‘SPECIATION AND ANTIBIOTIC SUSCEPTIBILITY OF ENTEROCOCCI ISOLATED FROM THE PATIENTS AND THE NORMAL POPULATION’

BY

DR SUDHAMANI, *MBBS*

DISSERTATION SUBMITTED TO

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UNDER THE GUIDANCE OF

Dr P M BEENA, *MD*

HOD AND PROFESSOR

DEPARTMENT OF MICROBIOLOGY

SRI DEVARAJ URS MEDICAL COLLEGE, KOLAR

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DR P.M.BEENA *MD*

HOD AND PROFESSOR,

DEPARTMENT OF MICROBIOLOGY,

SRI DEVARAJ URS MEDICAL COLLEGE, KOLAR

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SIGNATURE OF THE GUIDE

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PROFESSOR
DEPARTMENT OF MICROBIOLOGY

ENDORSEMENT BY THE HOD, PRINCIPAL/HEAD OF THE INSTITUTION

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HOD AND PROFESSOR

DEPARTMENT OF MICROBIOLOGY

SEAL & SIGNATURE OF THE HOD

DR BEENA P.M

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DR SUDHAMANI

POST GRADUATE STUDENT IN THE DEPARTMENT OF MICROBIOLOGY OF

SRI DEVARAJ URS MEDICAL COLLEGE

TO TAKE UP THE DISSERTATION WORK ENTITLED

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Date: *Signature of the Candidate*

Place: Kolar ***DR SUDHAMANI***

LIST OF ABBREVIATIONS

ATCC - American Type Culture Collection

CFU - Colony Forming Unit

HLAR - High Level Aminoglycoside Resistance

KDa - Kilo Dalton

MIC - Minimum Inhibitory Concentration

NCCLS - National Committee for Clinical Laboratory Standards

NNISS - National Nosocomial Infection Surveillance System

PBP - Penicillin Binding Protein

VRE - Vancomycin Resistant Enterococci

CLSI - Central Laboratory for Standard Information

GIT - Gastrointestinal Tract

PAI - Pathogenicity islands

NNIS - National Nosocomial Infection Surveillance System

CDC - Center for Disease Control

HLR - High Level Resistance

UTI - Urinary Tract Infection

ABSTRACT

INTRODUCTION:

Enterococci are the normal commensals of the human intestine. Sites less often colonized are oral cavity, genitourinary tract and perianal area.[65] Enterococci were previously considered as low grade pathogens but recently it gained importance because of changing trends in infections caused by different species of *Enterococci* and emergence of multidrug resistant strains. Most of the laboratories identify the organism only up to genus level.[16] Enterococci identification upto species level in the clinical microbiology is important because some of the Enteroccoci species have intrinsic resistance to several antibiotics like Beta-lactams (cephlosporins, low level aminoglycosides), and species like *E.gallinarium* and *E.classeliflavus* show intrinsic resistant to vancomycin.[16] So correct speciation can help to predict antibiotic resistance pattern and treatment.

MATERIALS and METHODS:

100 Enterococci were isolated from various clinical samples of Hospitalized patients and Out Patients attending R L Jalappa Hospital, Kolar. The various clinical samples were Urine, Pus, blood, body fluids, endotracheal aspirations and sputum. 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 Agar dilution and E-strip method.70 stool samples were collected from hospitalized patients who had Enterococcal infection and 70 stool samples were collected from the healthy normal individuals and processed.

RESULTS:

Most of the Enterococcal infections were seen in the age group 21-40 years and 41-60 years each accounting for 32%. Males accounted for 58% of the patients whereas female patients accounted for 42%. Maximum number of Enterococci were isolated from pus sample (50%) followed by urine (29%) and blood (13%). Enterococci isolation was from with post-operative wound infections (26%) followed by Urinary Tract Infection (12%) and bacteremia (13%). 43% of isolates were associated with polymicrobial infection. *E. faecalis* (55%) was the predominant species followed by *E. faecium* (45%). *E.faecium* showed 80% resistance to Penicillin and Ampicillin whereas *E.faecalis* showed 28% and 38% respectively. Both species showed resistance to High Level Gentamicin (60%). Urinary isolates of Enterococci were least resistant to Nitrofurantoin. All enterococci isolates in our study were 100% sensitive to Vancomycin, Linezolid and Teicoplanin. MIC for Vancomycin both by Agar dilution and E-strip methods showed that one strain of *E. faecium* isolated from blood sample was resistant to Vancomycin. Enterococcal colonization was found in 80% of healthy individuals and 42% of hospitalized patients. *E.faecium* was the common isolate found in 70% of hospitalized patients and 95% of healthy individuals without any VRE carriage among them.

Conclusion: We conclude that Enterococcal strains with high rate of resistance to multiple drugs are not only prevalent in the clinical environment but also in the gastrointestinal tract of the colonized patients and the healthy individuals.

Though the study showed there was no prevalence of fecal carriage of VRE, hospital should keep in mind to implement

Screening surveillance to detect fecal VRE carriage in the hospitalized and in the normal population as these colonized people contaminate themselves as well as environment

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1.INTRODUCTION

Enterococci are aerobic and facultative anaerobic gram positive cocci, previously considered as normal commensals of gastrointestinal tract of humans and other animals, has recently emerged as a medically important pathogen causing hospital as well as community acquired infections. [1] Recent years have witnessed an increasing interest in Enterococcus species not only because of their ability to cause serious infections like endocarditis, bacteremia, intra-abdominal infections, surgical site infection, but also because of their increasing resistance to many antimicrobial agents.[2] Enterococci are medically important as they far outweigh the relatively insignificant proportion of the total adult human commensals they represent. It is ranked as one of the leading organisms causing hospital associated infections .[3]

E.faecalis is the most common species isolated from hospital associated infections followed by *E.faecium*, which account for about 90-95% of infections caused by Enterococci and others like *E.gallinarum, E.casseliflavus, and E.durans* are isolated less frequently accounting for 5% of clinical infections.[4] However at present, prevalence of *E. faecium* is more common than *E. faecalis* in hospital acquired infections.[5] Enterococci and its species differ in drug susceptibility which emphasizes the need for identification of species in treating Enterococcal infection.[6] Enterococci are intrinsically resistant to many antibiotics. They can also acquire drug resistance by various mechanisms like mutation in the chromosome, acquisition of plasmids or transposons containing genetic sequences that confer resistance from other bacteria.[7]

