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Phenotypic Characterization and Antibiotic Susceptibility Pattern of Clinical Isolates of Enterococci with Special Emphasis on Vancomycin Resistance

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ABSTRACT

Aims And Objectives
Primary Objective:
To the isolates and identify the enterococci from various clinical specimens.
Secondary Objectives:
To determine the antibiotics susceptibility pattern of enterococci isolates.
Design: Prospective Study
Materials And Methods: 65 Clinical samples sent for bacteriological examination and culture sensitivity were taken up in this study. Preliminary findings and identification of Enterococci species was carried out using Gram staining, Catalase test, Bile esculin test and growth in NaCl, followed by Antibiotic sensitivity testing in Muller Hilton Agar. The resistant strains were subjected to agar dilution method, Vancomycin e-strip method and Vitek-2 automated system for phenotypic detection of Vancomycin resistance. The results were observed and analysed.
Result: Among 65 clinical specimens analysed, enterococci species were isolated from various specimens. Among which 50 isolates which were identified as E. faecalis from biochemical tests were further analysed for antibiotic susceptibility pattern.

Keywords: VRE, Enterococci, Phenotypic methods, Resistance, Enterococci, Speciation, Antibiotic resistance, High level gentamicin, Vancomycin.

INTRODUCTION

Enterococci are Gram-positive cocci which were originally classified as enteric gram-positive cocci. Enterococci form microbiota of humans and animals. They are survived in the gastrointestinal and biliary tract, as well as the vaginal and male urethra is lesser numbers. Since the mid-1980s, the taxonomy of Enterococcus species has drastically changed. Before the widespread use of genetic techniques for taxonomic analysis, enterococci were differentiated from streptococci in addition to taxa to their capacity to grow at 10°C and 45°C, growth in the presence of 6.5% NaCl, growth at pH 9.6, ability to hydrolysed esculin in the presence of 40% bile, and production of pyrrolidonyl arylamidase (PYR). E. faecalis is the most commonly isolated species, causing about 70% of human infections. However, enterococci acquired resistance to vancomycin (VRE, vancomycin-resistant enterococci) now account considerably 30% of enterococcal infections, and greater than 90% of VRE isolates are E. faecium. Enterococcus species are an important agent cause complicated UTIs, bacterial sepsis, endocarditis, intra-abdominal and pelvic infections, post-operative wound infections and soft tissue infections, neonatal sepsis and rarely meningitis. Enterococcal

bacteremias are most frequently hospital-acquired and can be connected to enterococcal infections in regions other than the urinary system (e.g., intravenous catheter infections, biliary tract infection, gastrointestinal/genitourinary infections). Intra-abdominal or pelvic abscesses, wounds, decubitus ulcers, or intravenous access devices both can allow organisms to enter the bloodstream. Bacteremias caused by E. faecium are associated with a poorer prognosis than those due to E. faecalis primarily because of increased antibiotic resistance in the former species and the inherent challenges in treating more resistant isolates.

Enterococcal infections have been referred to as difficult to treat infections. In the current history, there has been a growth in the number of occurrences of enterococcal infections in hospitals, with the introduction in antibiotics resistance strains causing special worry. Being second most prevalent organism after abdominal and pelvic infections, third in producing blood stream infections, incidence of CNS and newborn infections have also been identified.

Enterococci have a special ability to survive in the presence of antibiotics-saturated environment. Indeed, these organisms' susceptibility pattern to a variety of antimicrobial agents that makes them such feared pathogens. There are two types of antimicrobial resistance in Enterococci: inherent / intrinsic resistance and acquired resistance. Intrinsic resistance is a species-species trait that is found in all individuals of the species and is mediated by the chromosome. Acquired resistance, on the other hand, is it results of either a DNA mutation or acquisition of extra DNA.

