ORIGINAL RESEARCH PAPER

Medical Microbiology

ISOLATION OF ENTEROCOCCUS SPP. IN VARIOUS CLINICAL SAMPLE AND THEIR ANTIMICROBIAL RESISTANT PATTERN WITH MIC OF VANCOMYCIN IN PATIENT ATTENDING TERTIARY CARE CENTRE, RIMS, RANCHI, JHARKHAND

KEY WORDS: Blood agar, Mac Conkey agar , bile esculin agar, vancomycin resistant Enterococci , minimum inhibitory concentration, kirby-Bauer disc diffusion method.

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ABSTRACT

Introduction- Enterococci are part of normal intestinal flora of humans and animals but have also emerged as important pathogens responsible for serious infections in hospital and community acquired infections.it is second most common cause of nosocomial infections in gastrointestinal tract, wound and genitourinary tract. **Aim-** To process all the clinical samples from various department in our hospital, for isolation of Enterococci spp. To speciate the isolates & to have resistance pattern of the isolates of vancomycin **Material & Methods-** total 926 sample were collected from both out patients and in patient in all clinical departments and transported to microbiology laboratory. specimens were processed by inoculating on to blood agar, MacConkey Agar, nutrient agar, potassium tellurite agar and incubated at 37°C for24-48 hr. Enterococci were identified by their typical arrangement in and salt tolerance test Gram stain, bile esculin test and biochemical tests. Antimicrobial susceptibility patterns were determined by performing Kirby-Bauer disc diffusion method and Minimum inhibitory concentration (MIC) values were identified by tube dilution methods. **Result-** a total of 926 sample, 645 (69.72%) were culture positive and 281 (30.28%) were culture negative. Among 645 culture positive cases, 81(12.55%) were Enterococcus faecalis. Antimicrobial susceptibility & MIC done as per standard protocols. The E. Faecalis showed 99% sensitive to Vancomycin. the resistance to vancomycin was 1% & further confirmed by MIC via tube dilution methods. In which MIC was ≥32 µg/ml in one isolate. About 8 of Enterococcal strains showed MIC of 0.0125µg/ml. **Conclusions-** species level identification of Enterococcus is important for epidemiological study and also for analysis of drug resistant pattern. Effective detection of vancomycin resistance helps in reducing the morbidity and mortality of VRE in hospitalized patients.

INTRODUCTION

Enterococci are gram positive anaerobes that live as commensal habitant in the alimentary canal of a person. The genus Enterococcus included more than 17 species, but only a few causes clinical infections in humans [1]

In the 1930s, with the establishment of the Lancefield serological typing system, Enterococcus were classified as group D streptococci and were differentiated from the Non Enterococcal group D streptococci such as Streptococcus bovis by distinctive biochemical characteristics.

[2] Enterococcus faecalis and Enterococcus faecium are the most prevalent species cultured from humans, accounting for more than 90% of clinical isolates. Other Enterococcal species known to cause human infection include Entero coccus avium, Enterococcus gallinarum, Enterococcus casseliflavus , Enterococcus durans, Enterococcus raffinosus and Enterococcus mundtii. E faecium represents most vancomycin-resistant Enterococci (VRE) [1]. Infections commonly caused by Enterococci include urinary tract infections, endocarditis, bacteremia catheter-related infections, wound infections and intra-abdominal and pelvic infections.

Intestinal colonization with resistant Enterococcal strains is more common than clinical infection. Colonized patients are a potential source for the spread of organisms to the health care workers, the environment and other patients [3]. Enterococci can survive for long periods on environmental surfaces, contributing to their transmission. VRE have been isolated from all objects and sites in health care facilities [4] For colonization development and infection with VRE, antimi crobial and Nonantimicrobial risk factors have been identified [5]. Third-generation cephalosporins, aminog ycosides, aztreonam, ciprofloxacin, imipenem, clindamycin and metronidazole have been associated with VRE colonization. Non-antimicrobial risk factors (e.g., increased duration of exposure to individuals colonized with VRE and close proximity to other colonized patients) increase the likelihood of VRE exposure [6]. According to recent surveys, Enterococci remain in the top 3 most common pathogens that cause nosocomial infections. Nosocomial Enterococcal infections typically occur in very ill debilitated patients who have been exposed to broad-spectrum antibiotics. These emphasize the need for their identification from the clinical specimens and also differentiate them from other group D streptococci which are generally more sensitive to the antimicrobial agents.