Combination therapy with Penicillin and Gentamicin which has synergistic effect in treating Enterococcal infections was widely employed before. In the current scenario, the acquisition of high level resistance to Aminoglycoside (HLAR) has made this therapeutic combination ineffective.[8] Vancomycin is the drug of choice in

many bacterial infections caused by resistant strains of gram positive cocci, especially those caused by Enterococci. But recently there has been an increase in the number of Vancomycin Resistant Enterococci (VRE). This has posed a serious problem not only in the treatment of Enterococcal infections but also they can transfer this resistance determinant horizontally to other Vancomycin susceptible species.[9] Until recent times, Linezolid has been used as the drug of choice in treating the infections caused by VRE strain, but it is found that resistance to Linezolid is developing slowly.[10]

Several reports have documented the presence of Vancomycin resistant Enterococci (VRE) in the stool samples of asymptomatic individuals from the community who have neither recently been in the hospital nor received any antibiotics. Such findings are contrary to the existing belief that the infections caused by VRE are strictly hospital acquired infections of debilitated and immunocompromised patients.[11] According to epidemiological data, Enterococci are important reservoirs for transmission of antibiotic resistance genes among different species of bacteria. Thus, the occurrence of resistance to Vancomycin is a persisting clinical problem in health care facilities in all geographical areas.[12]The present study is aimed to identify the different species of Enterococci, their antibiotic susceptibility and to determine the prevalence of VRE among patients and normal healthy subjects in and around Kolar.

2.OBJECTIVES

- To isolate and speciate Enterococci from the clinical specimens and from the stool samples of hospitalized and healthy individuals.
- To detect their antibiotic susceptibility pattern.
- To detect the prevalence of VRE among the patients and normal healthy individuals.

3.REVIEW OF LITERATURE

Genus Description [13,14,15]

The genus Enterococcus are gram-positive oval cocci, measuring 0.6 - 2.0 x 0.6 - 2.5µ in size, they are arranged in pairs at an angle to each other or short chains. They are non-capsulated, non-sporing, non-motile cocci (except *E.casseliflavus* and *E.gallinarum*), aerobes and facultative anaerobes. They are heat and salt resistant organisms capable of growing at temperature ranging between 10^0C to 45^{o}C, in 6.5%NaCl and 40% bile salts. They possess Lancefield group D antigen in the cell wall. All the members of the genus are catalase negative, hydrolyze esculin and pyrrolidonyl-β-naphthylamide (PYR).They are usually non hemolytic, though some strains may show alpha or beta hemolysis. Currently more than 47 species have been grouped under this genus.[16]

Historical Perspectives

In 1899, Thiercelin coined the term Enterococcus to describe bacteria that are seen in pairs and short chains in faeces. Andrews and Harder in 1906, gave the name *Streptococcus faecalis* as they were isolated from faeces.[13] In 1919,Orla Jensen described *Streptococcus faecium*, which differed from the fermentation pattern of *Streptococcus faecalis*.[17]Later Sherman and Wing in 1935 identified third species

Streptococcus durans and proved that it was similar to *Streptococcus faecium* but had less fermentation activity. He also used the term Enterococcal group to describe Streptococci that grew at temperature ranging from 10^0C and 45^0C with pH9.6, in broth containing 6.5% NaCl and tolerated temperature of 60^0C for 30 minutes.[17]In 1967, Nowlan and Deibel added *Streptococcus avium* to the Enterococcal group[13]. In 1970, Kalina proposed that *Streptcoccus faecalis* and *Streptococcus faecium* can be included in genus Enterococcus based on phenotypic characteristics [13].

Shattock in 1962 and Deibel in 1964 described the two non-Enterococcal group D Streptococci: *Streptococcus bovis* and *Streptococcus equinus*[18]. In 1992, Pownall identified the motile Enterococci and named it as *Streptococcus faecium* subspecies *mobilis*, which was later renamed by Mundtt as *Streptococcus faecium var casseliflavus.*[19] Later hybridization studies have shown many other *Enterococcus* species; *E.avium, E.casseliflavus, E.durans, E.faecalis, E.faecium, E.gallinarum, E.hirae, E.malodoratus, E.mundtii,E.raffinosus and E.solitarius*[20]. Currently *Enterococcus* genus includes 47 species.[16]

HABITAT

Enterococci are widespread in nature which can grow and persist in harsh environments.[13]Hence, they are readily recovered from various environmental sources like waste and surface water and also from foods such as milk and meat products.[21]

Enterococcus species have been detected in the fecal microbiota of most animals, from insects to mammals.[13]They are found in the gastrointestinal tract of most healthy adults. Studies from Japan, Federal Republic of Germany, and Scandinavia reported

that Enterococci were found in stool specimens from 97% of healthy individuals.[17]The most frequently isolated species are *E. faecalis and E. faecium.* Many studies reported that *E. faecalis* was the more common isolate and was found in higher numbers than *E.faecium,* however, few studies have reported that E.faecium was found more often than *E. faecalis.*[17]

In humans, typical concentrations of Enterococci in stool are up to 10^8colony forming unit (CFU) per gram of faeces.[17] Although Enterococci is known to colonize the oral cavity and vaginal tract, recovery from these sites is as less as 20% .[22]

CULTURAL CHARACTERISTICS

Enterococci are not fastidious organisms as they grow readily on ordinary media like nutrient agar.[23]On blood agar, they grew as small colonies measuring 1-2mm, circular in shape , translucent, convex with smooth surface and an entire edge. Usually colonies are non-hemolytic, but some strains show α or β-haemolysis on blood agar prepared by using human, horse, cow and rabbit's blood, but not sheep blood agar. This is because, the sheep RBC's are refractory to cytolysin mediated lysis.[17,22]

On Mac Conkey agar, they form minute colonies measuring 0.5-1mm,deep pink/ magenta color due to lactose fermentation[23].They also grow on media containing high salt concentration of 6.5% NaCl, hydrolyse esculin in presence of 40% bile and tolerate heat at 60^0C for 30 minutes. [19,20,24]

Selective media; Specimens containing Enterococci that are heavily contaminated with gram negative bacilli may be easily isolated on media containing selective agents

like Sodium azide, carbohydrates and antibiotics such as Kanamycin or Gentamicin.[25,26]

Most often used selective media are:

- Bile esculin azide agar
- Kenner fecal Streptococcus broth
- Selective media with Vancomycin for VRE
- Kanamycin aesculin azide agar
- Eosin methylene blue agar
- Phenyl ethyl alcohol agar
- Cephalexin aztreonam arabinose agar.

