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Antibiotic Resistance and Susceptibility Profile, with Biofilm Forming Potential of Clinically Isolated *Enterococcus Specie* from Blood and Urine Samples

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ABSTRACT

Enterococcus spp. is Gram-positive cocci bacteria among the normal flora in human intestine. Common species are *Enterococcus fecalis* and *Enterococcus faecium*. They work as probiotics and help in digestion and immune system modulation. *Enterococci* are opportunistic pathogens and can cause nosocomial infections. Biofilm is one of the factors that facilitate the antibiotic resistance, which is complexEPS (exopolysaccharides) structure surrounding microbial cells, makingthem resistant from antimicrobial treatments. Due to acquisition of different virulence factors and resistance from antibiotics, infections of *Enterococcus spp*., have increased. In this study antibiotic resistance and sensitivity profile with their ability to form biofilm, has been determined.Human blood and urine samples were taken from Memon Medical Hospital Karachi. Culture and sensitivity test was performed for the major groups of antibiotics used for treating enterococcal infections with analysis of biofilm formation.Highest resistance was observed against the antibiotic levofloxacin 90% and all organisms were sensitive from linezolid 100%. Overall organisms were resistance from one and more antibiotics. All organisms were able to form biofilms as well. Antibiotic resistance has become an alarming challenge for the health care globally. Enterococcalresistance from major groups of antibiotics for their treatment has increased the rate of nosocomial infections too.

Key Words: Enterococcus Spp., Antibiotic resistance, Biofilm formation, Gram-positive Cocci, BSI, UTI

1. INTRODUCTION

Enterococci are group of lactic acid bacteria in different environments. They are found in, animals and human digestive system, and from natural biomes such as soil, sewage, water and arable land (Abdulla & Abdulla, 2006). They can be isolated from plants such as olive and in some wild plants. Some of their species are commensal, they can induce immune response, and responsible for the homeostasis of human GIT(Ahmed & Baptiste, 2018). *Enterococci* are also probiotic (diet supplement or therapeutic application) that support immune system and also play important role in food technology as their culture is involved in the fermentation of meats and cheeses along with food preservation, in prolonging their shelf life and improving their organoleptic properties (Krawczyk et al., 2021,Výrostková et al., 2021).

Genus *Enterococcus* is clinically important due to antibiotic resistance, contributing to the risk of colonization to establish an infection (Kristich et al., n.d.).*Enterococcus species* are commonly known to cause hospital acquired bloodstream infections (BSI) (Suppli et al., 2011).Most clinically important species are *Enterococcus faecalis* and *Enterococcus faecium*. *E. faecium* is more associated with Blood Stream Infections (BSI) with elevated rates of antimicrobial resistance in more severally ill patients, with higher mortality than *E. faecalis* BSI (Sattari-Maraji et al., 2019; Suppli et al., 2011). BSI caused by *Enterococcus*is often associated with intra-abdominal and endovascular sources, urinary tract, and older age, liver disease, renal impairment, diabetes, hematologic transplant, malignancy, male gender and prior treatment (Billington et al., 2014, Seo & Lee, 2013, Zhang et al., 2017).

Antibiotic resistance in *Enterococccus* is not a recent phenomenon but evidences have been reported decades ago from the developing countries as well, such as: an analysis of the data from Center for Disease Control (CDC) in the United States, between January 1989 and March 1993, revealed a twenty-fold increase in the prevalence of *Enterococci* resistant to vancomycin associated with nosocomial infections(Genaro et al., 2005). There are several factors involved in microbial drug resistance like misuse or overuse of antibiotics, bacterial mutation, insertion of eDNA (environmental DNA) or plasmid, and bacterial biofilm production(Bhandari et al., 2022, Costerton et al., 1987). Bacterial biofilm is a complex layer of EPS (exopolysaccharides) structure surrounding microbial cells (Fleming et al., 2016). Both, clinically important *Enterococcus Spp.* are capable of forming biofilm which is among the great cause of antibiotic resistance. Bacteria that form biofilms cause more than 65% of nosocomial infections and 80% of bacterial infections, which may become a severe concern in the area of antibiotic resistance (García-Solache & Rice, 2019, La Rosa et al., 2022). This property

enables the colonization of inert and biological surfaces as well as mediates the adherence to host cells(Cui et al., 2020, Shridhar & Dhanashree, 2019). The aim of this study is also to evaluate the possible evidences of antibiotic resistance and biofilm formation by clinically isolated *Enterococcus Spp.* from a hospital in Karachi, Pakistan.

2. MATERIALS AND METHODS

2.1 Ethical considerations of the study

This study had no direct interaction with the patients or attendants, so patient consent forms were waived off. Data publication approval letter was obtained from Memon Medical Hospital.

