ijlpr 2022; doi 10.22376/ijpbs/lpr.2022.12.2.L86-92

International Journal of Life science and Pharma Research

ISSN 2250-0480

Research Article

Microbiology for health care

Phenotypic Surveillance of *Enterococcus* Sp. From A Tertiary Care Regional Hospital

Jijo G Varghese[1] Anandharaj B[2] and Fiji E[3]

[1]*Research Scholar, Department of Microbiology, M.R. Government College (Affiliated to Bharathidasan University), Mannargudi-614001, Thiruvarur District, TamilNadu, India*

[2]*Department of Microbiology, M.R. Government College (Affiliated to Bharathidasan University), Mannargudi-614001, Thiruvarur District, TamilNadu, India*

[3]*Research Scholar, Department of Microbiology, M.R. Government College (Affiliated to Bharathidasan University), Mannargudi-614001, Thiruvarur District, TamilNadu, India*

Abstract: *Enterococci* are ubiquitous pathogens commonly present in gut microbiota in humans/animals that can able to form biofilms which leads to increased antimicrobial resistance. As the treatment of drug resistant Enterococci associated infections is difficult to treat and the mortality rate is rapidly increasing among clinical settings. The present study is aimed to investigate the antimicrobial resistance pattern, virulence genes activation and biofilm forming ability of *Enterococcus* species isolated from hospital wards in Kerala, India. From January 2018 to March 2019, we collected 100 samples and detected 93 Enterococci isolates which were confirmed by Gram staining and biochemical assay tests. Ability of the isolates to produce biofilms were also studied. In total of 93 enterococci strains, *Enterococcus faecalis* (85%) was observed as predominant species. Many isolates exhibited multidrug resistance; in particular, 91.30 % isolates of *E. faecium* and *E. faecalis* were resistant to five antibiotics. Notably, *E. faecalis* (88.6%) is more resistant against vancomycin than *E. faecium*. And, we detected 11virulence genes and 15 antibiotic resistance genes in the tested isolates. *E. faecium* had exhibit higher level of biofilm formation than *E. faecalis*. This is the first report to exhibit hospital wards (ICU wards) as reservoir for multidrug resistant enterococci with potential of virulence and biofilm formation. It may ease to transmit the organism from environments into humans. This study provides some useful data about enterococci and suggesting that a regular maintenance of aseptic condition and patients surveillance is needed to control the pathogenesis of Enterococci from hospitals.

Keywords: Enterococcus; hospitalised patients; antibiotic resistance; biofilm formation; Vancomycin; Nosocomial infections

***Corresponding Author**

Jijo G Varghese , Research Scholar, Department of Microbiology, M.R. Government College (Affiliated to Bharathidasan University), Mannargudi-614001, Thiruvarur District, TamilNadu, India

Received On 28 January, 2022
Revised On 23 February, 2022
Accepted On 28 February, 2022
Published On 4 March, 2022

Funding This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

Citation Jijo G Varghese, Anandharaj B and Fiji E , Phenotypic Surveillance of Enterococcus Sp. From A Tertiary Care Regional Hospital.(2022).Int. J. Life Sci. Pharma Res.12(2), L86-92 http://dx.doi.org/10.22376/ijpbs/lpr.2022.12.2.L86-92

