



PREVALENCE OF *Enterococcus* SPECIES FROM VARIOUS CLINICAL SPECIMENS IN SHRI SATHYA SAI MEDICAL COLLEGE AND RESEARCH INSTITUTE WITH SPECIAL REFERENCE TO SPECIATION & THEIR RESISTANCE TO VANCOMYCIN

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Abstract-

Introduction- *Enterococci* are part of the normal intestinal flora of humans and animals but have also emerged as important pathogens responsible for serious infections in hospital and community acquired infections. According to recent surveys, *Enterococci* remain in the top 3 most common pathogens that cause nosocomial infections.

Aim- To process all the clinical samples from various departments in our hospital, for the isolation of *Enterococci* sp.

To speciate the isolates & to have the resistance pattern of the isolates to Vancomycin

Methods and Material- A total of 928 samples were collected from both out patients and in patients in all clinical departments and transported to microbiology laboratory. Specimens were processed by inoculating on to Mac Conkeys agar, blood agar, nutrient agar and Pfizer selective media and incubated at 37°C for 24 - 48 hours. *Enterococci* were identified by their typical arrangement in Gram stain, Bile esculin test and salt tolerance test. Speciation was done by subjecting the isolates to a battery of biochemical tests. Antimicrobial susceptibility patterns were determined by performing Kirby-Bauer disc diffusion method and Minimum Inhibitory Concentration (MIC) values were identified by Tube & Agar dilution method.

Results- A total of 928 samples. Among these, 647 (69.72%) were culture positive with different isolates and 281 (30.28%) were culture negative. Among 647 culture positive cases, 100 (15.46%) were *Enterococcus faecalis*. Antimicrobial susceptibility & MIC done as per standard protocols. The *E. Faecalis* showed 99% sensitive to Vancomycin. The resistance to Vancomycin was further confirmed by MIC both Agar & Tube dilution methods, in which the MIC was: 32µg/ml in one isolate.

Conclusions- Species level identification of *Enterococcus* is not only important for epidemiological study, but also for analysing the drug resistant pattern. Effective detection of Vancomycin resistance in laboratory helps in reducing the morbidity and mortality due to VRE in hospitalized patients.

Key words- Blood agar, Mac Conkeys agar, Bile esculin, Vancomycin resistant *Enterococci*, Minimum inhibitory concentration.

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Introduction

Enterococci are part of the normal intestinal flora of humans and animals but have also emerged as important pathogens responsible for serious infections in hospital and community acquired infections. The genus *Enterococcus* includes more than 17 species, but only a few cause clinical infections in humans [1].

Enterococci were originally classified as enteric gram-positive cocci and later included in the genus *Streptococcus*. In the 1930s, with the establishment of the Lancefield serological typing system, *Enterococci* 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 *Enterococcus 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, antimicrobial and non antimicrobial risk factors have been identified[5].

Third-generation cephalosporins, aminoglycosides, 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 [2]. 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, Shri Sathya Sai Medical College & Research Institute, Kancheepuram. To study the prevalence of *Enterococcus* species from various clinical specimens in Shri Sathya Sai Medical College & Research Institute with special reference to speciation & their resistance to Vancomycin.

Aim and objectives

1. To process all the clinical samples from various departments in our hospital, for the isolation of *Enterococci* sp.
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.

Materials and Methods

A total of 928 samples were collected from both Out-Patient and In-Patient from all clinical departments of Shri Sathya Sai Medical College & Research Institute, during one year period.

Specimens collected

- Urine
- Pus

- Blood
- Endotracheal aspirates
- Sputum

Collection and transport of specimen

Data collection

Complete data about the patients such as name, age, sex, hospital number, date, time of collection, source of specimen and details about the clinical history was recorded before the specimen was processed.

All specimens for microbiological processing were collected in appropriate sterile containers. For urine collection the patients were given a sterile, screw capped, wide mouth, leak proof, labeled with name of the patient and number of the sample on the containers and patients were advised how to collect the midstream urine to avoid contamination.

For sputum collection a well coughed out sample was collected in sterile, wide mouthed, screw capped container labeled with name and number of the specimen.