BIOCEHMICAL REACTIONS AND SPECIATION:

Preliminary tests for identification of Enterococci are -

- Catalase test
- Bile esculin hydrolysis test
- Salt tolerance (6.5% NaCl) test
- Pyrrolidonyl β naphthylamide hydrolysis (PYR) test
- Heat tolerance test (60^0C for 30 minutes).

CATALASE TEST:

Enterococci do not have cytochrome enzymes and are thus catalase negative, although some strains occasionally produce pseudocatalase.[13,14]

Principle: Catalase is an enzyme that decomposes hydrogen peroxide (H_2O_2) into water and oxygen. Chemically, catalase is a hemoprotein which is similar in structure to hemoglobin, except that the four iron atoms in the molecule are in the oxidized Fe^{3+}

state rather than the reduced Fe^{2+}state. Excluding the streptococci, most aerobic and facultative bacteria possess catalase activity.[27]

Hydrogen peroxide forms as one of the oxidative end products of aerobic carbohydrate metabolism. If allowed to accumulate, it is lethal to bacterial cells where Catalase enzyme converts hydrogen peroxide into oxygen and water .[27]

BILE ESCULIN HYDROLYSIS TEST

It was first described in 1926 by Meyer and Schonfeld, later Facklams and Moody showed that, bile esculin test has high sensitivity (100%) and specificity (97%) in identifying group D Streptococci and Enterococci.[28]

Principle: Esculin is a glycosidic coumarin derivative (6βglucoside-7hydroxy cumarin). The two moieties of the molecule, glucose and 7-hydroxy coumarin are linked together by an ester bond through oxygen. For this test, esculin is incorporated into a medium containing 4% bile salts or 40% bile. Hydrolysis of esculin in the medium results in the formation of glucose and a compound called esculetin. Esculetin in turn reacts with ferric ions, supplied by the inorganic medium containing ferric citrate to form a black diffusible complex.[27]

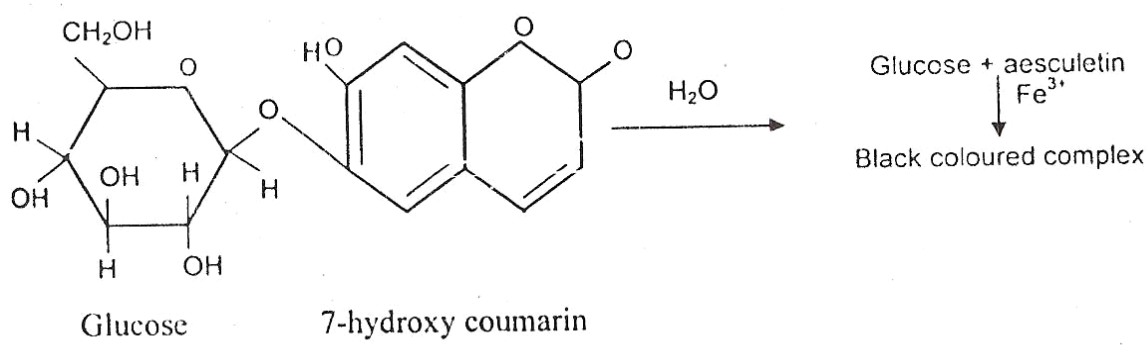


Figure: 1

Procedure: Colonies were inoculated on the slant of the bile esculin medium and incubated at 35°C for 24 – 48 hours. Diffuse blackening of more than half the slant within this period indicates hydrolysis of esculin.[27,29]

SALT TOLERANCE (6.5% NaCl) TEST

Wie Shing Lee in 1972 used a modified NaCl broth called D broth and interpreted results within 24 hours[24]

Principle: Enterococci tolerate and has the ability to grow in salt concentrations of 6.5% NaCl. This test is useful for presumptive identification of Enterococcal species and differentiates from other group D Streptococcus species, *S.bovis and S.equinus*[14,15]

Procedure: Inoculate 2 or 3 colonies into BHI broth with 6.5% NaCl and incubate overnight. Later subculture onto blood agar plate and incubate this plate for 24 hours at 37 ^{0}C and look for obvious bacterial growth .[27]

HEAT TOLERANCE TEST

In 1914, Ayers and Johnson showed that certain kinds of Streptococci are thermally resistant to pasteurization.[30] Houston,et al in 1918, recognized that the heat tolerance of Enterococci is a physiologic characteristic and can be used to differentiate these organisms from other Streptococci. Diebel in 1921 used heat tolerance characteristic of Enterococci to isolate these organisms from mixed culture.[18] In 1937 Sherman JM, et al showed that the high survival rate of few

Streptococci when subjected to 60^0C for 30 minutes was due to heat tolerance property of *Enterococci*, following which the heat tolerance test was standardized. [30,31]

Procedure: An overnight broth culture of the suspected colonies should be heated for 30 minutes at 60 ^{0}C and streaked on to blood agar plate. The blood agar plates should be incubated at 37 ^{0} C for 24-48 hours. Growth in the blood agar plate indicates that the organism is heat tolerant.[27]

PYRROLIDONYL B NAPHTHYLAMIDE HYDROLYSIS (PYR) TEST

Facklam and co-authors in 1982 described the PYR test and gained acceptance as a rapid test for the presumptive identification of both groups A β-hemolytic Streptococci and Enterococci. Initially the test was described as a 16-20 hour agar test, but subsequently PYR test formats included a 4 hour broth assay and several rapid tests.[27,32]In 1997, Chi-Hsiang Chen et al described a 2 min test format for this test, where PYR reagent is impregnated to the filter paper disc, which are inoculated with organisms to be tested.[33]

Principle: L-pyrrolidonyl-β–naphthylamide is hydrolysed by a specific bacterial aminopeptidase enzyme and release free β-naphthylamide, which is detected by the addition of N N-dimethyl aminocinnamaldehyde. This detection reagent couples with the free β napthylamide to form a red Schiff base.