1. INTRODUCTION

Nosocomial infections/health care associated infections (HAI)are considered as a major threat worldwide. World health organization reported that ~ 1.4 million people have suffered from nosocomial infections/HAI and about 80,000 deaths occurred every year. The antibiotic resistance patterns of each isolates were detected by Kirby-Bauer disk diffusion method and were assessed by clinical laboratory standards institute, CLSI, 2021[1]. The low income countries/highly populated nations like India are reservoir for HAI compared to developed countries. In India, 1 in 4 patients is suffered from HAI. The effect of HAI comprises prolonged hospital stay, severe illness and an economic burden experienced by health systems[2]. The primary causative agents of HAI globally are known as ESKAPE pathogens includes *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species since the antibiotic resistance is common[3]. Enterococci is a Gram-positive bacterium naturally distributed in the gut microbata of humans and animals[4]. Enterococci are able to survive in extreme conditions like acidic/alkaline pH, high salt concentrations and wide range of temperature (10 to 45 °C)[5]. Based on the review, the twelve Enterococci species are vulnerable to humans, of these *Enterococcus faecalis* is a primary pathogen followed by *Enterococcus faecium*. The moderate level of infections caused by *Enterococcus gallinarum*, *Enterococcus raffinosus*, *Enterococcus avium*, *Enterococcus casseliflavus*, *Enterococcus pseudoavium*, *Enterococcus malodoratus*, *Enterococcus mundtii*, *Enterococcus durans* and *Enterococcus hirae*[6,7]. Enterococci species are gradually increasing their resistance mechanism against various group of antibiotics such as beta-lactam, tetracycline, vancomycin, chloramphenicol etc. Enterococci species are majorly causing urinary tract infections, wound site infections, surgical site infections, endocarditis, bacteraemia, neonatal sepsis, catheter associated infections and strangely cause meningitis[8]. Unfortunately, many of the (mentioned above) infections are caused by pathogens with high-level resistance to numerous antimicrobial drugs, including, ampicillin, vancomycin, penicillin, methicillin, tetracycline, gentamicin, streptomycin and clindamycin[3]. In recent years, WHO declared vancomycin resistant Enterococci (VRE) as a severe global threat in ICU wards and general ward patients. Enterococcal meningitis is a rare complication of neurosurgery. Up to 90% of enterococcal infections in humans are caused by E. *faecalis*[9]. The majorities of the remaining are caused by E. *faecium*. The ability of *E. faecalis* to tolerate or adapt to harsh environments may act as an advantage over other species. Sensitive strains of this bacterium can be treated with Ampicillin, pencillin & vancomycin. UTI can be treated specifically with Nitrofurantoin even in case of vancomycin resistance[10]. An important feature of *enterococcus* is the high level of intrinsic antibiotic resistance. Some *enterococci* are intrinsically resistant to beta lactam based antibiotics and many aminoglycosides. Acquired resistance and virulence traitsare usually transposon or plasmid coded and are transferable. But intrinsic resistance is based on chromosomal genes, which are typically non – transferable[11]. Wide spread emergence and dissemination of ampicillin & Vancomycin resistance in *E. faecalis* would significantly confound a therapeutic dilemma.[12] In the last two decades, particularly virulent strains of *enterococcus* that are resistant to Vancomycin (Vancomycin Resistant *Enterococci* or VRE) have emerged in nosocomial infections[13]. Several studies have documented that the hospital wards majorly cause enterococcal infections. Enterococcal colonization on biotic and abiotic surfaces can stay for prolonged time[14]. Thus, the present study was investigated to determine antimicrobial resistance pattern of enterococci isolates collected from hospitalized wards in Sunrise Institute of Medical Sciences (SIMS) Kerala, India. The study on phenotypic characterisation and drug resistance pattern of the pathogen will help to design the antibiotic stewardship. The study on colonisation mechanisms like biofilm production will also help to prevent the development of such colonisation in hospital setting.

2. MATERIALS AND METHODS

2.1 Study design and area

The study was conducted at Sunrise Institute of Medical Sciences (SIMS), Kerala, India between January 2018 and March 2019. This study was reviewed and approved by the institutional ethical committee of Sunrise Institute of Medical Sciences (SIMS/IEC/02/2022).

2.2 Sample collection

The clinical specimens were collected from the hospital wards, ICU and surgical wards of Sunrise Institute of Medical Sciences and the specific sampling articles includes hospital beds, sheets, curtains, screen, surgical tools, and cottongauze cloth. During the sampling process sterile cotton swabs were used to scrap, and then placed them into 10 mL aseptic tubes. The collected samples were stored with ice packs and later shipped them to the laboratory within 24 h of collection for isolating bacteria.

2.3 Bacterial culture

The collected samples were placed into 10 ml of aseptic tubes containing BHI broth and incubated at 37°C for 24 h. The enriched culture was inoculated into enterococci selective Bile Esculinazide agar plates. One colony was selected for each sample.

2.4 Bacterial species identification by morphology and Biochemical test

Active bacterial species were identified by microbiological morphology test methods (Gram-staining, microscopic analysis and motility test) and biochemical tests.

2.5 Gram staining

The overnight bacterial cultures were subjected to perform Gram staining method as described earlier[15]. One drop of bacterial cultures was smeared the sterile glass slides and stained with crystal violet and kept for 60 sec. After, addition of decolourization agent to remove the excess stain, the smear was washed gently with running water and allowed to dry. Then secondary stain safranin was added. The safranin stained slides were washed with gentle running water after 30 seconds and dried. The bacterial groups were visualized by light microscope.