2.2 Sample collection

Random samples of human blood (2) and urine (09) were taken from the clinical Laboratory at Memon Medical Institute (MMI) Hospital and cultured on the respective soiled agar media plates (Billington et al., 2014).

2.3 Identification of organisms

Morphological, Biochemical and Microscopic characteristics of suspected organisms as *Enterococcus spp*. was done for identification (Kim et al., 2018).

2.3 Antibiogram Assay

Kirby-Bauer disk diffusion method was used for the mentioned antibiotics (Table 1). Briefly, Mueller Hinton agar (MHA) media plates were used for determination of Zone of Inhibition with 0.5% McFarland standard of culture broth suspension and incubated at 37°C for more 18 to 24 hours. Plate was check for the zone of inhibitions around the tested antibiotic discs. Based on these ZOIs, the tested bacterial strain was considered as sensitive, resistant of intermediate susceptible to that particular drug(Lee, 2013; Sattari-Maraji et al., 2019)

2.4 Screening of biofilm forming bacteria: Bacteria were tested for biofilm production with the help of Congo Red Agar (CRA) differentiating them as biofilm and non-biofilm forming. CRA was prepared with the standard protocol as mentioned by Freeman (Freeman et al., 1989). Organisms forming grayish to black color colonies were considered as biofilm former and pink color colonies as non-biofilm former.

2.5 Biofilm formation on 96 well microtiter plates

Microtiter-plate method was used for quantitative analysis of isolated cultures for biofilm formation (Costerton et al., 1987). Shortly, cultures were inoculated in 3–5 ml of Trypticase Soya Broth (TSB) following 24 hours incubation. Afterwards, dilution of cultures was done as 1:100 in fresh broth, added with 0.2% glucose. 200 µL diluted culture was taken into each well of flat-bottom 96-well microtiter plate, incubated (covered) for 48 hours. Free floating filling were removed and wells were thrice washed with PBS. All tests were performed in triplicate.

2.6 Biofilm Quantification

Wells were stained for 10 min with 125 µLof 0.2% Crystal Violet solution. Subsequently, Plate was washed with clean water, and left to air dry. Subsequently, 200 µL of Ethanol (95%) was added to all tested wells and left for 10 to 15 minutes at room temperature (Shukla & Rao, 2017). Control was kept as blank TSB. OD (Optical density) of wells was observed at 595nm using a 96well-plate reader (Diatek Dc-200Bc).

3. RESULTS & DISCUSSION

The study findings revealed a significant phenomenon of emerging drug resistant in *Enterococcus Spp.*, isolated from human blood and urine samples. Grown cultures were first identified as *Enterococcus* Spp. and their antibiogram assay was observed against common groups of antibiotics used as treatment options for the Enterococcal infection. Eleven human blood (2) and urine (9) samples were tested for their antibiogram analysis by using Ampicillin, Amox + Clave (Augmentin), Levofloxacin, Vancomycin, Nitrofurantoin, Meropenem, Teicoplanin, Linezolid (Table 1). Almost all organisms were resistant from one or more antibiotics. Highest resistance was observed against the antibiotic levofloxacin 90% and all organisms were sensitive from the antibiotic linezolid 100%. Overall resistance was 27.24% whereas sensitivity was 72.72%. Vancomycin, Nitrofurantoin, Teicoplanin, Linezolid and Meropenem were among the effective antibiotics with a lesser resistant organism sensitive from all antibiotics were 9.09%, resistant from one antibiotic were 45.45%, resistant from two antibiotics were 18.18% and multidrug resistant were 27.27 %(Figure and table 4). Screening of Biofilm forming isolates was done by Congo Red Agar (CRA) method on the basis of color of colonies on agar plates. Organisms forming colonies of grayish to black color were considered biofilm former and the others forming pink color colonies were non biofilm former. All isolates were biofilm former as they have grown with grayish to black color colonies. Biofilm formation is among one of the tools bacteria adopt to escape from the

antimicrobial treatments. *Enterococcus Spp*. is also a well-known biofilm former and in among the emerging antibiotic resistant organisms. Biofilm forming potential of isolated Enterococcus was also observed qualitatively and quantitatively. Optical density (OD) of cells attached to the bottom of 96-well microtiter plate was measured with the help of the method mentioned above. OD range was observed as 0.196 to 0.907 at 595nm.In view of current findings of this research it is observed that there is a higher antibiotic resistance risk in the organisms which are capable of forming antibiotics. There has been significant evidence of both biofilm formation and antibiotic resistance in isolated organisms. All categories of antibiotic resistance, such as resistant from one antibiotic, resistant from two antibiotics, and multidrug resistant were noticed (Figure and table 4) from the major groups antibiotics used as treatment option for treating enterococcal infection.