Pus, other aspirates were collected in a test tube and pus from the site of lesion like abscesses, boils, infected wounds etc. was collected by using at least two sterile cotton swabs

Blood sample was collected carefully by first choosing the vein and then the skin was prepared by disinfecting with 70% alcohol, dried and then 2% of tincture iodine was applied and allowed it to dry. Then the vein was punctured with sterile needle and 5 ml of blood was collected without touching the site. Collected blood sample was then inoculated into blood culture bottles and then transported to the laboratory immediately for the incubation.

Endotracheal aspirates were collected by using sterile 12 Gauge endo tracheal suction catheter tube. Suction catheter was connected to the suction pump and passed through endotracheal tube. In case of purulent secretion the endotracheal suction catheter tips were collected using sterile scissors into the sterile container (screw capped). In case of thick or dry secretions with clinical diagnosis of Pneumonia, sterile normal saline is pushed into endotracheal tube and then suction was done for the aspirate. The samples were transported to microbiology laboratory within 15 minutes. Specimens were processed as soon as they were obtained.

Processing of samples

The samples were plated on to Mac Conkeys agar, blood agar, nutrient agar, Pfizer selective media and incubated at 37°C for 24 - 48 hours.

The plates were examined for growth after 24 hours. If growth was observed, colony morphology and Gram stain morphology were studied. If no growth was observed, the plates were further incubated for the next 24 hours.

The colonies were further identified by hanging drop preparation and biochemical reactions. The Gram positive and Gram negative organisms were differentiated and identified by a battery of tests as per standard protocols. The Gram negative organisms are excluded and not further processed as the study is on only *Enterococci*.

Identification of *Enterococci*

Enterococci are identified microscopically by their typical arrange-

ment in Gram stain in which they are arranged in pairs and in obtuse angles and Catalase test is done to differentiate between *Staphylococci* and Group D *Streptococci*.

Further to differentiate between other groups of *Streptococci*, tests like Salt tolerance tests, Heat resistance test at 60°C for 30 minutes, Bile esculin test where *Enterococci* can grow in the salt concentration of 6.5%, Pfizer selective media, they also can tolerate the temperature of 60° for 30 minutes and grows on 40% bile where all the other groups of *Streptococci* are inhibited (Fig. 1) ⁽⁶⁾.

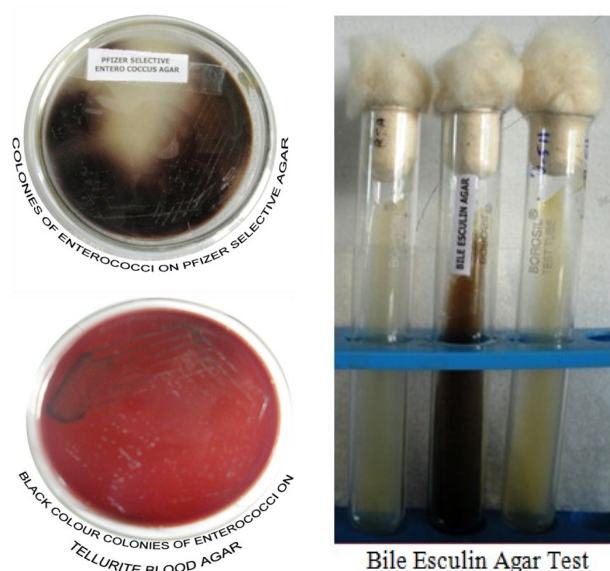


Fig. 1-

Identification of species

The speciation is done by subjecting the isolates with a battery of biochemical and other tests like sugar fermentation (Glucose, Sucrose, Mannitol, Raffinose, Arabinose and Sorbitol). L-Arginine decarboxylase, Pyruvate fermentation, Motility testing is done and results interpreted (Fig. 2 and 3 a & b)