The initial cases of Vancomycin resistant Enterococcus (VRE) were recorded in the 1980s, followed by an upsurge in the transmission of the bacteria. Followed by an upsurge in the transmission of the bacteria Van A, Van B and Van C. Van A and Van B are the main phenotypes linked to its dissemination Van A and Van B are mostly linked to E. faecalis and E. faecium. In Van C is mentioned, in E. gallinarum and E. casseliflavus is a type of casseliflavus. Van A is primarily associated with resistant strains among the phenotypes. VRE strains are usually isolated from patients with recurrent bacteremia, endovascular infections, which lead to an increase in mortality rates. Resistance both Vancomycin and penicillin frequently coexist, making therapy more difficult [11]. Resistance to glycopeptide antibiotics develop in Enterococcus, which then spreads another bacterial pathogen such Staphylococcus aureus, Streptococci, Listeria monocytogenes.

Enterococci have acquired prominence as a result of the recent occurrence in conjunction with serious symptoms infections and the development of resistance. Studies are needed, particularly in tertiary care hospitals to properly isolates, identify and speciate them. Antibiograms should also be followed up on as well, depending on the species. The spread of VRE in the hospital environment must be controlled and inhibited. The CDC also emphasizes the controlled of VRE in hospitals by teaching personnel on how to spot VRE early, report them, and implement a strategic plan quickly.

MATERIALS & METHODS

The present titled "Study of phenotypic characterization and antibiotic susceptibility pattern of clinical isolates of enterococci with special emphasis on vancomycin resistance" was carried out in the Department of Microbiology, JSS Academy of Higher Education and Research, Mysuru for over a duration of 6 month (October 2020-March 2021).

MATERIALS

All Enterococci isolates isolated from various clinical samples such as pus, urine and Blood, ET and other clinical specimens sent to microbiology over a duration of 6 months were a part of the study.

All samples were processed for isolated on solid agar, identification of enterococci by biochemical tests, antimicrobial susceptibility testing and detection of vancomycin resistance by using Agar Dilution Method.

Inclusion criteria: 1. Enterococci isolates from various clinical samples.

Exclusion criteria: 1. Isolates other than Enterococci. 2. Enterococcal isolates from the stool Samples

HISTORY TAKING AND EXAMINATION

A detailed History was taken with reference to Name, Age, Sex, Address, date of Birth, ward, microscopy, identification details, antibiotic susceptibility pattern, relevant clinical information including chief complaints, and any previous history of treatment and recorded on a pre designed proforma.

COLLECTION OF SAMPLES

All the clinical Specimens that were received in the microbiology laboratory were processes using a standard protocol of the laboratory.

PROCESSING OF THE SAMPLES

1) Pus and wound swabs

The swab was spread in the blood agar, MacConkey agar and colistin nalidixic acid (CAN) are incubated at 37°C for 24 hours.

2) Blood

In adults about 10 ml of blood and in children 1-5 ml of blood was collected by venipuncture and inoculated into automated blood culture bottles and transported to the laboratory and incubated into BacTALERT automated blood culture machine immediately.

3) Urine

A sterile, dry, wide-necked, leak-proof container was used to collect clean catch mid-stream urine. In the case of catharized patients, urine was collected 30 minutes after clamping the catheter with a syringe and needle placed proximal to the clamping site under strong aseptic conditions. As a wet mount, a loopful of the uncentrifuged specimen was tested for presence of pus cells and bacteria. Loopful of specimen were inoculated into UCA. For 24 hours, Plate was incubated at 37°C for 24 hours.

4) Gram Staining:

Gram's stain was used to create smears for each specimen. Gram's stain smears are viewed to identify the presence and type of cells in the samples at low magnification (10x objectives) specimen, and then under an oil immersion on lens to study and recover the relative quantity of microorganism and their shapes.

Culture:

Except for the blood, all of the samples were inoculated into blood agar, Blood agar, and MacConkey agar plate. All plates are incubated aerobically at 37°C for 24 hours, and growth was observed.

IDENTIFICATION AND SPECIFICATION OF ENTEROCOCCI

Speciation of Enterococci was performed by using following culture media and biochemical tests

1. On the basis of culture characteristics on

- ✓ Blood Agar: In blood agar plate, after an overnight incubation period, Enterococcus colonies appeared as grey colonies 0.5-1mm in diameter with alpha, beta, or gamma hemolysis.
- ✓ MacConkey Agar: In MacConkey agar Following 18–24 hours of growth on agar the colonies were around 0.5-1mm in size, with a smooth surface and convex borders, and small magenta-coloured colonies.

