Section: Microbiology

Original Article

ISSN (O): 2347-3398; ISSN (P): 2277-7253

Study of Correlation between Virulence Factors and Drug Resistance among the Clinical Isolates of Enterococci

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Abstract

Background: The objectives is to study the correlation between virulence factors and drug resistance. **Subjects and Methods:** Detection of Virulence factors Hemolysin by using blood agar Gelatinase by using gelatine agar Biofilm by using tube adherence method, Detection of Drug resistance Detection of MIC by agar dilution method. **Results:** Virulence factors were produced by majority of VRE, LRE and HLGR strains. **Conclusion:** Virulence factors like hemolysin, Gelatinase and Biofilm were produced by majority of VRE, LRE and HLGR strains. This Proved that there is a significant association between the virulence factors and drug resistance.

Keywords: Virulence factor, drug resistance, biofilm, Hemolysin, Gelatinase, MIC.

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Received: 06 October 2021 **Revised: 19 November 2021** **Accepted: 28 November 2021** **Published: 31 December 2021**

Introduction

Enterococci once considered a harmless commensal has emerged as a medically important multi drug resistant virulent pathogen causing outbreaks of many nosocomial infections. NNIS (National Nosocomial Infection Surveillance System) reported that enterococci to be the second most common pathogen associated with Nosocomial Urinary tract Infection and it can cause dangerous infections such as bactremia, endocarditis, intra-abdominal and pelvic infection, surgical site infection and diabetic foot Infection.[1]

Among the enterococcal isolates E. faecalis and E.faecium are the leading cause of nosocomial infection. Many virulent factors have been identified in enterococci but none has been established as having a major contribution to virulence in humans.[2]

Hemolysin, Gelatinase and biofilm are most important virulence factors produced by enterococci. This virulence factors are responsible for drug resistance in enterococcal spp. Hemolysin is a post translationally modified protein toxin that occurs in many strains of enterococci.[3]

Gelatinase is an extra cellular protease produced by enterococci capable of hydrolysing gelatine, casein, collagen, Hemoglobin and other peptides.[4] Biofilm is an another virulent factor produced by enterococci on abiotic surfaces.[5]

Subjects and Methods

Virulence factors of clinical isolates of enterococcus were idenfied by human blood agar, Gelatine agar and tube adherence method. Drug resistance tested by detection of MIC by agar dilution method.

Detetction of Virulence Factors: Hemolysin (CYTOLYSIN):

Hemolysin production will be detected by inoculating enterococci onto freshly prepared blood agar plates. Plates were incubated at 37°C and evaluated after 24 and 48 Hrs. A clear zone of haemolysis around the stab or streak on human blood agar was considered to be a positive indication of Hemolysin production.[6]

Gelatinase

Gelatine agar is prepared by adding gelatine to nutrient medium. The organism is inoculated in gelatine agar plate and incubated at 37°C for 24 hours. Then the plate is flooded with mercuric chloride and clear zone around the colonies indicate Gelatinase production.[6]

Biofilm

Figure 1: Haemolysis on blood agar

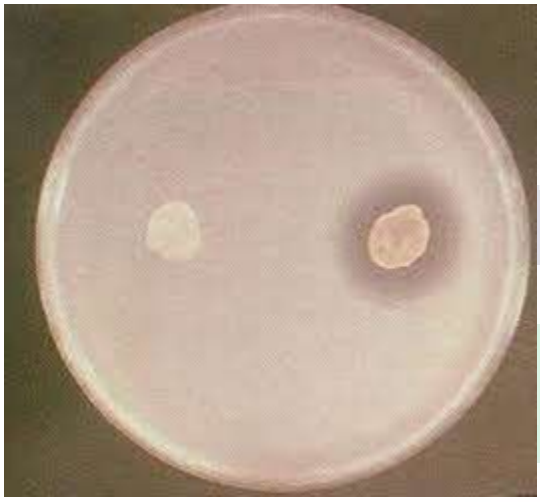

Figure 2: Gelatinase production

Tube Adherence Method:

Trypticase soy broth 10 ml was taken in sterile test tubes and was inoculated with loopful of microorganism from overnight culture plates and incubated for 24 hours at 37°C.

The tubes were decanted and washed with phosphate buffer solution (PH 7.3) and dried. The dried test tubes were stained with crystal violet (0.1%).