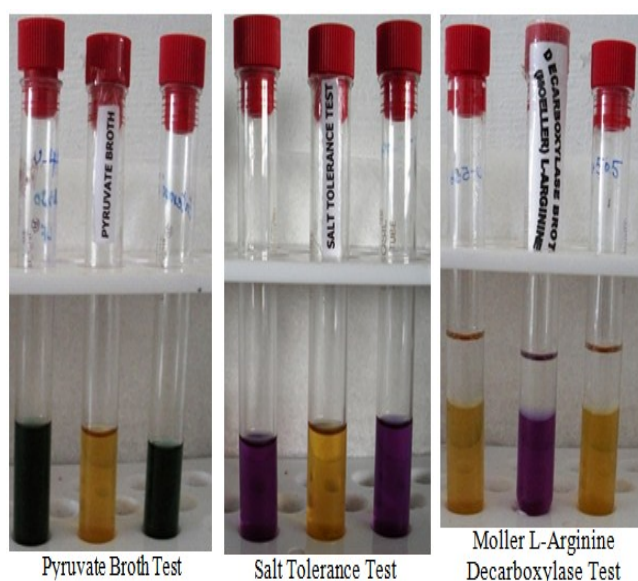


Fig. 2-

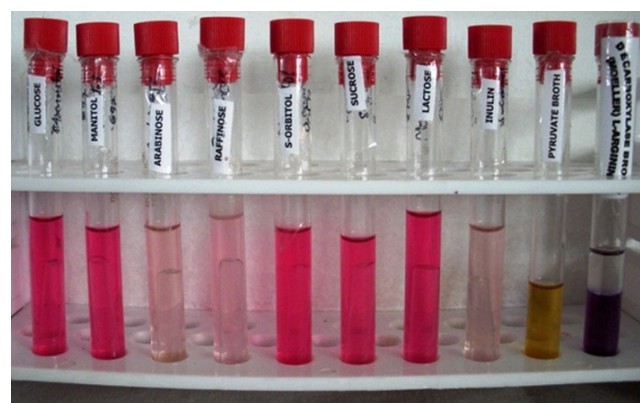


Fig. 3a- Sugar Fermentation Test for Enterococcus

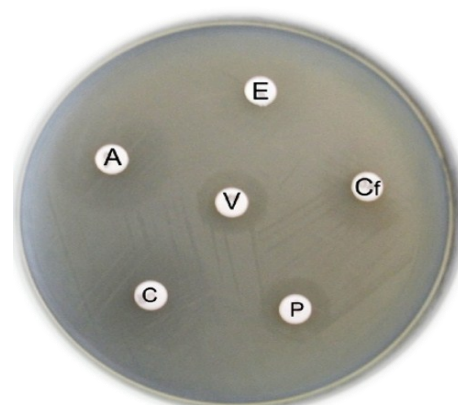


Fig. 3b- Antibiotic Sensitivity Test by Kirby – Bauer Disc Diffusion Method

Detection of Anti microbial susceptibility pattern

Disc diffusion method

Anti microbial susceptibility pattern was done in Mueller Hinton agar plate by Kirby Bauer Disc diffusion method as recommended by NCCLS.

Requirements

Mueller Hinton Agar

- Bacterial inoculum adjusted to 0.5 Mac - Farland Standard
- Incubation time and temperature- 37°C 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 Mc Farland standard. After inoculum has dried specific antibiotics discs were placed 2 cm apart from each other with sterile forceps and was incubated for 18-24 hours at 37°C 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).

Dilution methods- (Tube & Agar dilution)

Materials required

Sterile graduated pipettes of 10ml, 5ml, 2ml and 1ml.
Sterile capped 7.5 x 1.3 cm tubes, Pasteur pipettes, overnight broth culture of test and control organisms (same as for disc diffusion tests), Vancomycin antibiotic in powder form is obtained from the Hi-Media manufacturer accompanied by a statement of its activity in mg/ per ml solvent for the antibiotic, sterile Distilled Water and nutrient broth medium for testing the isolate.

Preparation of stock solutions

Stock solutions were prepared using the formula $(1000/P) \times V \times C = W$, where P = potency of the antibiotic base, V=volume in ml required, C=final concentration of solution and W=weight of the antimicrobial to be dissolved in V.

Medium used for broth and Agar dilution

Brain heart infusion broth is used for testing the isolates by broth dilution. 5ml of broth is added after sterilization to sterile test tubes. Mueller Hinton Agar is used for agar dilution testing.

Preparation of broth and agar dilutions

Stock solution

Stock solution can be prepared using the formula

$$\frac{1000}{P} \times V \times C = W$$

P

Where P=Potency given by the manufacturer in relation to the base

V= Volume in ml required

C=Final concentration of solution (multiples of 1000)

W= Weight of the antimicrobial to be dissolved in the volume V.