2. Morphology on Gram staining: Smears stained with gram-positive cocci showed oval gram-positive cocci arranged singly, pairs, or in short chains.

3. Growth in presence of 6.5% NaCl (Sodium chloride): The colonies were inoculated and incubated overnight in a broth containing 6.5% NaCl. The tubes were tested next day for any turbidity increases.

4. This was tested by subculturing the bacteria on agar plates. The presence of an organism was proved by its growth. The lack of turbidity suggested that the bacterium could not grow in this concentration.
5. Catalase test: On the slide, a little amount of catalase reagent (3% hydrogen peroxide) was applied. An applicator stick was used to apply the growth to it. The presence of bubbles was considered a positive test. The absence of bubbles, which showed a negative test, indicated a negative test for enterococcal colonies
6. Bile Esculin test: The colonies from the 24-hour culture had to be inoculated in the media obtain 40% bile. The color of the media changes to black, indicating a good reaction to Enterococci.
7. Fermentation of sugars: Sugar consumption was used to further species identification by inoculating the microorganism with 1% sugars and Andrade's indicator in peptone the sugars examined were mannitol, arabinose, raffinose, sorbose, sorbitol, lactose, and sucrose. If the test tubes turn pink, it means the sugar fermentation test was positive. The lack of color shift was interpreted as a negative.
8. Pyrrolidonyl arylamidase (PYR): First we had to prepare PYR agar and pour into the petri plate then we had to incubated those plate at 37℃ for 24 hours for the sterility check. The next day colonies were incubated in PYR agar and incubated for 24-hours. The next day Later 3 drops of reagent were added, development of red color was considered positive indicated Enterococci, absence of color changes indicated negative test.
9. Detection of motility: The colony was cultured in a semi-solid medium, and the spread of the organism into the media confirmed locomotion. Non-motile strains are those that do not spread into the media. For proven motility, tubes were cultured for up to 7 days.

Antibiotic Susceptibility Testing Methods

The Enterococci were tested by using the Kirby-Bauer disc diffusion method for antibiotic susceptibility patterns. Three to four three hours were spent incubating pure colonies in peptone water. 0.5 Mc Farlands was used to match the increasing turbidity. In Muller Hinton agar, a lawn culture of the broth was performed with a sterile swab, and the discs were placed at a distance of 24mm. Each 90 mm petri plate holds six discs. ✓ Ampicillin (10 µgm) ✓ Penicillin G (10µgm ✓ Nitrofurantoin (300 µgm) ✓ Norfloxacin (10 µgm) ✓ High Level Gentamicin (120 µgm) ✓ Linezolid (30 µgm) ✓ Vancomycin (30 µgm) ✓ Teicoplanin (30 µgm) ✓ Tetracycline (30 µgm) For isolates from urine sample Norfloxacin and Nitrofurantoin were taken. All of the discs were purchased from HIMEDIA. According to CLSI standards, zones of inhibition were measured and documented, and the organism was classified as sensitive or resistant. Enterococcus faecalis ATCC 29212 was utilized as a quality control.

RESULT

The current Research titled “Study of phenotypic characterization and antibiotic susceptibility pattern of clinical isolates of enterococci with special emphasis on vancomycin resistance.” was arranged in the Department of Microbiology, JSS Academy of Higher Education and Research Institution, Mysuru over a period of 6 months from October 2020 - March 2021. During the 6-months duration (October 2020 - March 2021) study period, total of the 65 enterococci species was isolated from various specimens. Among which 50 isolates which were identified as E. faecalis from biochemical tests were further analysed for antibiotic susceptibility pattern.

TABLE 1: GENDER WISE DISTRIBUTION OF THE E. FAECALIS ISOLATES

In the present study, among the 50 clinical E. faecalis samples taken from

patients, the Male to Female ratio observed was 3:1 i.e., about 34(68%) samples were collected from males about 16(32%) included females as shown in Table 1 Graph 01.