This test can be done with PYR broth which contains Todd Hewitt broth with 0.01% L-pyrrolidonyl-β-naphthylamide and PYR reagent which is 0.01% P-dimethyl aminocinnamaldehyde. After 16-20 hours of incubating the test organism in PYR broth, addition of PYR reagent will lead to the development of cherry red color,

which is considered as positive and development of yellow or orange color is considered as negative reaction.[15,27,]

Recently disc tests were developed, where the substrate-L-pyrrolidonyl-β – naphthylamide is impregnated into the paper discs which are moistened and inoculated with the test organism. After 2 min to allow for the hydrolysis, the PYR reagent is dropped onto the disc. If pink color appears within 1 min then the test is positive.[33]

SPECIES IDENTIFICATION:

SUGAR FERMENTATION TESTS:

In 1921 Diebel and in 1924 Ayers and Johanson used carbohydrates like arabinose, maltose, sucrose, lactose, trehalose, raffinose, inulin, glycerol, mannitol, sorbitol and salicilin along with the other tests to speciate the Enterococci.[19,20]Fermentation test is done with 1% sugar in brain heart infusion broth with bromocresol purple indicator or peptone water with Andrade's indicator.[20]Enterococci are fermenters and as they lack Kreb's cycle and respiratory chain, they do not produce gas.[25]In 1972,Facklam RR presented a summary of twenty six tests to differentiate the species of Enterococci[31]But with above scheme, *E.avium, E.faecium, E.casseliflavus* and *E. equinus* species could not be identified. In 1975, Gross et al added three more tests to the above scheme, like pyruvate utilization, deamination of arginine and acidification of sorbose broth .[18,20]Later in 1989, Facklam RR and Collins MD incorporated the above three tests, along with other 14 tests (from the scheme of Facklam RR in 1972) to present a species identification scheme by conventional tests.[20]

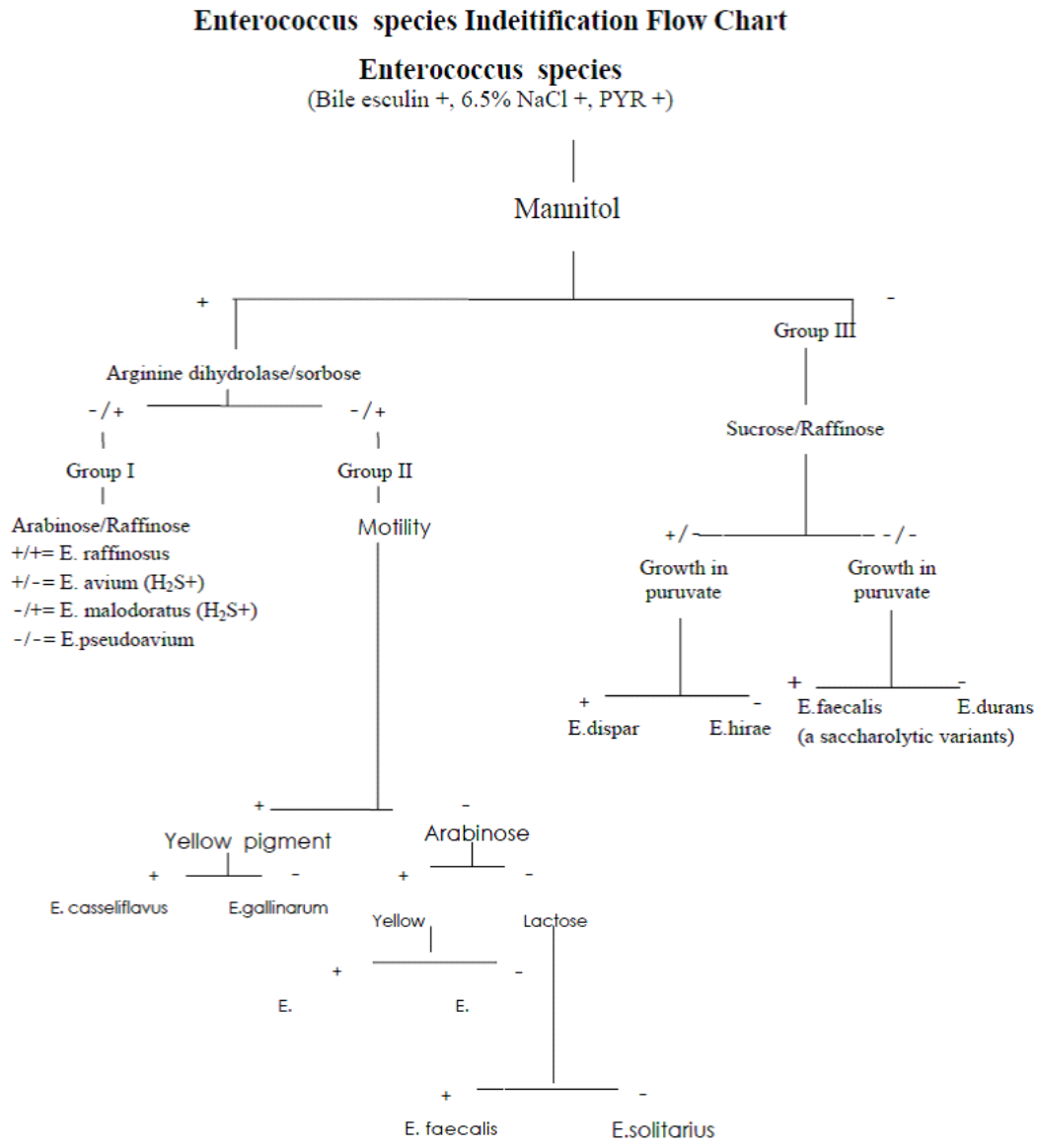


Figure:2 [14]

Principle: This test is used to determine the ability of an organism to ferment a specific carbohydrate that is incorporated in a basal medium, producing acid with or without visible gas. Enterococci do not produce gas.[14,25]

ARGININE DIHYDROLASE TEST:

In 1955, Moeller developed and used the amino acid decarboxylase media to detect the production of arginine dihydrolase. Davis and Edwards, compared Moeller's decarboxylase medium to Falkows decarboxylase medium and they found that Moeller's method as the standard or reference method for determining the decarboxylation reactions.[34] In 1975, Gross K showed that, for speciation of Enterococci, arginine hydrolysis can be used as a dependable test [19.]