Keeping these in mind the present study has been undertaken in the Department of Microbiology, Rajendra institute of Medical Science RIMS RANCHI. To study the prevalence of Enterococcus species from various Clinical specimen and their AST PATTERN with MIC of Vancomycin.

AIM AND OBJECTIVES

1. To process all the clinical samples from various departments in our hospital, for the isolation of Enterococci spp.
2. To speciate the isolated Enterococci & to have the resistance pattern of the isolates to Vancomycin
3. To know the prevalence of Enterococcal infections in our hospital.

4. To know the resistance pattern of the isolates to Vancomycin.

MATERIAL AND METHODS-

A total 926 samples were collected from both out patient & in patient from all clinical department of RIMS RANCHI during 1 December 2021 to 30 May 2022 at microbiology department of tertiary care hospital were assessed.

Clinical Isolates

The various clinical isolates were blood, urine, pleural fluid, cerebrospinal fluid, pus, ascetic fluid, etc. All received isolates from patients admitted in various wards like; surgery, medicine, orthopaedics, gynaecology, paediatrics, etc. in mentioned study duration of 6 month were included as a part to retrospective data analysis. The samples were processed for microscopy, culture and sensitivity testing according to standard.

Specimen processing

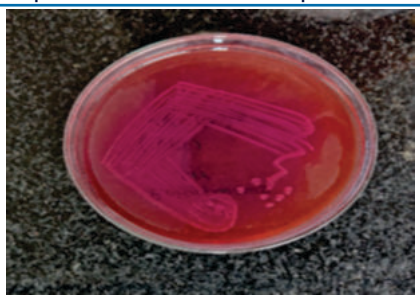
Enterococci were recognized by standard biochemical tests like; bile esculin hydrolysis test, arabinose fermentation test and affirmed by optochin and bacitracin plate test. Bile esculin test helps in differentiating group D streptococci from other streptococci. Group D streptococci have peculiar ability to hydrolyse esculin in availability of bile salts. Antimicrobial susceptibility testing was finished by Kirby Bauer plate dispersion strategy on Muller Hinton agar as indicated by standard CLSI guideline. Vancomycin resistance was distinguished by disc diffusion method and affirmed by Minimum Inhibitory Concentration Test (MIC).

Identification of enterococci

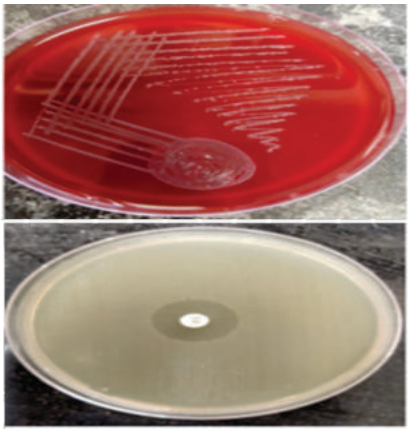
Enterococci are identified microscopically by their distinguished characteristics in Gram stain. In microscopic examination they are found in pairs and at obtuse angles and later catalase test are done. Catalase test helps in primary differentiation of Enterococci from Staphylococci. Staphy lococci are gram positive whereas Enterococci are gram negative. 4 The further speciation was done by subjecting the isolates with a panel of biochemical and various tests like sugar fermentation (Glucose, Sucrose, Mannitol, and Arabinose) and was subjected to motility for result interpretation. A specific carbohydrate containing basal medium was used for sugar fermentation. A production of acid carbohydrate complex helped in identification of Enterococcus. Appearance of yellow colour indicated acid production and was considered as positive response. Appearance of reddish pink colour indicated negative result. In negative result medium remained purple. Appearance of orange colour indicated delayed response. In such scenarios after comparing with un-inoculated tube re-incubation was carried out.