TABLE 1: GENDER WISE DISTRIBUTION		
Male	34	68
Female	16	32

TABLE 02: NUMBER OF E. faecalis IN RELATION TO AGE GROUP

Of the total 50 samples, highest number of patients were under the age group of 61-70 years accounting for 13(26%), followed by the age groups 51-60 years 10(20%), 71-80 years 8(16%), 0-10 years 7(14%), 41-50 years 6(12%), and least number of cases in the age group of 31-40 years 4(8%),21-30 years 2(4%) and 11-20 years 0(0%) which is represented in Table 02 and Graph 02

TABLE 02: AGE-WISE DISTRIBUTION		
AGE	**NUMBER**	**PERCHATAGE**
0-10	7	14%
11-20	0	0%
21-30	2	4%
31-40	4	8%
41-50	6	12%
51-60	10	20%
61-70	13	26%
71-80	8	16%

TABLE 03: SAMPLE-WISE DISTRIBUTION

Among the total 50 clinical E. faecalis isolates, highest number of isolates were isolated from Urine 42 (84%), followed by Blood 4 (8%), Pus 3(6%), and Endotracheal secretions (2%), which is represented in TABLE NO 3 GRAPH 03.

TABLE NO 3 SAMPLE-WISE DISTRIBUTION		
SAMPLE	**NO. OF CASES**	**PERCENTAGE**
URINE	42	84%
ET	1	2%
BLOOD	4	8%
PUS	3	6%

TABLE NO 05: AST PATTERN OF E. faecalis BY DISC-DIFFUSION TEST

Of 50 clinical isolates from urine sample maximum resistance was seen to Norfloxacin 78%(n=39), followed by tetracycline 72%(n=36), High Level Gentamicin 68%(n=34), Nitrofurantoin 30%(n=15), Penicillin 52%(n=26), teicoplanin 8%, Ampicillin 52%. All the isolates were sensitive to linezolid, 100 %(n=50) cases showed vancomycin sensitive, 96 %(n=48) cases showed moderate sensitivity which is represented in TABLE NO 5 GRAPH 05

TABLE NO 05: AST PATTERN IN URINE SAMPLE FOR E. faecalis BY DISC DIFFUSION METHOD.						
DRUGS	**S**	**%**	**I**	**%**	**R**	**%**
NITROFURANTOIN (42)	27	54%	0	0	15	30
NORFLOXACIN	14	28	0	0	36	72
HIGH LEVEL GENTAMICIN	15	30	0	0	35	70
PENICILLIN-G	18	36	0	0	32	64
AMPICILLIN	24	42	0	0	26	52
TETRACYCLINE	13	26	1	2	36	72%
TEICOPLANIN	46	92%	0	0	4	8
LINEZOLID	50	100	0	0	0	0
VANCOMYCIN	48	96	0	0	2	4

In the present study, maximum number of samples were obtained from Medicine ward (20%) and Neurology ward (12%) accounting for 20.83% each. Followed by COV-MICU 5 (10%), NAC 5 (10%), OBG, NICU and SICU-SD all three are same 4(8%), MICU, URO, PEAD, RICU, ICU, GERI and GERI all accounting for 1 each i.e. (2%), SICU 3(6%) and GEN WARD 2(4%).

Vancomycin Susceptibility Comparison By The Disc Diffusion Method And Agar Dilution Method

Among 50 E. faecalis isolated, 48(96%) were sensitive to vancomycin whereas 2(4%) were resistance by vitek-2 method. However, when these isolates were tested with Agar Dilution Method 42(92%) were sensitive whereas 8(16%) were resistance with the MIC 6µg/ml.