Excess stain was removed and tubes were washed with deionised water. Tubes were then dried in inverted position and observed for Biofilm formation. The Biofilm formation was positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of Biofilm formation. Tubes were examined and amount of Biofilm formation was scored as 0-absent, 1- week, 2-moderate, 3-strong.[6]

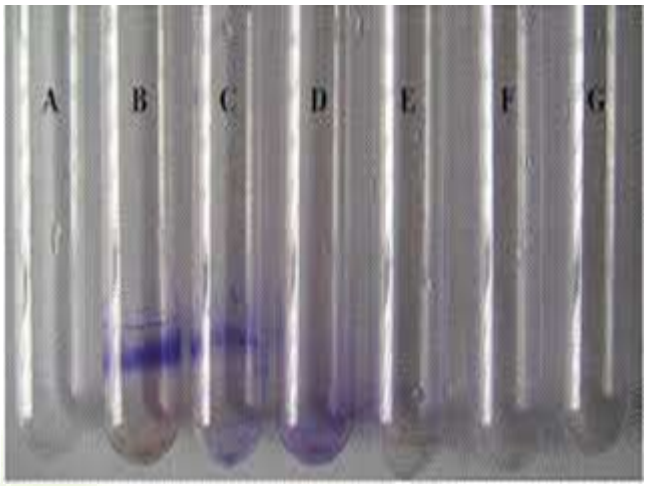

Figure 3: Tube Adherence method for Biofilm

Detection of drug resistance:

Detection of MIC by agar dilution method:

Minimum inhibitory concentration (MIC) detection for High level gentamicin, Linezolid and Vancomycin were tested by agar dilution method.

Antibiotics used:

High level gentamicin Linezolid Vancomycin

Procedure:

Label a sterile Petri dish on the base for each concentration required. Prepare the dilutions in water, placing 1ml of each in the appropriate dish. Pipette 19 ml melted agar, cooled to 55°C to each plate and mix thoroughly.

After the plates have set they should be well dried at 37°C for 20 to 30 minutes in an incubator. They are then inoculated with a wire loop or platinum loop calibrated to deliver 0.001ml spread over a small area.

The culture should be diluted to contain 105 to 106 organisms per ml, this can be obtained approximately by adding 5μl of an overnight broth culture to 5ml broth or peptone water. Each doubling dilution of an antimicrobial agent is incorporated into a single agar plate so that testing a series of dilutions of one drug. By this method one or more bacterial isolates are tested per plate. After incubation the plates are examined for growth and the MIC is the lowest concentration of anti-microbial agent in agar that completely inhibits the visual growth. The MIC break points are applied for interpretation of agar dilution methods.

The sensitivity of Vancomycin was confirmed with MIC by agar dilution method, the resistance was indicated by $\geq$ 32μg/ml, intermediate was indicated by 8-16μg/ml and the sensitive was indicated by 4μg/ml of concentration of Vancomycin. The minimum inhibitory concentration (MIC) for Enterococcal spp tested against Linezolid were as follows $\leq$ 2μg/ml for the susceptible category, 4 μg/ml for the intermediate category and $\geq$8 μg/ml for the resistant category. The MIC for Enterococcal spp tested against high level gentamicin was 500μg/ml (CLSI 2013).

Figure 4: MIC detection by agar dilution method

All isolates were detected for virulence factors production. Common virulence factors in Enterococcal spp are Hemolysin, Gelatinase and Biofilm. Among 89 E. faecalis 46 (51.68%) were producing Hemolysin, 39 (43.8%) were producing Gelatinase and 42 (47.19%) were producing Biofilm. Out of 11 E.faecium 3(27.27%) producing Hemolysin, 2 (18.18%) were producing Gelatinase and 4 (36.36%) were producing Biofilm. A total of 49(49%) Hemolysin, 41(41%) Gelatinase and 46(46%) Biofilm were produced.

In our study we highlighted only on High level gentamicin, Linezolid and Vancomycin sensitivity. Among 89 E.faecalis 14(15.73%) were resistant, 5(5.61%) were intermediate and 70(78.65%) were sensitive to high level gentamicin. Among 89 E.faecalis 1(1.12%) was resistant, 4(4.49%) were intermediate and 84(94.38%) were sensitive to Linezolid. Among 89 E.faecalis 3(3.37%) were resistant, 3(3.37%) were intermediate and 83 (93.25%) were sensitive to Vancomycin. Among 7 VRE strains, all 7(100%) were producing Biofilm, 4(57.14%) were producing Hemolysin and 5(71.42%) were producing Gelatinase. Out of 3 LRE strains, all 3(100%) were producing Biofilm and Gelatinase but only one (33.33%) was producing homolysing. Among 16 HLGR strains, 15(93.75%) were producing Biofilm,13(81.75%) were producing Hemolysin and 14(87.5%) were producing Gelatinase. Out of 26 resistant strains, 25(96.15%) were producing Biofilm, 18(69.23%) were producing Hemolysin and 22(84.61%) were producing Gelatinase.

Discussion

Enterococci once considered a harmless commensal survive in the gastrointestinal tract of human has emerged as a medically important multidrug resistant virulent pathogen causing outbre aks of many dangerous nosocomial infections like bacteraemia, endocarditis, urinary tract infections, intra - abdominal and pelvic infection, surgical site infection and diabetic foot infection.[7]

The recent increase of Vancomycin resistant (VRE) E.faecium strains in clinical isolates is especially a cause of serious concern because this glycopeptides type antibiotic often remains the last treatment available in life threatening infections.