Sugar fermentation test

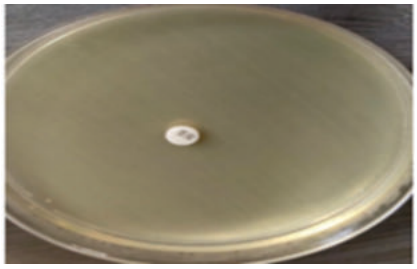
Enterococcus colonies on Mac Conkey agar



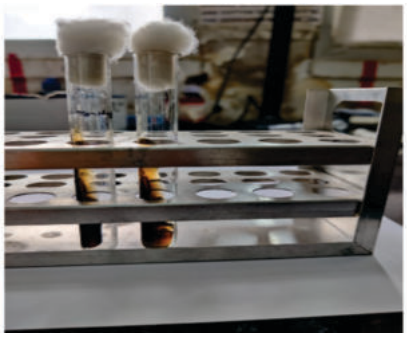
Enterococcus colonies on blood agar



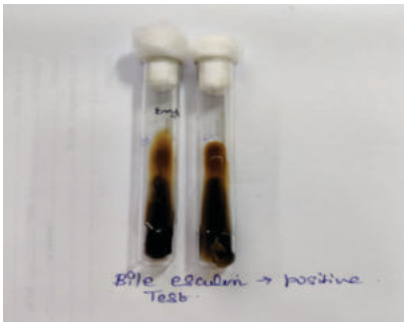
Enterococcal Strain Showing Susceptibility To Vanco mycin (30mcg)disk



Enterococcal strain showing resistance to vancomycin (30mcg) disk



Bile Esculin Agar Test



Antimicrobial susceptibility testing of enterococcal isolates

All the Enterococcal species were subjected to modified Kirby Bauer plate diffusion as per standard procedure suggested by CLSI guidelines. Antibiotic susceptibility testing was done by Kirby-Bauer disk diffusion method using antibiotic disks and Mueller Hinton Agar as per formed protocol by CLSI. 4

Vancomycin was used for detection of vancomycin resistance. After complete 24 hour of incubation the Mueller Hinton agar containing the vancomycin antibiotic was observed with naked eye-using transmitted light for presence or absence of zone of inhibition around the disk. On presence; Inhibition zones, were calculated with ruler. Any identified growth within the zone of inhibition was also observed.

Ethical approval was obtained from Institutional ethical committee.

Requirements

Mueller Hinton Agar

- Bacterial inoculum adjusted to 0.5 McFarland Standard Incubation time and temperature- 370c for 16 -18 hours.

Lawn culture of the organism is made over the Mueller Hinton agar (Hi Media). with the suspension of organism cultured in peptone water which is standardized with 0.5 McFarland standard. After inoculum has dried specific antibiotic discs were placed 2 cm apart from each other with sterile forceps and was incubated for 18-24 hours at 37oC aerobically. The zone size was interpreted according to the reference chart provided by the manufactures, according to NCCLS standards for each organism. The Antibiotic discs used for Gram positive cocci were: Penicillin G (10 units/disc), Erythromycin (15mg/disc), Ciprofloxacin (30mg/disc), Gentamicin (50mg/disc), Nalidixic acid (30mg/disc), Vancomycin (30mg/disc).

MIC was performed using broth microdilution technique from the standard operating procedure of august 2020 national programme on antimicrobial resistance containment national centre for disease control, INDIA AUGUST 2020

BROTH MICRODILUTION SUSCEPTIBILITY TESTING

Preparation of drug stock solution of vancomycin- the formulation of reference standard powder used for AST is vancomycin hydrochloride as per CLSI standard

Potency calculation- If potency of vancomycin powder available in vancomycin hydrochloride salt mentioned in the CAS/CoA Is 975µg/mg.