VANCOMYCIN SUSEPTIBILTY COMPARISON BY THE DISC DIFFUSION METHOD AND AGAR DILUTION METHOD		
PATTERN	**VITEK 2 METHOD**	**AGAR DILUTION METHOD**
VANCOMYCIN SENSITIVITY (50)	48(96%)	42(92%)
VANCOMYCIN RESISTANCE (50)	2(4%)	8(16%)

DISCUSSION

Enterococci, traditional commensal bacteria, enterococci, are now recognized as entities capable of producing life-threatening infection in humans, particularly in the nosocomial setting. Enterococci's ability to play two roles is enhanced by their intrinsic and acquired resistance to essentially all the antibiotics now in uses. Enterococci have emerged as a significant nosocomial pathogen, with increasing vancomycin resistance and increasing mortality rates. [5,8]

The fast emergence of resistance in Enterococci, as well as rising incidence of the infection with VRE has becoming a major health concern for health care settings. Enterococcus faecalis and Enterococcus faecium are the two species to cause health-associated infections. Our study included 65 isolates of enterococci isolated during the study duration of 6 month from October 2020 to March 2021 in which,50 isolates were identified as Enterococcus faecalis by standard biochemical test and they were further analysed for antibiotic resistance by disc diffusion method.

In our study, among the 50 E. faecalis clinical isolates,34(68%) isolates were from male and 16(32%) isolates were from female individuals Whereas in the study conducted by Mehta Pooja B et.al [41] included 52.9% of samples from male individuals and 47.1% of samples from female individuals remarking that a greater number of male individuals were infected similar to our study.

Sl. No	Study Series	Year	Age Group More Effected	Percentage
1	Anjana talker et.al	2012	0-20 year	54%
2	Srivastava	2013	21-40 year	40.0%
3	Abdulhakim Abamecha et.al	2014	28-55 year	73%
4	Bhatt P el.al	2014	>60 year	30.5%
5	Present study	2021	61-70 year	26%

In our study, the most of the isolates were from patients of age group 61-70 years which accounted for 13(26%) Similar demographic Observations were made in a study done by Srivastava P et al. [42] where in maximum number of cases were in the age group of 21- 40yrs, remarking significant differences in both the studies. In the present study majority of the isolates were included from Urine 42(84%) sample, Comparison to the studies done by Ira praharaj et al, Mathur P et al, Yasliani S et al, Sharma R et al, Golia S et al, Srivastava P et al,[37] emphasizing on the fact that the UTI is the most common infection caused by enterococcus. However, in a study done by Mohanty S et al, Enterococci was predominantly isolated from blood (36.1%), followed by urine (35.2%). [14]. Majority of the enterococci isolated in the study were from urine sample collected from were non-catheterized 35(70%) and rest of the sample were collected from catheterized 15(30%), patients similar to the studies done by Golia S et al, Mehta Pooja B et.al, Ujjwala B et al,[43] on that fact UTI is the most common infection caused by Enterococcus. However, in a study done by Archana Rao k [30], the Enterococci was predominantly isolated from non-Catheterized urine sample in which 30 out of total 46 (65.2%) were isolated.

In present study bacterial coinfection was observed with E. faecalis. Out of total E. faecalis isolates the other bacterial coinfection was observed with E. faecalis. 43 samples (86%) yielded growth of E. faecalis alone, 1 sample (2%) yielded growth of E. faecalis+Morganella morganii, 2(4%) were E. faecalis + E. coli, 2(4%) were E. faecalis+ k. pneumoniae,1(2%)

were E. faecalis + Pseudomonas aeruginosa,1(2%) were E. faecalis + A. baumannii. Urine tract infection is the most important causes of infectious disease caused by the Enterococci, both in community and hospital set ups. Enterococci was reported the third most frequent uropathogen in intensive care unit-acquired urinary tract infections after Escherichia coli and Pseudomonas aeruginosa. In the present study among 42(84%) urine samples yielding the growth of Enterococci. The higher infection rate of urinary tract could be because of instrumentation or catheterization, and previous use of antibiotics. In some cases, such as in patients with catheterization, ability to form biofilms by Enterococci may be the cause. Enterococci survive in adverse conditions, exhibit both intrinsic as well as acquired antibiotics resistance pattern. The conditions in the hospital settings that play an important role in acquiring the drug resistance include (i) Indiscriminate use of antibiotics, (ii) Prolonged hospital stay, (iii) Severity of illness and (iv) immune-suppression are mainly responsible for nosocomial acquisition of drug resistant enterococci. [30,31]