Principle: Decarboxylases are specific enzymes that are capable of reacting with the carboxyl group of acid forming alkaline amines. This reaction is known as decarboxylation where Pyridoxal in the medium acts as coenzyme factor for decarboxylases. In Arginine test, the conversion of arginine to citrulline is a dihydrolase reaction in which amino group is removed from arginine as a first step. Citrulline in next converted into ornithine, which then undergoes decarboxylation to form putrescine. Decarboxylation occurs under anaerobic conditions. Hence the medium should be over layered with ½ or 1/4thinch of sterile mineral oil. During initial stages of incubation, the medium turns yellow owing to the fermentation of small amount of dextrose. Later once the amines are formed due to the decarboxylation medium returns to original purple colour[18,19,20,27]

POTASSIUM TELLURITE (0.04%) REDUCTION:

Diebel in 1964 used 0.05% potassium tellurite reduction as one of the test to differentiate Enterococci[19]. In 1971, Facklam employed 0.04% potassium tellurite to heart infusion agar with defibrinated rabbit blood and consider as one of the batteries

of tests to speciate the strains of Enterococci. On examination of these plates revealed black color colonies due to reduction of tellurite to tellurium was recorded as positive after any time interval up to 3 days [18,20,35].

Principle: Tellurite (0.04%) inhibits the growth of most other bacteria acting as a selective agent. Enterococci reduce tellurite to metallic tellurium which is incorporated in the media, giving them a gray or black colour. *E.faecalis* reduce tellurite, whereas *E.faecium* do not reduce the potassium tellurite.[27,35]

VOGES-PROSKAUER (VP) TEST:

In 1987, Fertally SS and Facklam RR showed certain strains of Enterococcus produce acetyl methyl carbinol[36.] They described all the four methods of VP testes i.e. Coblentz, Barritt's, O'Meara's, Barry and Feeney's methods and also compared the results with rapid Strip system results.[32]Finally concluded that Coblentz method will give the best results and thus helps in differentiating the species of Enterococci [36].

PYRUVATE UTILIZATION /FERMENTATION TEST:

Pyruvate fermentation medium was prepared as per recommended method. Enterococcal isolates were inoculated into the broth medium and incubated at 37 ^{0} c for 2-7 days .Change in color of the indicator bromothymol blue from green to yellow indicated pyruvate utilization with acid production.[14]

VIRULENCE FACTORS

Virulence of an organism is regulated by virulence coding genes which are present on the genome in special regions which are called pathogenicity islands

[PAI].[9] The PAI of Enterococcus was first identified in the genome of multi-drug-resistant strain of *E. faecalis* that had caused an outbreak of nosocomial infection in the 1980s. [10]

Enterococcus species inhabit the GIT and is considered as a normal commensal flora, however certain predisposing conditions may allow this organism to acquire newer characters making it more virulent and enables it to invade extra intestinal regions and cause infections.[3]There are many factors which is responsible for the virulence of the organism. Many studies have identified different virulence factors, most important among them being haemolysin, gelatinase, enterococcal surface protein [Esp], aggregation substance [AS], MSCRAMM Ace (Microbial surface component recognizing adhesive matrix molecule adhesin of collagen from Enterococci), serine protease,cell wall polysaccharide and superoxide.[16]

Hemolysin/Cytolysin

In the late 1960 and early 1970, studies by Granato and Jackson established that cytolysin was bicomponent in nature, namely 'L' for lysin and 'A' for activator on the basis of the kinetics of interaction. It occurs as extra chromosomal elements and encoded by PAD1 plasmid.[22]

It is a cytolytic protein capable of lysing human, horse and rabbit erythrocytes. About 20% of humans are normally colonized with cytolytic enterococci. Haemolysin producing strains of Enterococci have been shown to be more virulent in human infections and to be associated with increased severity of infection. Haemolysin production can be detected by inoculating enterococci on freshly prepared beef heart infusion agar supplemented with 5% horse blood. When plates are incubated overnight at 37° C in a carbon dioxide chamber and evaluated

after 24 and 48 hours, it shows a clear zone of β haemolysis around colonies on horse blood agar is taken as positive.[3]

Aggregation substance (AS)

It is a pheromone inducible surface protein of *E. faecalis* which promotes formation of conjugation tube [3.]Under electron microscope; it appears as hair like structure embedded in the cell wall.[19] Aggregation Substance (AS) mediates efficient Enterococcal donor- recipient contact to facilitate plasmid transfer.[3]

In vivo, aggregation substance may contribute to the pathogenesis of Enterococcal infection through a number of mechanisms. Enterococci expressing AS were found to resist phagocytosis by inhibition of the respiratory burst by production of reactive oxygen species {ROS} in the macrophages.[3]

Surface Adhesions [Enterococci Surface Protein (ESP)]

Enterococcal surface protein [ESP] is a cell wall associated protein in *E. faecalis* isolates. Frequency of gene coding for ESP has been higher among clinical isolates than commensal isolates. ESP is shown to enhance the persistence of enterococci in urinary bladder in case of urinary tract infection as it help the organism adhere to bladder epithelium through specific components of the bladder wall such as mucin or uroplakin.[3]

Lipoteichoic acid

It constitutes the group D antigen of Enterococcal cell wall.[37] Membrane associated lipoteichoic acids are amphipathic polymers composed of a hydrophilic polyglycerol phosphate backbone linked via an ester bond to hydrophobic glycolipid

tail. Within single organism, lipoteichoic acid exists in varying forms especially it varies in its glycerophosphate chains.[22]

Protease/Gelatinase

Suet et al sequenced this protease gene – gel E and demonstrate the production of protease in semi-solid media which is supplemented with 3% gelatin or 1.5% skimmed milk. In 1975, Gold et al detected protease as one of the virulence factor of Enterococcal infection.[22]

It is an enzyme produced by Enterococci that is capable of hydrolyzing gelatin, casein, haemoglobin and other peptides. Gelatinase producing strains of *E. faecalis* have been shown to contribute to virulence of endocarditis in an animal model. Gelatinase production in the laboratory can be detected by inoculating the *Enterococci* on freshly-prepared peptone-yeast extract agar containing gelatin. When plates are incubated at 37ºc overnight and cooled to ambient temperature for two hours, shows a turbid halo or zone around the colonies, if it is positive for gelatinase production.[3,16]

Hyaluronidase

Hyaluronidase is a cell surface associated enzyme, which cleaves the mucopolysaccharide moiety of connective tissue or cartilage and leads to dissemination of microorganisms. Hence hyaluronidase plays a role in invasive diseases.[22]

Extra-cellular Superoxide

E. faecalis isolates from blood stream are unique in their ability to produce superoxide. Superoxide production was observed to enhance in vivo survival of *E. faecalis* in mixed infection with *Bacteroides fragilis* in a subcutaneous infection.[3]

INFECTIONS:

The *Enterococci* are a dominant bacterial group in the intestinal flora of human and animals and it is recognized that they cause serious infections such as endocarditis, septicemia and UTI.