Preparation of stock solutions and various concentrations of dilutions for both tube dilution and agar dilution are mentioned below.

ANNEXURE - I

Preparation of antibiotic solution for broth and agar dilutions

Stock solution I,II,III

Stock solution – I (10,000mg/ml)-

20 ml solution is prepared by mixing 210.52mg of powder base whose potency is 950 mg per gram, with sterile distilled water

$$W = \frac{1000}{950} \times 20 \times 10 = 210.52\text{mg}$$

Stock solution – II (1000mg/ml)-

1ml of solution from the stock solution-I is transferred to 9 ml of distilled water (stock solution-II) which gives a concentration of 1000mg/ml.

Stock solution – III (100mg/ml)-

0.1 ml of stock solution- I is transferred to 9.9 ml of distilled water (stock solution-III) which gives a concentration of 100mg/ml.

Preparation of various drug concentration for tube dilution-Inoculum preparation

Stock sol-I	Ist tube(128mg/L)	IInd tube(64mg/L)	IIIRD tube(32mg/L)	IVth tube (16mg/L) 32
10,000mg/L	256µl from soln-I	128µl from soln-I	64µl from soln-I	µl from soln-I
Stock sol-II	Vth tube(8mg/L)	VIth tube(4mg/L)	VIIth tube(2mg/L)	
II 1000mg/L	160µl from soln-II	80µl from soln-II	40µl from soln-II	
Stock sol-III	VIIIth tube(1mg/L)	IXth tube(0.5mg/L)	Xth tube (0.25mg/L)	XIth tube (Control) Free
III 100mg/L	200µl from soln-III	100µl from soln-III	50µl=	from Antibiotics

At least three to five well-isolated colonies of the same morphological type are selected from an agar plate culture. The top of each colony is touched with a loop and the growth is transferred into a tube containing 4 to 5 ml of a Nutrient broth medium.

The broth culture is incubated at 37°C until it achieves or exceeds the turbidity of the 0.5 McFarland standard 2 - 6 hours.

The turbidity of the actively growing broth culture is adjusted with sterile saline or broth to obtain turbidity optically comparable to that of the 0.5 McFarland standards. Finally suspension containing approximately 1×10^6 CFU/ml for *Enterococci* ATCC 29212, isolates is compared with 0.5 McFarland standards in adequate light with a white background and contrasting black lines.

Procedure

1- 2 µl of inoculum is transferred to the agar plates which give a final concentration of 10^4 cfu/ml, similarly 0.1ml of inoculum is aseptically transferred to each of the different concentration test tubes. A control tube and an agar plate also inoculated which does not have the antibiotics. The plates and tubes are incubated at 37°C for 24 hours along with controls.

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 both broth and 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.

Results

A total of 928 samples were collected from both out and in patients. Among these 647(69.72%) were culture positive and 281 (30.28) were culture negative (Table-1). Gender wise distribution of samples were, males 511(55.06%) and females 417(44.94%) (Table-2). Age wise distribution of samples, the youngest patient was 1 year and oldest was 80 years old (Table- 3). Distribution of different types of samples like Urine, Pus, ET-aspirates and Blood etc (Table- 4). Prevalence of *Enterococci* and other isolates in the total number 647 of positive samples; *Enterococci* were 100 (15.46%) (Table-5). Prevalence of *Enterococci* among different samples *Enterococci* was isolated more from urine sample 59% (Table-6). All 100 isolates of *Enterococci* were identified as *Enterococcus faecalis* (Table- 7).

Antimicrobial resistance of *Enterococcus faecalis* isolates (100) by disk diffusion method by battery of antibiotic (Table- 8). Among all the isolates (100), one isolate showed MIC of 34µg/ml in both macrodilution (Table- 9 & Fig. 4) and agar dilution methods (Table - 10 and Fig. 5) which is considered as resistance.

