- lactams, aminoglycosides and glycopeptides. The biofilm formation is also responsible for developing antibiotic resistance at very higher rates. In the current study, 99% of Enterococcal isolates were sensitive to linezolid followed by 98% to vancomycin and 96% to teicoplanin. The higher rate of resistance was found to be 76% to tetracycline and 58% to HLG and the lower rate of resistance was found to be 1% to linezolid. The study conducted by F. Fallah et.al in 2017 (4) reported 87.8% resistance rate to tetracycline. Their study reported a higher resistance rate to tetracycline and lower resistance rate to linezolid, which correlated with our study. In our study, the biofilm formation by Enterococci was detected by TCP method. Among 100 Enterococcal isolates, 61% were found to be non/weak biofilm producers, 15% were found to be moderate biofilm producers and 24% were strong biofilm producers. This study correlated with a similar study conducted by Yomna Hashem et.al. (5) which reported a higher rate of non/weak biofilm producers. Many factors influence the biofilm formation in Enterococci like agg, gelE, esp, fsr locus and environmental factor. Esp codes for Enterococcal surface protein, responsible for the initial attachment during biofilm formation. In this study, isolates of Enterococci were screened for the presence of Esp gene by real -time PCR. Out of 50 isolates, 35 (70%) were found to be positive for Esp gene and 15 (30%) were negative. A similar study conducted by Y. Seno et.al. in 2005 (6) reported that, 72.2% of the isolates harbored Esp gene, which correlated with our study. Some studies have suggested that Esp gene enhances the biofilm production but some studies have suggested that Esp is not much necessary for the biofilm production in Enterococci. Even-though Esp helps in initial adhesion during biofilm formation but it does appear to be the only virulence factor which induce significant biofilm formation in Enterococci. However higher rate of Esp gene was seen in our study suggesting its role in biofilm formation by facilitating initial attachment.

CONCLUSIONS

Enterococci with the ability to form biofilm have developed both intrinsic and acquired resistance to many clinically used antibiotics, making treatment difficult. This study found a significant correlation between biofilm formation and increasing antibiotic resistance among biofilm forming Enterococcal isolates. The study concludes that, although Esp is required for biofilm formation, other virulence factors are also important for biofilm formation in Enterococci.

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