Urinary Tract Infections (UTI):

The role of Enterococci in urinary tract infections (UTIs) was first reported by Andrewes and Horder in 1906.[17] Studies has been reported that enterococci as the third most common cause of nosocomial UTIs.[38]*Enterococci* have been associated with cystitis, pyelonephritis, prostatitis and perinephric abscess due to ascending infection.

Risk factors for UTI are[39]

- Anatomical anomalies of urinary tract
- Frequent instrumentations
- Prolonged hospital stay with catheterization
- Age more than 50 years.
- Concurrent acute respiratory failure
- Concurrent gastrointestinal hemorrhage
- Chronic debilitated patients
- Presence or absence of mixed infections

Endocarditis:

In 1899, an organism isolated from a patient with endocarditis was called "*Micrococcus zymogenes*", but was later thought to be *S. faecalis var. zymogenes* [17]. Andrewes and Horder called the organism *Streptococcus faecalis* in 1906; Hicks in 1912 and a number of subsequent workers confirmed the association of Enterococci with endocarditis.[17]Enterococci cause 5-20% of all cases of endocarditis and they are fifth most common cause of prosthetic valve endocarditis.[14,] *E.faecalis* is the most common species isolated from endorcarditis however other species like *E. avium, E. casseliflavus, E. durans, E. gallinarum,* and *E. raffinosus* are isolated less commonly.[20]

Common risk factors are elderly men with underlying valvular disease or with prosthetic valves, genitourinary infection and instrumentation, biliary portal infections or other manipulation or trauma in the preceding 3 months and intravenous drug addicts.[13]

Bacteremia:

According to Nosocomial surveillance data which was conducted in October 1986-April 1997, Enterococci as the third most common cause of nosocomial bacteremia, accounting for 12.8%. The organism enters into the blood stream through the urinary tract, intra-abdominal or pelvic sepsis, wounds, decubitus ulcer, and intravenous access devices. So risk factors for development of bacteremia include immunosuppression, diabetes, malignancy and deep seated infection, prior gastrointestinal, genitourinary or respiratory tract instrumentation, long term hospitalization, and the use of broad spectrum antibiotics. Community-acquired

Enterococcal bacteremia is more commonly associated with endocarditis than nosocomial bacteremia.[40]

Intra-abdominal and pelvic infection[41,42]

Enterococci are part of the normal intestinal flora of human beings and are found in about 17% of routine vaginal cultures, hence the need for empirical or prophylactic treatment of the Enterococcal intra-abdominal and pelvic infections remains somewhat controversial. Enterococci do not cause sepsis when injected intra-peritoneally alone but can act synergistically with other indigenous aerobic and anaerobic organisms or substances to cause abscess formation. [17] Despite the difficulty in establishing pure Enterococcal infections, it is clear that Enterococci can cause and contribute to abdominal and pelvic abscess and sepsis. Enterococci have also caused acute salpingitis, peripartum maternal infection (such as endometritis) with bacteremia and abscess formation following Cesarean section.[43]

Wound and soft tissue infections

Enterococci causes surgical wound infections, ulcers, diabetic foot infections, burn wound infections and osteomyelitis. It is usually found in wound and soft tissue infections with other facultative anaerobic and anaerobic bacteria. *E. faecalis* accounts for up to 5 % of isolates from skin and soft tissue infections.[14]

Central nervous system (CNS) infection

About 0.3%–4% of all meningitis cases are caused by *Enterococcus* species. Most cases of Enterococcal meningitis have been reported in adults following some neurosurgical procedure. However, a few cases of spontaneous meningitis have also

been reported with some co-morbid conditions such as diabetes, malignancies or immunosuppression. Other predisposing factors for CNS infection are intracranial shunt device, invasive procedure of CNS, long term primary disease of CNS, prior antibiotic therapy and head injuries.[44] *Enterococcus* meningitis has been reported in *Strongyloides stercoralis* hyper infection.[45]The commonest species isolated from cases of meningitis is *Enterococcus faecalis*. Other species such as *E. faecium*, *Enterococcus casseliflavus* and *Enterococcus gallinarum* have also been isolated from CSF samples. There are very few antimicrobials effective against serious VRE infection, thus treatment of meningitis caused by VRE is a therapeutic challenge for clinicians. Antimicrobial agents such as Chloramphenicol and Linezolid have been used to treat patients with VRE meningitis. Zeana *et al*. described a case of VRE meningitis in a patient with *Strongyloides stercoralis* hyperinfection which was successfully managed with Linezolid. Linezolid is an oxazolidinone with good CSF penetration and has the advantage of being available in oral formulations. It has been used in quite a few cases of VRE meningitis although there have been a few reports of clinical failure of Linezolid treatment in cases of post-neurosurgical meningitis.[44]

Respiratory tract infections

Enterococci are less frequently isolated from sputum and other specimens obtained from respiratory tract. These bacteria rarely cause respiratory tract infections (including pneumonia) except in the most debilitated patients.[14]

Neonatal sepsis:

Enterococci cause neonatal sepsis in premature or low birth weight neonates, in infants with nasogastric tubes or intravascular devices and in post-surgical

conditions.[46] Neonatal Enterococcal sepsis is characterized by fever, lethargy and respiratory difficulty accompanied by bacteremia or meningitis.[22]

ANTIBOITIC THERAPY

Recently Enterococci have gained importance not only because of their increasing role in nosocomial infections, but also because of their remarkable and increasing resistance to antimicrobial agents.

When the patient has normal host defense, majority of Enterococcal infections can be cured with bacteriostatic antibiotic therapy alone. Penicillin, Ampicillin, Piperacillin and Imipenem has good activity against Enterococcal infections as single agent but infections like endocarditis and meningitis required both bacteriostatic and bactericidal combination drugs.

ANTIMICROBIAL RESISTANCE PATTERN:

There are three reasons for the emergence of multidrug resistant Enterococci[17]

- Intrinsic resistance to several antimicrobial agents
- Acquired resistance through plasmids and transposons and chromosomal exchange
- The transferability of resistance.

Antimicrobial resistance can be divided into two general types, one which is an inherent or intrinsic property and other which is acquired.

Antimicrobial resistance due to intrinsic property.

Inherent or intrinsic property:

The term inherent or intrinsic resistance refers to resistance, which is a usual species characteristic present in all or most of the strains of that species. The genes for intrinsic resistance appear to present on chromosomes.