To prepared stock solution of 1000µg/ml(1mg/ml) weigh 10 mg of this vancomycin hydrochloride powder with potency of 975µg/ml and add9.75ml of autoclaved distilled water. This is the 1mg/ml primary stock solution.

Preparation of working stock solution-for making 128µg/ml (4x working stock solution) from original stock solution (1000µg/ml)

Preparation of dilution of vancomycin-add 500µl from 128 µg/ml WSS to 500 µl MHB medium in MCT & perform 2fold serial dilutions (in 9 MCTS containing 500 µg MHB) to get drug concentrations as 64 µg/ml, 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml and so on. Dilute 0.5 McFarland suspension 1:75 times by adding 10 µl to 740µl of autoclaved MHB medium. From this diluted suspension, take 25 µl and add to each of the wells in column 1 to 11 already containing 75 µl (50µl MHB + 25µl antibiotic) to yield bacterial concentration of approximately 5 x104CFU/well.

Table 1-analysis Of Total Samples

TOTAL SAMPLES	NO OF SAMPLES	PERCENTAGE
NO OF CULTURE POSITIVE	645	69.71
NO OF CULTURE NEGATIVE	281	30.29
TOTAL	926	100

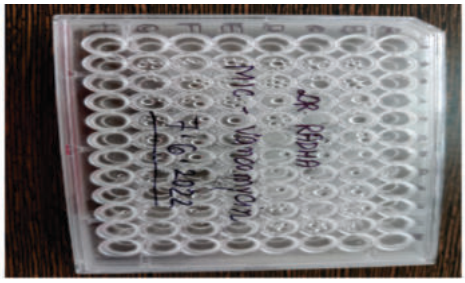
Table 2- GenderWise Distribution Of Samples

GENDER	NO OF SAMPLES	PERCENTAGE
MALES	510	55.05
FEMALES	416	44.95
TOTAL	926	100

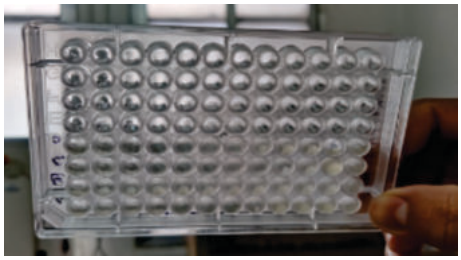
Table 3- Distribution Os Different Types Of Samples In The Study

TYPE OF SAMPLE	NO OF SAMPLE	PERCENTAGE
URINE	378	40.30
PUS AND OTHER BODY FLUIDS	298	31.90
ET ASPIRATES	90	9.80
BLOOD	160	18.00
TOTAL	926	100

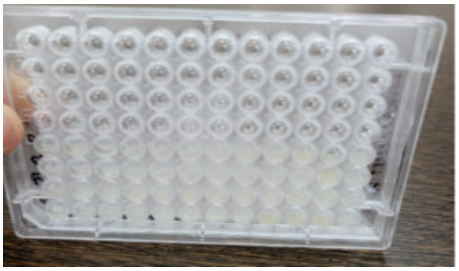
Antimicrobial resistance profiles of Enterococcus faecalis isolates by DISK-DIFFUSION METHOD



Enterococcal strain showing susceptibility to vancom ycin by MIC



Mic Of Vancomycin Resistance Enteroccocus Strain



S. No.	Result	Inhibition zone (mm) Susceptible	Intermediate	Resistant
1	Vancomycin Disk	≥17	15-16	≤14 and 1 or any discernable growth within zone of inhibition
2	Vancomycin MIC	<4	8-16	>32

Vancomycin resistance characteristics(MIC break points)

Table 5: Interpretive categories and MIC Breakpoints (µg/ml) for *Staphylococcus aureus* and *Enterococcus* species against vancomycin