2.6 Light microscopy

The overnight bacterial cultures were subjected to evaluation

of the bacterial morphology by light microscope[15]. The gram stained smears were visualized under the light microscope at 100X magnification.

2.7 Biochemical analysis

2.7.1 Catalase test

The 16 hr old bacterial cultures were smeared on a sterile glass slide and added 3 % H_2O_2 drop by drop and allowed to react for 30 sec. Then the presence and absence of bubbles formation were recorded[15].

2.7.2 Indole test

The tryptone broth was dispensed into the tubes and sterilized. The test organisms were inoculated into the tubes and one was left uninoculated as control. The tubes were inoculated at 37 °C for 48h. After incubation 1ml of KOVAC's reagent was added to all the tubes including control. The tubes were shaken gently and allowed to stand for 1-2 min. The tubes were observed for formation of cherry red ring[16].

2.7.3 Salt tolerance assay

The selected colonies were picked and inoculated into BHI broth supplemented with 6.5% sodium chloride and bromocresol purple as a pH indicator. The test tubes were incubated at 37 °C for 24 h[15].

2.7.4 Citrate utilization test

The 18 h bacterial cultures were streaked on Simmons citrate agar slants and incubated for 24 – 48 h at 37°C. After incubation, the colour changes were recorded[15].

2.7.5 Bile esculin assay

The selected colonies were picked and inoculated the slant of the bile esculin medium with an S-shaped motion. The test tubes were incubated at 37 °C for 24 h[15].

2.7.6 Litmus milk decolourization test

Overnight bacterial cultures were inoculated into skim milk media (contains: skim milk, litmus, sodium sulphite and pH 6.8) and incubated at 37°C for seven days. The changing colour observation was recorded[15].

2.7.7 Arginine hydrolysis test

Overnight bacterial cultures were inoculated into 1 ml of BHI media with argine amino acid and the test tubes were incubated at 37°C for ten days. The modified colour changes have been observed[17].

2.7.8 Motility assay

Motility behaviors of the isolates were evaluated by motility assay. Briefly, 3µl of 1 OD of overnight grown isolates culture was inoculated at the centre of Swimming agar plates (tryptone 1 % (w/v), NaCl 0.5, agar 0.3; g/100 ml) then plates were incubated at 37 °C for 24 h and migration movement was then recorded[18].

2.8 Determination of antibiotics susceptibility (by Disc diffusion method)

The antibiotic sensitivity test of clinical isolates was assessed by disk diffusion method and assessed the antibiotic resistance pattern by CLSI guidelines. The disk diffusion assay was performed in brain heart infusion agar (BHI). The overnight cultures of all clinical isolates were sub-cultured in BHI broth until to reach the turbidity of 0.5 Mcfarland (1 x 10^8 CFU/ml) standards. The sterile cotton swabs were used to uniformly spread the isolates on the agar plates.The known antibiotics discs such as gentamicin, ciprofloxacin, ampicillin, penicillin, erythromycin, streptomycin, imipenem, vancomycin, clindamycin, bacitracin B, norfloxacin, tetracycline, carbenillin and clarithromycin (Hi-Media, Mumbai, India) were placed over the swabbed plates and incubated at 37 °C for 24 h. After incubation, the zone of inhibition was measured at mm scale[19].

2.9 Determination of minimum inhibitory concentration of Vancomycin

MIC was determined by micro-broth dilution test using sterile 96-well microtitre plates. Antibiotic stock solution was prepared by dissolving vancomycin powder in sterile distilled water, and the concentration was adjusted to 512 µg/ml. A 1:10 dilution of 0.5 McFarland Standard was used; 50 µl each of antibiotic dilutions and organism suspension were mixed and incubated at 37 °C for 24 hrs. The highest dilution which inhibited growth was considered MIC. MIC ≥32 µg/ml was considered to be indicative of resistant isolates[20].

2.10 Phenotypic identification of virulence traits

2.10.1 Hemolytic Activity

For hemolytic activity, fresh culture of clinical specimens were streaked on Columbia agar plates containing 5% (w/v) sheep blood and incubated for 48 h at 37 °C. Blood agar plates were examined for signs of *β*-hemolysis (clear zones around colonies). *E. faecalis* ATCC 29212 were used as a positive control for *β*–hemolysis assay[21].