All isolates were tested for virulence factors production. Common virulence factors in Enterococcal spp are Hemolysin, Gelatinase and Biofilm. In our study only 3 (3%) isolates showed resistance to Linezolid. Only one (1.12%) E.faecalis and 2(18.18%)E.faecium isolates showed resistance to Linezolid. MIC detected by agar dilution method showed the same result. A study in Iran by Yasliani et al reported that 2% of Enterococcal isolates were resistant to Linezolid and also reported 23.5% of VRE isolate were resistant to Linezolid.

In our study total of 18 (18%) isolates showed resistance to high level gentamicin by (Kirby Bauer) disc diffusion method. Out of these 14 (15.73%) isolates were from E.faecalis and 4 (36.36%) isolates were from E.faecium.

MIC detection by agar dilution method, 13(14.6%) isolates of E.faecalis and 3(27.27%) isolates of E.faecium showed MIC of gentamicin exceeds 500μg, which showed resistance to High level gentamicin.

Agarwal J from India reported that 8.3% of Enterococcal isolates showed resistance to high level gentamicin and the frequency of resistance to high level gentamicin was higher in E.faecium than E.faecalis.

The low prevalence of resistance to high level gentamicin suggests, gentamicin should used to get synergistic effect with cell wall active agents for the treatment of serious Enterococcal infections. High level gentamicin in combination with β lactams are used to treat Vancomycin resistant Enterococci.[8] Jang H C et alreported that bacteraemia caused by high level gentamicin resistant Enterococci was associates with more severe underlying disease and higher mortality.[9]

In the year 2012, HasaniA et al reported that 60.45 % of Enterococcal isolates were identifies as high level gentamicin resistant. Out of them 59.4% were E.faecalis and 40.6% were

Table 1: Prevalence of virulence factors inEnterococcal spp

Virulence factors	E.Faecalis n=89	E. Francium n=11	Total n=100
Haemolysin	46 (51.68%)	3 (27.27%)	49(49%)
Gelatinase	39 (43.8%)	2 (18.18%)	41(41%)
Biofilm	42 (47.19%)	4 (36.36%)	46(46%)

Table 2: Correlation between virulence factors and drug resistance

Resistance	Biofilm		Hemolysin		Gelatinase	
	+ve	-ve	+ve	-ve	+ve	-ve
VRE (n=7)	7(100%)	0	4(57.14%)	3(42.85%)	5(71.42%)	2(28.57%)
LRE (n=3)	3(100%)	0	1(33.33%)	2(66.66%)	3(100%)	0
HLGR (n=16)	15(93.75%)	1(6.25%)	13(81.25%)	3(18.75%)	14(87.5%)	2(12.5%)

E.faecium.[10] Among the total VRE isolated from our study, only one strain showed Linezolid resistance this proved that still Linezolid can be the drug of choice for VRE. Among 7 VRE strains, all 7(100%) were producing Biofilm, 4(57.14%) were producing Hemolysin and 5(71.42%) were producing Gelatinase.

Out of 3 LRE strains, all 3(100%) were producing Biofilm and Gelatinase but only 1(33.33%) was producing hemolysin. Among 16 HLGR strains, 15(93.75%) were producing Biofilm, 13 (81.75%) were producing Hemolysin and 14(87.5%) were producing Gelatinase. Out of 26 resistant strains, 25(96.15%) were producing Biofilm, 18(69.23%) were producing Hemolysin and 22(84.61%) were producing Gelatinase.

High level gentamicin resistance associated with the presence of Gelatinase among E.faecalis and the Biofilm was detected as a predominant virulent factor of E.faecium.[11]

In our study 81.25% of High level gentamicin resistant strains were haemolytic but the study by Mundy LM et al reported that only 45% of the High level gentamicin resistant strains were haemolytic.[12]

We also noted that, High level gentamicin resistance was more in E.faecalis than E.faecium but another Indian study showed that E.faecium was more commonly associated with High level gentamicin resistance.

Our study showed that the virulence factors like Hemolysin, Gelatinase and Biofilm were produced by majority of VRE, LRE and HLGR strains. This proved that there is a significant association between the virulence factors and drug resistance.

Conclusion

1. The prevalence of resistance to High level gentamicin, Linezolid, Vancomycin Was found low among the clinical isolates.
2. Significant association between virulence factors and drug resistance was found.

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How to cite this article: Ravichandran L, Umadevi S, Pramodhini S, Srirangaraj S. Study of Correlation between Virulence Factors and Drug Resistance among the Clinical Isolates of Enterococci. Asian J. Med. Res. 2021;10(4):1-5.

DOI: dx.doi.org/10.47009/ajmr.2021.10.4.MB1

Source of Support: Nil, **Conflict of Interest:** None declared.