In the present study the High-level Gentamicin in E. faecalis was 35(70%). However, in the other study karmarkar MG et al [44] Ujjwala B et al [24], showed high in High-Level Gentamicin. The resistance to High level Gentamicin could be because of the indiscriminate use of aminoglycosides as prophylaxis before surgeries and also as a part of chemotherapy. In the present study, of 50 isolates the AST pattern of E. faecalis by disc diffusion test maximum resistance was observed to Norfloxacin 78%(n=39), followed by tetracycline 72%(n=36), High Level Gentamicin 68%(n=34), Nitrofurantoin 30%(n=15), Penicillin 52%(n=26), Teicoplanin 8%, Ampicillin 52%. All of the isolates were sensitive to linezolid,100%(n=50) [38] In the present study, among the E. faecalis showed more resistance to antibiotics like Norfloxacin, Penicillin-G and Tetracycline. Similar study was done by Deepa C et al. [27] and Mohanty S et al. [19] in which E. faecalis showed more resistance to antibiotics s like tetracycline, chloramphenicol, erythromycin, ciprofloxacin and Nitrofurantoin.

The ability to understand the organism's resistance pattern help in the development of an empirical treatment. Each geographical area and hospital setting has its own pattern of resistance. Due to the widespread usage of broad-spectrum antibiotics, resistant pathogens are common in medical settings. This indiscriminate use maintains a pool of resistant bacteria. Because there is a lack of consistency in antibiotic treatments for individuals infected with the numerous resistant to Enterococci, preventing the spread of these organisms is essential.

In the present study,2 of the E. faecalis isolates were resistant to Vancomycin by the disc diffusion methid,8 of the isolates was resistant to the vancomycin by agar dilution test. Comparison with other studies done by Mohanty S et al, Moses V et al. [42] Jain S er al. in contrary Salem-Bekhit MM et al. [40] reported 3.9% and Ujjwala B et al. [43] reported 5% of resistance. In the present study, vancomycin when tested by Kirby Bauer disc diffusion method, 96% (n=48) E. faecalis were sensitive, 4% (n=2) showed resistance. Similar study was done by Mohanty S et al, (119) Moses V et al. (7) Jain S et al. (1) in contrary Salem-Bekhit MM et al. [25] reported 3.9% and Ujjwala B et al. [24] reported 5% of resistance to Vancomycin. In our study, Kirby Bauer disc diffusion method showed 96% of E. faecalis isolates to be sensitive strains, which were sensitive when tested Agar Dilution Test also. The discordant results between disc diffusion and agar dilution were may be due to error in preparation or performance of agar dilution. Similarly, in a study done by Sreeja S et al., [12] 73% were susceptible, 27% were moderately susceptible by disc diffusion method

CONCLUSION

Enterococci, known to be commensals in human intestine, can cause wide variety of illnesses, including UTI, Bacteremia, Wound Infections, and Lower Respiratory Tract Infections. E. faecalis is the prevalent species to cause infection in humans. Correct speciation is very important since there is variation in resistance to antibiotics by particular enterococcal species. E. faecium is well-known for its multi drug resistance. Enterococcus showed resistance to various commonly used antibiotic like Ampicillin, Ciprofloxacin, Erythromycin and Tetracycline. Knowing the antibiotics resistance pattern among Enterococci helps in formulating the antibiogram of the Hospitals which further helps in better management of the patients and prevention of the drug resistance. Vancomycin resistance in Enterococci is posting a major problem management of the patients admitted to intensive care unit. Identification of vancomycin resistance Enterococci is crucial not only in treatment but also prevent transfer of vancomycin resistance between Enterococci and Staphylococcus. Detection of vancomycin resistance by disc diffusion method is easy to perform and can be done in peripheral settings and less expensive though technical error can result in wrong results. Detection of Vancomycin MIC by automated method is more reliable than disc diffusion method, the major advantage of this method is that multiple isolates can be testes simultaneously although preparation of media is cumbersome and technical errors can interfere with the results.

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