2.10.2 Gelatinase tests

Gelatinase activity of the clinical specimens were assessed by previous described method[21]. GelE-positive colonies on gelatine medium were surrounded by a turbid halo after 2 days of incubation at 37 °C. To measure the hydrolyzed gelatine in the agar plates, 0.5-1.0 mL of Frazier solution (mercuric chloride, 15.0 g; hydrochloric acid (37%), 20 mL; distilled water, 100 mL) was poured on the surface of the medium to precipitate the unhydrolyzed gelatine. *E. faecalis* ATCC 29212 was used as a positive control.

2.10.3 DNase tests

DNase activity was tested using DNase agar medium. The plate was inoculated with the appropriate strain by streaking a thick line of inoculum across the plate. After incubation at 37°C for 24-48 h, the surface of the DNase test agar plate was flooded with Toluidine Blue solution. DNase activity is indicated by a pink zone surrounding growth. The color of the medium remains unchanged if the test is negative. *S. aureus* ATCC 6538 was used as a positive control[21].

2.10.4 Lipase Tests

Briefly, the isolates were inoculated in MLB (tryptone 1%; 0.5% yeast extract; 0.5% NaCl) agar supplemented with 2.0 g/L of CaCl2 and 10 g/L of Tween-80. Plate was incubated at 37 °C for 24-48 h. A positive reaction was indicated by a clear halo around the colonies. *S. aureus* ATCC 6538 was used as a positive control[22].

2.10.5 Biofilm Formation

Biofilm assays were performed according to the guidance of a reported method[23]. First, the purified Enterococcus colonies were resuspended in 10 mL of Tryptic Soy Broth (TSB) supplemented with 1% glucose, incubated at 37 °C for 18–20 h. Then, for each strain tested, 20 ml of bacterial suspensions were transferred to three wells of sterile 96- well polystyrene microtiter plates containing 180 ml of TSB supplemented with 1% glucose. *E. faecalis* (ATCC 29212) was used as the positive control, and 200 ml of broths (TSB with 1% glucose) were used as the negative control. The microtiter plates were incubated for 24 h at 37 °C, washed with sterile phosphate-buffered solution (PBS), dried at 28 °C, and stained with crystal violet for 30 min. The wells were washed twice with sterile deionized water and dried. Crystal violet dye bound to adherent cells was resuspended in 150 mL of 99% ethanol. The OD readings from respective wells were determined at 570 nm[23,24]. Each assay was determined three times.

3. RESULTS

3.1 Sample collection and the species of Enterococci

In this study, a total of 100 non – duplicated fresh specimens were collected from the Sunrise Institute of Medical Sciences. Of which 93 enterococci were identified. Among the enterococci (n= 93) E. *faecalis* (84.94 %), *E. faecium* (12.90 %), E. *raffinosus* (1.07 %) and E. *avium* (1.07 %) were noted to be common and *E. faecalis was predominant* (Table 1). In addition to the conventional methods. The Automated Microbiology System Vitek 2 from Biomerieux was also employed to confirm the isolates of Enterococcus spp.

3.2 Antimicrobial susceptibilities of Enterococcal isolates

The percentage pattern of Antimicrobial resistance of Enterococci isolates were given in Table. 1. It was observed that most of the test enterococci isolates were resistant against conventional antibiotics groups such as aminoglycosides, carbapenems, glycopeptides, lincosamides, macrolides, β-Lactam, quinolones, polypeptides and others groups.

3.3 Detection of vancomycin resistant strains

The *E. faecium* and *E. faecalis* isolates were resistant to vancomycin and other isolates *E. raffinosus* and *E. avium* were susceptible to vancomycin. The *E. faecium* 12 isolates and *E. faecalis* 75 isolates were resistance to vancomycin. The resistance pattern of the isolates against various antibiotics are illustrated in Table .2

3.4 Physiological test

The results showed the presence of different enzymatic virulence activity such as hemolysis, gelatinase, DNase and lipase activity. The maximum level of hemolysis activity was exhibited by *E. faecium* (71 %). Gelatinase enzymatic activity was exhibited by *E. faecium* (68%) followed by *E. faecalis* (32%). And DNase activity was observed in58 % of *E. faecium* strains followed by *E. faecalis* (17. 6%.) Lipase activity was exhibited by *E. faecium* strains (43.7%) only. The obtained results are showed in Table 3.