Entrococuus drug resistance is a warning concern for the medical sciences as well as food and drug designing industries. Previously its use as a probiotic has also now become controversial due to the increase rate of antibiotic tolerance (Boccella et al., 2021). Not only *Enterococcus* but overall if we evaluate for the other clinically pathogenic bacteria, antimicrobial resistance has grown over the time in past few decades. That is why health-care, food and pharmaceutical industries are more concerned on the research based approaches to reduce the emergence of drug resistance globally (Výrostková et al., 2021). There are several factors responsible for antibiotic resistance discussed previously (Miller et al., 2014). Our study is based upon the evaluation of antibiotic resistance along with biofilm forming capability of clinically isolated *Enterococcus ssp.*

Table 1: sensitivity and resistance profile, R: resistance, S: Sensitive

Specimen	Organism	Ampicillin	Amox + Clave (Augmentin)	Levofloxacin	Vancomycin	Nitrofurantoin	Meropenem	Teicoplanin	Linezolid
Urine	*Enterococcus Spp.*	S	S	R	S	S	S	S	S
		S	S	S	S	S	S	S	S
		S	S	R	S	S	S	S	S
		R	S	R	S	S	S	S	S
		S	S	R	S	S	S	S	S
		S	S	R	S	S	S	S	S
		R	R	R	R	S	S	R	S
		R	R	R	R	R	R	R	S
		S	S	R	S	S	R	S	S
Blood		R	R	R	S	S	S	S	S
		S	S	R	S	S	S	S	S
No of organisms resistant		4	3	10	2	1	2	2	0
No of organisms sensitive		7	8	01	9	10	9	9	11
Total no of Organisms		11							

Table 2: Percentage of Resistance and Sensitivity

Percentage of Resistance and Sensitivity	Ampicillin	Amox + Clave (Augmentin)	Levofloxacin	Vancomycin	Nitrofurantoin	Meropenem	Teicoplanin	Linezolid
Sensitive	63.63	72.72	9.09	81.81	90.90	81.81	81.81	100
Resistance	36.36	27.27	90.90	18.18	9.09	18.18	18.18	00

Table 3: Optical Density of Biofilm Results

Sample	organism	Biofilm OD (optical density)
Urine	*Enterococcus Spp.*	organism
		0.308
		0.313
		0.349
		0.352
		0.434
		0.436
		0.523
		0.625
Blood		0.349
		0.907

Table 4: Categories of antibiotic resistance and sensitivity

Total organisms	Sensitive from all antibiotics	Resistant from one antibiotic	Resistant from two antibiotics	Resistant from two antibiotics
11	1	5	2	3
%	9.09	45.45	18.18	27.27

100%. Overall resistance from the isolated organisms was 27.24% whereas sensitivity was 72.72%. On the basis of different categories organisms sensitive from all antibiotics were 9.09% resistant, from one antibiotic were 45.45%, resistant from two antibiotics were 18.18% and multidrug resistant were 27.27 % (Figure, table 4). Vancomycin, Nitrofurantoin, Teicoplanin, Linezolid and Meropenem were among the effective antibiotics. In case of antibiotic sensitivity our results are somewhat comparable to the findings of Abdilla et al who concluded that vancomycin, Teicoplanin, Nitrofurantoin and Chloramphenicol are also significantly effective on their isolates (Abdulla & Abdulla, 2006) and opposite to Ahmed & Baptiste, according their review about vancomycin-resistant enterococci (VRE) (Ahmed & Baptiste, 2018) On the other hand antibiotic resistance of our findings against levofloxacin is similar to the findings of Gilho Lee, who concluded high rate of fluoroquinolone resistance in *Enterococcus Spp* (Lee, 2013, Yasufuku et al., 2011). Long ago fluoroquinolone (Ciprofloxacin) was considered to be effective and safe in the treatment of serious urinary tract infections. Because the concentrations of the drug in urine exceed the MICs for virtually all potential urinary pathogens and it used to be most useful for treating infections caused by multidrug resistant organisms (Fass, 1987), but at present several researches have demonstrated the elevated resistant pattern against fluoroquinolone drugs (Kristich et al., n.d.).

Biofilm formation is among one of the tools bacteria adopt to escape from the antimicrobial treatments (Cui et al., 2020). *Enterococcus Spp*. is also a well-known biofilm former and among the emerging antibiotic resistant organisms, in our quantitative analysis by observing optical density (OD) of cells attached to the bottom of 96-

well microliter plate confirmed biofilm formation by the isolates and OD values were 0.196 to 0.907 at 595nm. More likely our findings match to the conclusion of Cui et al, that is 60.37% isolated strains of enterococci from yaks were MDR and 9 antibiotic-resistant genes were detected in his study with the great frequency of biofilm formation and virulence genes. AMR genes and virulence genes in enterococci from yaks is a serious concern because they could be transmitted to humans through the food chain(Cui et al., 2020). Another research revealed the emergence of antibiotic resistance in enterococci limits the therapeutic use of antimicrobials and a threat to hospitalized patients. Their findings also showed biofilm formation in the MDR isolates (Khalil et al., 2022). Alternatively, in Shridhar & Dhanashree, finding there was no significant correlation between drug resistance and biofilm production in both Enterococcus species. Thus, biofilm formation is not always associated with drug resistance in *Enterococcus spp* (Shridhar & Dhanashree, 2019). Antimicrobial resistance comprises of a combination of divers mechanisms adopted by microorganisms and among those biofilm formation is common in different type bacterial specie. To reduce and control the spread of MDR multi-drug resistant organisms, novel approaches towards better and effective treatment options should be made.