Antimicrobial Agent	Interpretive Categories and MIC breakpoints, µg/ml					
	Staphylococcus aureus			*Enterococcus* spp.		
	S	I	R	S	I	R
Vancomycin	≤2	4-8	≥16	≤4	8-16	≥32

Fig. 1: Preparation of vancomycin dilutions

	(1)	1:2 (2)	1:2(3)	1:2 (4)	1:2(5)	1:2(6)	1:2(7)	1:2(8)	1:2(9)	1:2(10)
MCT	872 sterile MHB + 128µl working stock solution	500 sterile MHB	500	500	500	500	500	500	500	500
Vancomycin conc. (µg/ml) in MCT	128	64	32	168	8	4	2	1	0.5	0.25
From MCTs take 25µl and add to all wells in corresponding microtitre plate column containing 50µl CaMHB										
Final conc. in microtiter plate wells	32	16	8	4	2	1	0.5	0.25	0.125	0.06

Reading of result MIC is expressed as the lowest dilution, which inhibits the growth which is judged by lack of turbidity in the tube. Because very faint turbidity may be given by the inoculum itself, the inoculated tube kept in the refrigerator overnight may be used as the standard for the determination of complete inhibition. Standard strain of known MIC value Enterococci ATCC 29212, also run along with the test to check the reagents and conditions. The results were recorded in a standard chart for tube dilution and interpreted.

Quality control of media and other tests-

Each batch of medium was tested for sterility by selecting a plate at random and incubated at 37°c for 24-48 hours. Further the plates were inoculated with the positive and negative control organisms to know the performance of the media.

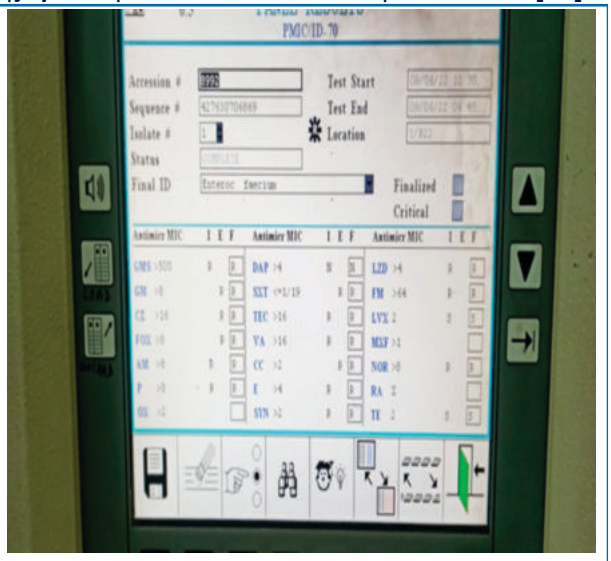
RESULT-

A total 926 samples were analysed at microbiology department of RIMS RANCHI during 6 months period, among these 645 were culture positive & 281 were culture negative. Gender wise distribution of sample were -males 510 (55.05 %) &females 416 (44.95%).

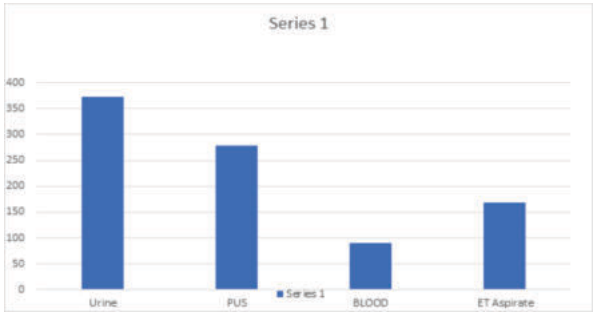
Distribution of different types of samples like urine- 372, pus - 298, ET 90, Blood 168 etc prevalence of Enterococci in total no. of 654 positive samples was 81 which is-12.55% . in our study 74 Enterococci isolates are isolated which shows the Highest isolation of Enterococci from the urine sample followed by pus and other body fluids- 4 blood 3 isolates respectively.