3.5 Detection of biofilm formation

Biofilm formation of the Enterococci isolates were asfollowed; non-formers, 40/93 (43 %), weak formers, 16/93 (17.2 %), moderate formers, 25/93 (26.8 %) and strong formers, 6/93 (6.4 %). 47/93 (50.5 %) of isolates were biofilm formers. Biofilm formation of the tested *E. faecalis* (82 %) was statistically significant higher than that of *E. faecium* (48 %) strains ($p < 0.001$). The Enterococcus species biofilm formation was shown in Figure 1 and in Table 4.

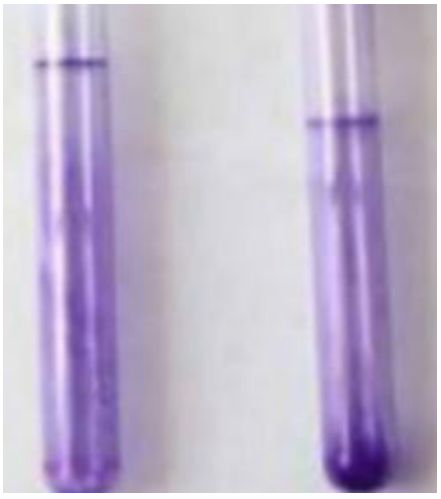

Fig 1. Biofilm activity of Enterococci isolates

Table 1 Numbers of isolates of Enterococcal species from hospital wards

Species	Isolates	Percentage of rate
E. faecalis	79	84.94 %
E. faecium	12	12.90 %
E. raffinosus	1	1.07 %
E. avium	1	1.07 %

Retrospective data of the isolates obtained from clinical specimens

Table 2 Antibiotic resistance profile of *Enterococcus species* by Kirby–Bauer disc diffusion method

Antibiotics	*E. faecium* N = 12	*E. faecalis* N = 79	*E. raffinosus* N = 1	*E. avium* N = 1	No. (%) isolate Total no.
Gentamycin	10	70	0	0	86 %
Streptomycin	11	69	1	1	88.1 %
Imipenem	9	69	1	0	84.9 %
Vancomycin	12	75	0	0	93.5 %
Clindamycin	12	72	1	1	92.4 %
Erythromycin	11	68	0	0	84.9 %
Clarithromycin	12	69	0	0	87 %
Ampicillin	12	79	1	1	100 %
Penicillin	12	75	0	0	93.5 %
Nitrofurantoin	10	70	0	0	86 %
Ciprofloxacin	10	70	0	0	86 %
Levofloxacin	12	71	0	0	89.2 %
Tetracycline	12	77	0	0	95.6 %
Linezolid	10	69	0	0	84.9 %
Tegicyclin	8	68	0	0	81.7 %

Antibiogram of the various isolates which explains the percentage of resistance.

Table 3 Phenotypic virulence factors of Enterococci isolates

Strains	Hemolysis			Gelatinase activity	DNase activity	Lipase activity
	Alpha	Beta	Nill			
E. faecium	8/12 (66.6 %)	-	4/12 (33.3 %)	9/12 (75 %)	7/12 (58.3 %)	5/12 (41.6 %)
E. faecalis	0/79 (0 %)	0/79 (0 %)	-	22/79 (27.8 %)	12/79 (15.1 %)	-
E. raffinosus	0/1 (0 %)	0/1 (0 %)	-	-	-	-
E. avium	1/1 (100 %)	-	-	-	-	-

Presence of various virulence factors in four strains isolated

Table 4 Detection of enterococci species biofilm-forming strength

Species	Weak N = 17	Moderate N = 27	Strong N = 6	Non-former N = 43	No. (%) isolate Total no.
E. faecium	4	5	3	0	12 (12.9 %)
E. faecalis	13	22	3	41	79 (84.9 %)
E. raffinosus	0	0	0	1	1.07 %
E. avium	0	0	0	1	1.07