Table 1- Analysis of Total samples

Total samples	No. of samples	Percentage
No of culture positives	647	69.72
No of culture negatives	281	30.28
Total	928	100

Table 2- Gender wise distribution of samples

Gender	No of samples	Percentage
Males	511	55.06
Females	417	44.94
Total	928	100

Table 3- Age wise distribution of total samples

Age	No. of samples	Percentage
0 – 10	57	6.14
11 – 20	82	8.84
21 – 30	102	10.99
31 – 40	147	15.84
41 – 50	170	18.32
51 – 60	162	17.46
61 – 70	133	14.33
71 – 80	75	8.08
Total	928	100

Table 4- Distribution of different types of samples in the study

Type of sample	No of samples	Percentage
Urine	374	40.30
Pus and other body fluids	296	31.90
ET aspirates	91	9.80
Blood	167	18.00
Total	928	100

Table 5- Prevalence of enterococci and other isolates in the total no. of positive samples

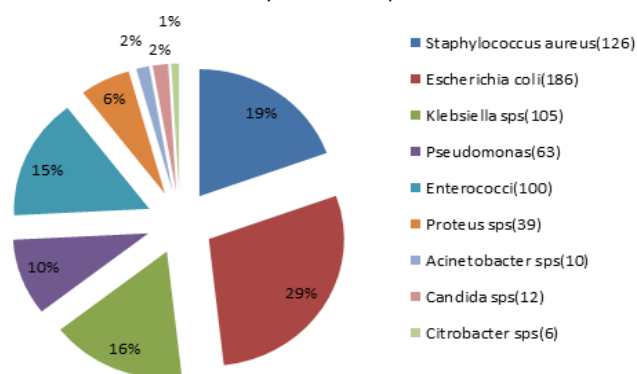


Table 6- Prevalence of enterococci in different samples

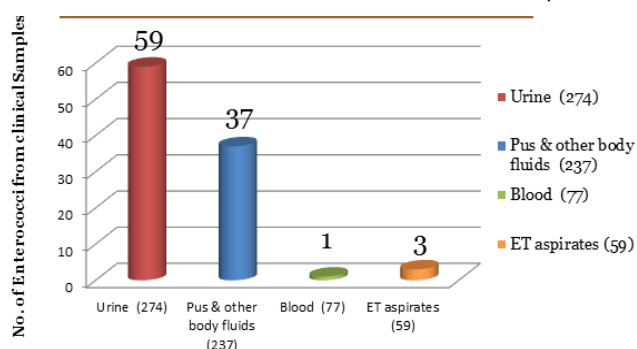


Table 7- Species identities of Enterococci from total isolates

Species	No. of isolates	Percentage
E. faecalis	100	100
Total	100	100

Table 8- Antimicrobial resistance profiles of E. faecalis isolates by Disk-diffusion method (n=100)

Antibiotics	Resistance	Percentage
Penicillin	78	78
Erythromycin	82	82
Ciprofloxacin	82	82
Gentamicin	71	71
Nalidixic acid	91	91
Vancomycin	1	1

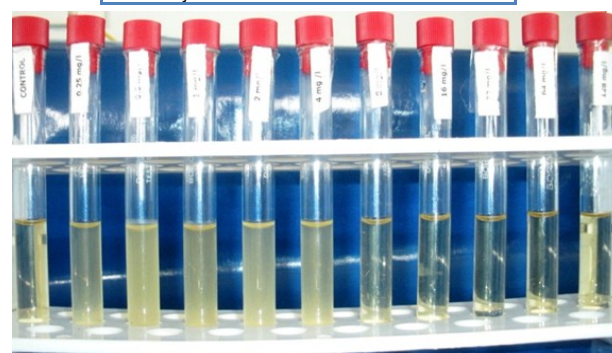


Fig. 4- Minimum Inhibitory Con. Test by Macrodilution Method for Enterococcus ATCC 29212



Fig. 4- Minimum Inhibitory Con. Test by Macrodilution Method for Enterococcus

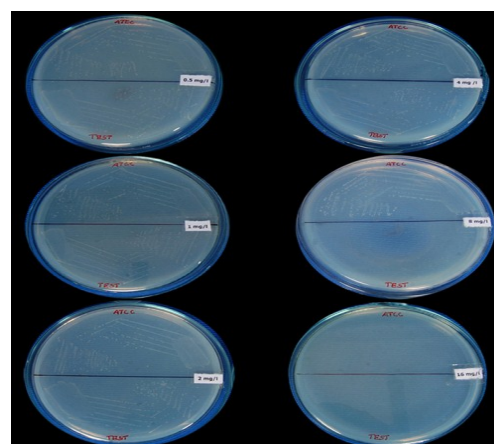


Fig. 5- Minimum Inhibitory Concentration For Test Enterococci and ATCC 29212 by Agar Dilution Method

Discussion

Vancomycin-resistant *Enterococci* have been increasingly reported worldwide since its first description in 1987. The present study documents the phenotypic character and resistant pattern of *Enterococci* to vancomycin over 1-year period from different clinical samples at Shri Sathya Sai Medical & Research Institute.