The intrinsic traits expresses by Enterococci are [17,48, 49]

- Resistance to β-lactams, semisynthetic penicillinase resistant Penicillins and Cephalosporins.
- Resistance to low levels of Aminoglycosides.
- Resistance to low levels of Clindamycin, Triemthoprim, Sulfamethoxazole and Fluroquinolones.

β– Lactams

Enterococcus has complete or relative resistance to β-lactams due to production of low affinity penicillin binding protein (PBP) and also constitutive production of β-lactamase. Compared to other Streptococci, *E.faecalis*is shows 10 to 100 times less susceptible to Penicillin and inhibited by more concentration of Penicillin/Ampicillin (1-8 μg/ml), while *E.faecium* is at least 4-16 times less susceptible than *E.faecalis*, requiring an average of 16-64μg/ml of penicillin.[47] Penicillin resistance is directly proportional to the production of PBP5 (a specific PBP). B-Lactamase-producing Enterococci are infrequently isolated. Unlike most staphylococci,where β-lactamase production is inducible; in Enterococci it is constitutively present in low level and inoculum dependent. [48]

Aminoglycoside resistance [48]

Enterococci showed resistance to Gentamicin and Streptomycin due to different mechanisms, it is important to test susceptibilities to both the agents. Early studies demonstrated that two types of Streptomycin resistance occur in Enterococci:

- Moderate-level resistance(MIC 62 to 500 mg/ml), because of low permeability, which can be overcome with a penicillin (which increases the cellular uptake of the Aminoglycoside);
- High-level resistance (MIC, 2,000 mg/ml), which is either ribosome mediated or due to the production of Aminoglycoside-inactivating enzymes.

Aminoglycosides resistance is predominantly due to the inactivating enzyme 20-phosphotransferase-69-acetyltransferase conferring resistance to Gentamicin, Tobramycin, Netilmicin, Amikacin and Kanamycin. Hence, Gentamicin resistance is a good predictor of resistance to other Aminoglycosides except Streptomycin. Streptomycin resistance is encountered mainly in Enterococcal strains that produce streptomycin adenyl transferase but these strains remain susceptible to Gentamicin. Penicillin-Aminoglycoside synergy does not occur in high-level Aminoglycoside-resistant Enterococci (Streptomycin-MIC-2,000 mg/ml: Gentamicin-MIC-500 mg/ml).[17,47, 48,]

Clindamycins:

Enterococci also has intrinsic resistance to clindamycin and lincomycin. MICs for most strains are 12.5 to100µg/ml. But Moellering and Krogtad et al showed low level resistance to clindamycin.[17]

Trimethoprim-Sulfamethoxazole (TMP/SMX):

Enterococci has intrinsic resistance to Trimethoprim-Sulfamethoxazole (TMP/SMX) though it shows in vitro susceptible. Media containing thymidine allow the bacteria escape from the inhibitory action of TMP/SMX due to thymidine phosphorylase which converts thymidine to thymine. Another potential problem is that Enterococci can use exogenous folinic acid, dihydrofolate, and tetrahydrofolate. [49]

ACQUIRED RESISTANCE:

Enterococcus has acquired resistance as results of either a mutation in existing DNA or acquisition of new DNA. It often acquired antibiotic resistance through exchange of resistance encoding genes carried on conjugative transposons [50.]

Three different conjugative transfer systems have been reported in enterococci [17]

1. Broad-host-range plasmids- can transfer resistance among *E.faecalis* and many other organism.
2. Narrow-host-range plasmids –found so far only in *E. faecalis* that transfer at a high frequency.
3. Conjugative transposons –resistance occurs at a low frequency.

Acquired resistance is seen to-

- High concentration of β-lactams, through penicillin binding proteins or β-lactamase.
- High level Clindamycin, Aminoglycoside, Glycopeptides (Vancomycin) and Lipopeptide (Teicoplanin).
- Penicillin by means of penicillinase.

β- Lactamase:

β -lactamase producing Enterococci were first reported in 1983. β- Lactamase production has been encoded by transferable plasmid. The resistance of beta-lactamase-producing strains is not detected by routine disk susceptibility testing because of an inoculum effect. When a low inoculum is used, strains appear susceptible, but at a high inoculum (e.g. 10^7 CFU/ml) strains appear resistant. It is due to the fact that low numbers of cells do not produce sufficient beta-lactamase to cause resistance. The Enterococcal beta-lactamase hydrolyzes penicillin, ampicillin, and piperacillin (and other ureidopenicillins), which correlates with resistance to these compounds; there is little or no inactivation of penicillinase-resistant semisynthetic penicillins, cephalosporins or imipenem. [48,51]

Penicillin resistance without β- lactamase:

Enterococci with higher penicillin resistance with MIC>25μg/ml are now being more common. The increased MICs of Penicillin for these strains may be an extreme intrinsic resistance property or due to low affinity of PBP or may be because of acquired resistance [47].

High level aminoglycoside resistance (HLAR):

In 1976, French investigators first described high level Gentamicin resistance in Entrococci at the Pasteur Institute.[17] Enterococci acquired HLAR through plasmid and have potential for the further dissemination[7]. The synergistic bactericidal effect of a Penicillin\Vancomycin and Aminoglycoside combination used to treat serious infections has been eliminated because of HLAR.[52]

Tetracycline resistance

Enterococci showed 60-80% resistance to Tetracycline due to several different gene like tetL, tetM, tet N and tet O. These various genes confer resistance by two different mechanisms; tetL mediates active efflux of tetracycline from cells, while tetM and tetN mediated resistance is by a mechanism that protects the ribosome from inhibitory action by tetracycline. An interesting feature of tetL is that, the resistance genes duplicate or amplify when the host is grown in sub inhibitory concentrations of tetracycline.[17]

Chloramphenicol, clindamycin, and Erythromycin.