The strength of biofilm produced is described in terms of percentage

4. DISCUSSION

Enterococci are an exclusive pathogen of humans/animals, and the rapid increase in enterococci resistance against antibiotics leads to increasing the mortality and morbidity in clinical settings[5]. The present study investigated the prevalence of enterococci and their antimicrobial resistance patterns isolated from the hospital wards including ICU that possess high risk for enterococci colonization in health care centre including Sunrise Institute of Medical Sciences, Kerala, India. Multidrug resistant *E. faecium* cause invasive infections, not only *E. faecium*, *E. faecalis* also cause nosocomial infections[5]. But the available data reveal that *E. faecium* caused a large number of nosocomial infections compared to *E. faecalis*[25]. But in contrast to an early reported study[25], the predominant isolates obtained from the present study were *E. faecalis* (84.94 %) followed by *E. faecium* (12.90 %) while *E. raffinosus* and *E. avium* observed with (1.07 %) (Table. 1). And the findings were comparable to the distribution of enterococcal species in other studies in other studies by[26,27] which says *E. feacalis* is the most prevalent species in clinical infections, approximately 80 - 90%. The present study showed that *E. faecalis* is the predominant enterococci species followed by *E. faecium*, which is in contrast with a previous study from the Egypt recorded that *E. faecium* as a dominant species followed by *E. faecalis*[28]. A total of 15 antibiotics groups were used to detect the enterococci antimicrobials susceptibility. The enterococci isolates has the

ability to resist against penicillin and ampicillin. Besides, *E. faecium* was less susceptible than *E. faecalis* to β-lactam antibiotics, as enterococci cell wall shows low affinity to penicillin binding proteins[29]. The present study also exhibited a similar observation. *E. faecalis* resistance rate was lower than the *E. faecium*. The result was compared with previous study[7] which also documented that *E. faecalis* has lower resistance than the *E. faecium* against ampicillin. Although *E. faecium* and *E. faecalis* isolates were resistant to penicillin which is similar level of resistance reported from the previous study[30]. In accordance with Mathai, 1994 we also found a high rate of High level aminoglycoside resistant (HLGAR) strains. Approximately 35% of isolates were HLGAR. Similar reports were obtained from other studies in India before[31]. Even though the vancomycin resistance (VRE) incidence rate is low, the emergence of VRE is to be considered very seriously. The antibiotic resistance pattern for VRE isolates of our study reveals that the 50% of them were resistant to teicoplanin also. This is a significant pattern to be studied as most of the earlier studies showed lower teicoplanin resistance[32]. Biofilm formation is an important virulence factor, which is responsible for causing severe infections and improvise pathogenesis colonization on any environment. Several studies clearly documented that enterococci were able to form biofilms[8,33]. In this context, the enterococcal biofilm forming ability detection was required to be done and was found that 57 % (121/212) enterococci isolates was able to form biofilms. Among them, *E. faecium* isolates showed higher level of biofilm formation compared to E. *faecalis*. In contrast, previous study recorded that *E. faecalis* was the dominant species in forming biofilms and virulence genes than *E. faecium*[34], the present findings indicated that *E. faecium* has domination in biofilm production over *E. faecalis*. In Sardinia, Italy, biofilm production was identified among 87% of *E. faecalis* clinical isolates and 16% of *E. faecium* clinical isolates[35], but in contrast we found that the major biofilm producer among clinical isolates was *E faecium.*

5. CONCLUSION

The findings of the study revealed that 89.1 % of the test strains was multidrug resistant. Among various virulence factors studies including hemolytic activity Gelatinase activity, DNase activity and Lipase activity, high level of biofilm formation was recorded among the isolated enterococci. The results of this study revealed that the extensive biofilm production leads to the spread of enterococcal nosocomial infections. Hence proper control of biofilm formation by disinfection of hospital premises can restrict the spread of infection. And the study also showed the prevalence in increased drug resistance. Preparation and application of a proper antibiotic usage plan is necessary in every hospital setting to ensure the wise usage of antibiotics.

6. ACKNOWLEDGEMENT

The authors acknowledge the management and staffs of Sunrise Institute of Medical Sciences and staffs of MR Government Arts College, Mannargudi.

7. AUTHOR CONTRIBUTION STATEMENT

Mr. Jijo G Vaghese conceived and planned the experiments. Mr. Jijo G Vaghese and Ms. Fiji E carried out the experiments with the support from Dr. B. Anandharaj All authors discussed the results and contributed to the final manuscript. Dr. B. Anandharaj supervised the entire project.