The study was conducted to know the prevalence of *Enterococci* sp in our hospital, to know the resistance pattern of the isolates to Vancomycin.

Recently *Enterococci* are being isolated more frequently from the clinical specimens and are gaining upper hand in causation of nosocomial infections. CDC indicates that enterococci are the second leading cause of nosocomial infection, joining *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* in the list of most prevalent pathogens. During the 12 months of our study, *Enterococci* were isolated from various clinical specimens with the prevalence rate of 15.46%. The wide variety of infectious material from which *Enterococci* was isolated was found similar to those obtained in other studies.⁽⁷⁾

In prevalence *Escherichia coli* remains at top of the table with 186 (28.75%) followed by *Staphylococcus aureus* 126 (19.46%), *Klebsiella* sp 105 (16.23%), *Enterococci* sp 100 (15.46%), *Pseudomonas* sp 63 (9.74%) and *Proteus* sp 39 (6.03%). The percentage in our study correlated with the study done by P.J.Desai et al where they reported a prevalence rate of 22.19%. Dr. Abdel -Moti Khyri Al Jarousha Mr. Ahmed Mohammed Saed / Hassan Afifi which is 18 %⁽⁸⁾.

P.J.Desai et al reported 8.92 % of *Enterococci* isolation in urine which correlates well with the present study where 59 *Enterococci* isolates are isolated, (9.12%), which shows the frequent and higher isolation of *Enterococci* is from urine sample followed by pus and other body fluids 37 isolates (5.72%), endotracheal aspirates 3 isolates (0.46%) and from blood 1 isolate (0.15%) respectively. The species isolated in the study was *Enterococcus faecalis* (100%), Incidence of *Enterococcal* infection is higher in the age group, between 51-60 years.

By Kirby Bauer disk diffusion method, Erythromycin and ciprofloxacin resistance was 82% and 83.2% respectively which is alarmingly high percentage. Penicillin and Gentamicin showed 78% and 71 % resistance. 99 strains showed 100% sensitive to Vancomycin by KBDDM.

One strain showed Vancomycin resistance in our study with 1% and 15% intermediate resistance in our present study which is also reported by other Indian studies⁽⁹⁾. In contrast to reports from U.S.A where Vancomycin resistance is more common. In our study, about 12 of *Enterococcal* strains showed raised MIC of 8 µg/ml.

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 implementa-

tion of 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.

Effective detection of vancomycin resistance in laboratory helps in reducing the morbidity and mortality due to VRE in hospitalized patients.

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Table 9- MIC range of *E. faecalis* for selected drug Vancomycin (Macrodilution method n=100)

Drug conc (µg/ml)	0.25 (µg/ml)	0.5 (µg/ml)	1 (µg/ml)	2 (µg/ml)	4 (µg/ml)	8 (µg/ml)	16 (µg/ml)	32 (µg/ml)	64 (µg/ml)	128 (µg/ml)
Control ATCC (29212)	---	1	---	---	---	---	---	---	---	---
MIC value (No. of isolates)	---	---	---	61	23	12	3	1	---	---
Percentage %	---	---	---	61	23	12	3	1	---	---

Table 10- MIC range of *E. faecalis* for selected drug Vancomycin (Agar dilution method n=100)

Drug conc (µg/ml)	0.25 (µg/ml)	0.5 (µg/ml)	1 (µg/ml)	2 (µg/ml)	4 (µg/ml)	8 (µg/ml)	16 (µg/ml)	32 (µg/ml)	64 (µg/ml)	128 (µg/ml)
Control ATCC (29212)	---	1	---	---	---	---	---	---	---	---
MIC value (No. of isolates)	---	---	---	64	24	9	2	1	---	---
Percentage %	---	---	---	64	24	9	2	1	---	---