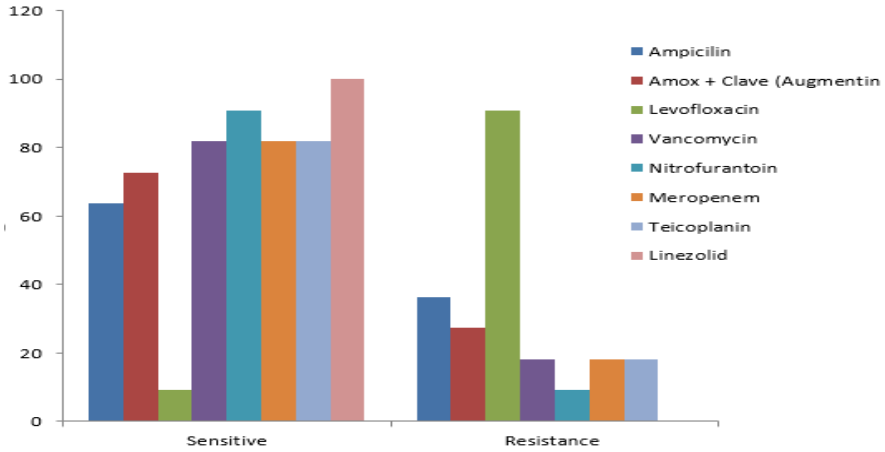

Figure 1: Overall Resistance and Sensitivity of organisms

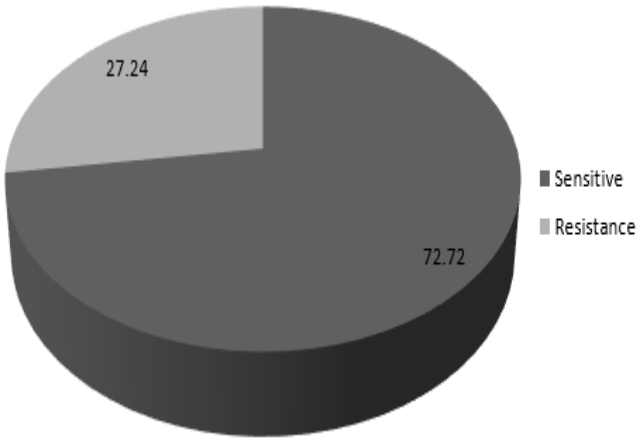

Figure 2:% of Resistance and Sensitivity

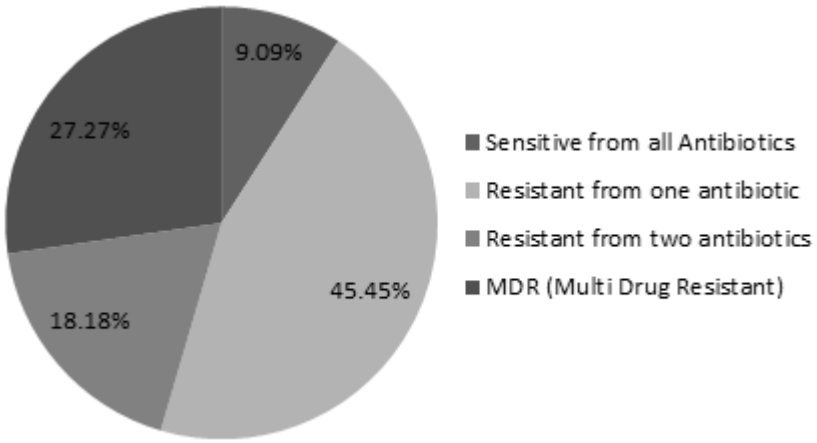

Figure 3: % of Different categories of antibiotic resistance and sensitivity

4. CONCLUSIONS

This study represents a possible relationship between antibiotic resistance and biofilm formation with the least effective use of levofloxacin for the treatment of enterococcal infections in Urinary tract and Blood Streams. As all organisms were biofilm former so drugs with ability to interfere with biofilm progression should be studied and designed.

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Authors Contribution: All authors have equally contributed throughout during the study, and agreed for the publication of manuscripts.

Ethical Statement: This study procedure did not include any direct contact to the patient or their attendants.

Informed Consent Statement: Not applicable

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