8. CONFLICT OF INTEREST

Conflict of interest declared none

9. REFERENCES

1. CLSI. Performance standards for antimicrobial susceptibility testing. CLSI approvedstandard M100-Ed 31. Wayne, PA: Clinical and Laboratory Standards Institute; 2021.
2. World Health Organization (WHO). Global priority list of antibiotic- resistant bacteria to guide research, discovery, and development of new antibiotics. Geneva: WHO.; 2017.
3. Marothi YA, Agnihotri H, Dubey D. Enterococcal resistance-an overview. Indian J Med Microbiol. 2005;23(4):214-9. doi: 10.1016/S0255-0857(21)02524-X, PMID 16327115.
4. García-Solache M, Rice LB. The Enterococcus: a model of adaptability to its environment. Clin Microbiol Rev. 2019;32(2). doi: 10.1128/CMR.00058-18, PMID 30700430.
5. Arias CA, Murray BE. The rise of the Enterococcus: beyond vancomycin resistance. Nat Rev Microbiol. 2012;10(4):266-78. doi: 10.1038/nrmicro2761, PMID 22421879.
6. Narayanaswamy A, Rajalakshmi K, Varadharajan M. Speciation and antimicrobial susceptibility pattern of Enterococci from a tertiary health care center of south India. J Pharm Res. 2011;4(4):989-90.
7. Abamecha A, Wondafrash B, Abdissa A. Antimicrobial resistance profile of Enterococcus species isolated from intestinal tracts of hospitalized patients in Jimma, Ethiopia. BMC Res Notes. 2015;8(1):213. doi: 10.1186/s13104-015-1200-2, PMID 26036911.
8. Ch'ng JH, Chong KKL, Lam LN, Wong JJ, Kline KA. Biofilm-associated infection by enterococci. Nat Rev Microbiol. 2019;17(2):82-94. doi: 10.1038/s41579-018-0107-z, PMID 30337708.
9. Kayaoglu G, Ørstavik D. Virulence Factors of Enterococcus faecalis: relationship to endodontic Disease. Crit Rev Oral Biol Med. 2004;15(5):308-20. doi: 10.1177/154411130401500506, PMID 15470268.
10. Zhanel GG, Hoban DJ, Karlowsky JA. Nitrofurantoin is active against vancomycin resistant *Enterococci*. Antimicrob Agents Chemother. January 2001;45(1):324-26. doi: 10.1128/AAC.45.1.324-326.2001, PMID 11120989.
11. Padmasini E, Padmaraj R, Srivani Ramesh S. High level aminoglycoside resistance and distribution of aminoglycosides resistant genes among clinical isolates of enterococcus species in Chennai, India. Sci World J. February 4 2014.
12. Huycke MM, Sahm DF, Gilmore MS. Multiple drug resistant *Enterococci*: the nature of the problem and an agenda for the future. Emerg Infect Dis.*Infect.diseases*. 1998;4(2):239-49. doi: 10.3201/eid0402.980211, PMID 9621194.
13. Kurup A, Chlebicki MP, Ling ML, Koh TH, Tan KY, Lee LC, Howe KBM. Control of a hospital-wide

vancomycin resistant *Enterococci* outbreak. Am J Infect Control. 2008;36(3):206-11. doi: 10.1016/j.ajic.2007.06.005, PMID 18371517.