The species isolated in the study was Enterococcus faecalis 99%,1% Enterococcus faecium was also isolated. By kirby Bauer disk diffusion method, erythromycin &ciprofloxacin resistance was 78% &85%respectively which is alarming high percentage. penicillin &gentamycin showed 77% &72% .99 strains showed 100% sensitive to vancomycin by KBDDM.

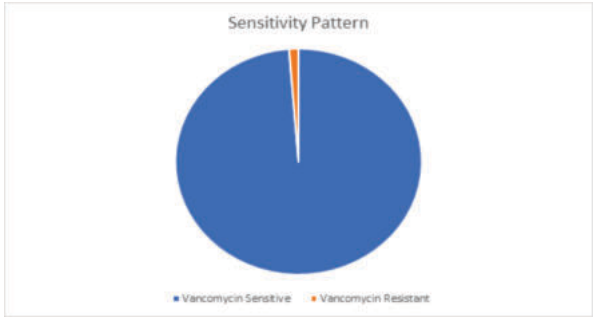
One strain showed vancomycin resistance in our study with1%, which is also reported by other Indian studies.in contrast to reports from U.S.A where vancomycin resistance is more common. About 8 of Enterococcal strains showed raised MIC of 0.125% µg/ml.



Enterococcus faecium strain showing resistance to vancomycin via BD-PHOENIX



Prevalence of Enterococci in different clinical samples



Distribution of Enterococcus according to vancomycin resistance

CONCLUSION-

Precise identification of Enterococci to species level enables us, to assess the species-specific antimicrobial resistance characteristics, apart from knowing the epidemiological pattern and their clinical significance in human infections. Further as shown in our study, the increase in the rate of prevalence of the Enterococcus species and the emergence of multidrug resistance among them, highlights the significance of rapid and accurate identification of Enterococci to the species level for initiating appropriate therapeutic regimen and reemphasize the importance of the implementationto appropriate infection control measures to limit the nosocomial spread of these Enterococci species in any nosocomial setting. Vancomycin in combination with an aminoglycoside has synergistic activity against Enterococci and is recommended as the drug of choice in patients with ampicillin- and penicillin-resistant.

However, Enterococci are becoming increasingly resistant to traditional antibiotic therapy. In addition to high-level

aminoglycoside resistance and ampicillin resistance, rapid spread of vancomycin resistance has resulted in limited therapeutic alternatives. Important to maintain regular surveillance of antibiotic susceptibilities so that changes in their pattern can be detected early. As MIC detection is a laborious procedure all Enterococcal isolates can be screened by vancomycin agar screen method containing 6µg/ml which is suggested by NCCLS and only those isolates which are positive by this method can be tested further for vancomycin MIC, as both the methods correlated well in this study.

Conflict of interest- none

REFERENCES

[1] Ssusan L. Fraser Infectious Diseases Service, Walter Reed Army Medical Center, Julia Lim, Army Medical Center; Curtis J Donskey, Case Western Reserve University, Robert A Salata, Case Western Reserve University School of Medicine.
[2] Yesim Cetinkaya, Pamela Falk and Glen Mayhall C. Department of Healthcare Epidemiology and Division of Infectious Diseases, University of Texas Medical Branch at Galveston, Galveston, Texas.
[3] Boyce J.M. (1997) Infect. Dis. Clin North Am. 11, 367-384.
[4] Robin Patel. Infectious Diseases Research Laboratory, Mayo Clinic and Foundation, 200 First St. S.W. Rochester, MN 55905, USA.
[5] Handwerger S., Raucher B., Altarac D., Manka J., Marchione S., Sing K.V., Murray B.E., Wolff J. and Walters B. (1993) Clin. Infect. Dis. 16, 750-755.
[6] Topley Wilsons microbiology and microbial infections 10th edition volume 2 Bacteriology 883-902. [7] Desai P.J., Pandit D, Mathur M. and Gogate A. (2001) Indian Journal of Medical Microbiology, 19(3), 132-13