14. Boost M, Lai L, O'Donoghue M. Drug resistance in fecal enterococci in Hong Kong. J Infect Chemother. 2004;10(6):326-30. doi: 10.1007/s10156-004-0337-z, PMID 15614455.
15. Cappuccino J, Sherman N. Microbiology: A laboratory manual. 6th ed; 2002 San Francisco Benjamin cummingspearson education.
16. Hemraj V, Diksha S, Avneet G. A review on commonly used biochemical test for bacteria. Innovare J Life Sci. 2013;1(1):1-7.
17. Kalhotka L, Manga I, Přichystalová J, Hůlová M, Vyletělová M, Šustová K. Decarboxylase activity test of the genus Enterococcus isolated from goat milk and cheese. Acta Vet Brno. 2012;81(2):145-51. doi: 10.2754/avb201281020145.
18. Packiavathy IASV, Priya S, Pandian SK, Ravi AV. Inhibition of biofilm development of uropathogens by curcumin–an anti-quorum sensing agent from Curcuma longa. Food Chem. 2014;1 48:453-60. doi: 10.1016/j.foodchem.2012.08.002, PMID 24262582.
19. Humphries RM, Ambler J, Mitchell SL, Castanheira M, Dingle T, Hindler JA, Koeth L, Sei K. CLSI methods development and standardization working group best practices for evaluation of antimicrobial susceptibility tests. J Clin Microbiol. 2018;56(4). doi: 10.1128/JCM.01934-17.
20. Hombach M, Mouttet B, Bloemberg GV. Consequences of revised CLSI and EUCAST guidelines for antibiotic susceptibility patterns of ESBL-and AmpC β-lactamase-producing clinical Enterobacteriaceae isolates. J Antimicrob Chemother. 2013;68(9):2092-8. doi: 10.1093/jac/dkt136.
21. Chakchouk-Mtibaa A, Elleuch L, Smaoui S, Najah S, Sellem I, Abdelkafi S, Mellouli L. An antilisterialbacteriocin BacFL31 produced by Enterococcus faecium FL31 with a novel structure containing hydroxyproline residues. Anaerobe. 2014; 27:1-6. doi: 10.1016/j.anaerobe.2014.02.002, PMID 24583094.
22. Tiago I, Teixeira I, Silva S, Chung P, Veríssimo A, Manaia CM., &Manaia. Metabolic and genetic diversity of mesophilic and thermophilic bacteria isolated from composted municipal sludge on poly-epsilon-caprolactones. Curr Microbiol. 2004;49(6):407-14. doi: 10.1007/s00284-004-4353-0, PMID 15696616.
23. Stepanović S, Vuković D, Hola V, Di Bonaventura GD, Djukić S, Ćirković I, Ruzicka F. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS. 2007;115(8):891-9. doi: 10.1111/j.1600-0463.2007.apm_630.x, PMID 17696944.
24. Vijayakumar K, Ramanathan T. *Musa acuminata* and its bioactive metabolite 5-hydroxymethylfurfural mitigates quorum sensing (las and rhl) mediated biofilm and virulence production of nosocomial pathogen *Pseudomonas aeruginosa* in vitro. J Ethnopharmacol. 2020;246: 112242. doi: 10.1016/j.jep.2019.112242.
25. Mohanty S, Jose S, Singhal R, Sood S, Dhawan B, Das BK, Kapil A. Species prevalence and antimicrobial susceptibility of enterococci isolated in a tertiary care hospital of North India. Southeast Asian J Trop Med Public Health. 2005;36(4):962-5. PMID 16295552.
26. Jones RN, sader HS, Erwin ME, Anderson SC. Anderson SC and *Enterococcus* study group. Emerging multiple resistant *Enterococci*. Diagn Microbiol Infect Dis. 1995;21(2):85-93. doi: 10.1016/0732-8893(94)00147-o, PMID 7628198.
27. Murray PR, Baron EJ, Pfaller MA, Yolken RH. Manual of clinical microbiology. 8th ed. Washington, DC: ASM Press; 2003.
28. Moemen D, Tawfeek D, Badawy W. Healthcare-associated vancomycin resistant Enterococcus faecium infections in the Mansoura University Hospitals intensive care units, Egypt. Braz J Microbiol. 2015;46(3):777-83. doi: 10.1590/S1517-838246320140403, PMID 26413060.
29. Sood S, Malhotra M, Das BK, Kapil A. Enterococcal infections & antimicrobial resistance. Indian J Med Res. 2008;128(2):111-21. PMID 19001673.
30. Kapoor L, Randhawa VS, Deb M. Antimicrobial resistance of enterococcal blood isolates at a pediatric care hospital in India. Jpn J Infect Dis. 2005;58(2):101-3. PMID 15858289.
31. Mathai E, Margaret A, George V, Brahmadathan KN. Identification of gram positive cocci from urine. Indian J Med Res. 1994; 100:10-4. PMID 7927545.
32. Karmarkar MG, Gershom ES, Mehta PR. Enterococcal infections with special reference to phenotypic characterization & drug resistance. Indian J Med Res. May 2004; 119;S uppl:22-5. PMID 15232156.
33. Igbinosa EO, Beshiru A. Antimicrobial resistance, virulence determinants, and biofilm formation of Enterococcus species from ready-to-eat seafood. Front Microbiol. 2019; 10:728. doi: 10.3389/fmicb.2019.00728, PMID 31057497.
34. Cui P, Feng L, Zhang L, He J, An T, Fu X, Li C, Zhao X, Zhai Y, Li H, Yan W, Li H, Luo X, Lei C, Wang H, Yang X. Antimicrobial resistance, virulence genes, and biofilm formation capacity among Enterococcus Species From Yaks in Aba Tibetan autonomous prefecture, China. Front Microbiol. 2020; 11:1250. doi: 10.3389/fmicb.2020.01250, PMID 32595625.
35. Duprè I, Zanetti S, Schito AM, Fadda G, Sechi LA. Inci dence of virulence determinants in clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates collected in Sardinia (Italy). J Med Microbiol. 2003;52(6):491-8. doi: 10.1099/jmm.0.05038-0, PMID 12748268.