Zimmerman and Ray Croft for the first time demonstrated **chloramphenicol** resistance in enterococci due to of transferability of resistance genes. And studies have shown that resistance also has been mediated by chloramphenicol acetyl transferase. Several studies have reported that 20 to 42% of enterococci are chloramphenicol resistant. [17]

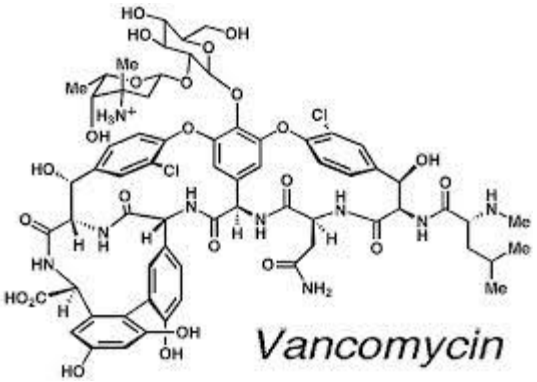
Erythromycin resistance plasmids and transposons are also commonly found in *Enterococci*. Strains with high-level Erythromycin resistance have shown a steady

increase in many places. Erythromycin resistance occurs as part of the macrolide-lincosamide-streptogramin B resistance phenotype.[17]

Enterococcus also confers HLR to **clindamycin** due to methylation of an adenosine residue in the 23S rRNA. Different erythromycin resistance determinants exist, but an especially common one (ermB) is carried by Tn917 which is widespread in human and animal isolates of *Enterococci*.[17]

Vancomycin Resistant Enterococci (VRE):

Figure: 3 Molecular structure of Vancomycin.



Courtesy www.Scripps edu-600x24

Vancomycin was the first glycopeptide antibiotic to be discovered as early as 1950.[52] Vancomycin and Teicoplanin(Lipopeptide) interfere with cell wall synthesis in gram positive bacteria by interacting with the D alanyl –d-alanine (D-Ala-D-Ala) group of the pentapeptide chains of peptidoglycan precursors.[52]

The most recent resistance trait to emerge in Enterococci is resistance to Vancomycin. Glycopeptide antibiotics, Vancomycin and Teicoplanin, are used in the treatment of serious infections caused by Enterococci, in cases of resistance or allergy

to β-lactams. Despite more than 30 years of clinical use of Vancomycin, glycopeptide resistance in Enterococci has rarely been detected. However, resistant strains responsible for colonization or infections have been isolated with an increasing frequency from patients in the presence or absence of glycopeptide therapy. [54]

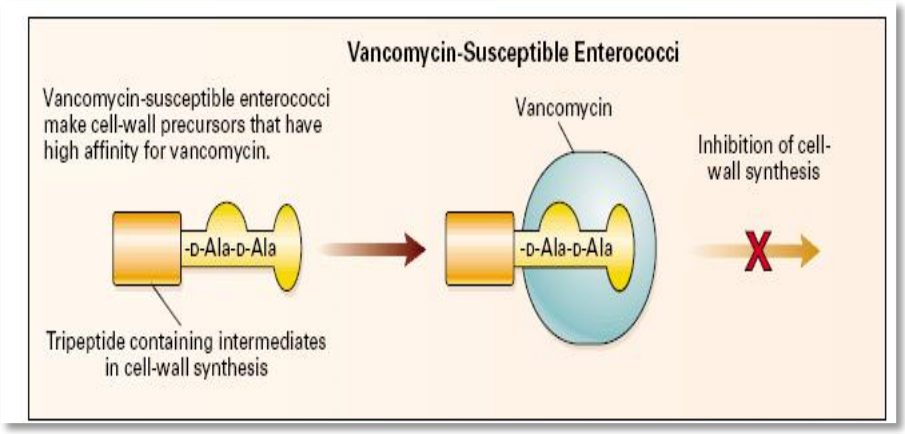
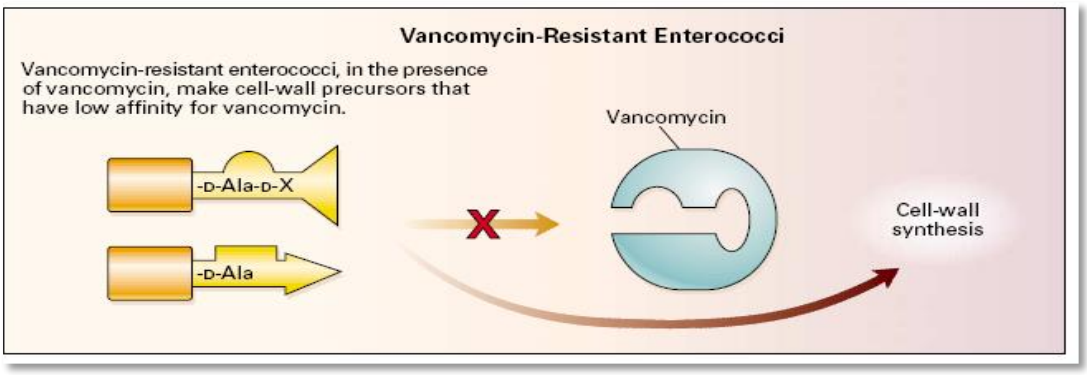


Figure:4 Mechanism of action of Vancomycin.*Courtesy www.jci.org-700x492*

In 1988, Uttley et al first reported Vancomycin resistant *E. faecalis* and *E. faecium* in England. From 1989 to 1993, the percentage of nosocomial infections (due to VRE) reported to CDC, NNISS (National Nosocomial Infection Surveillance System) increased from 0.3% to 7.9%.[55] The acquisition of VRE by hospitalized patients has been associated with number of factors, including the length of hospital stay, underlying disease, broad spectrum antibiotics and parenteral Vancomycin. This increase was directly reported to a rise in VRE infections in intensive care units and in non-intensive care units setting.[55]

Genes and mechanism of Vancomycin resistance

Figure : 5 Mechanism of vancomycin resistance *Courtesy www.jci.org-700x492*



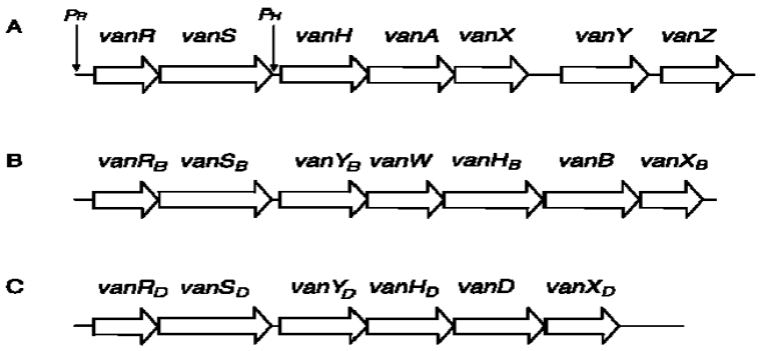
Glycopeptide resistant Enterococci are divided into **5 phenotypes** on the basis of their patterns of resistance to specific drugs. Van A and Van B are most common phenotype and primarily found in *E.faecalis* and *E.faecium*.

CHARACTERISTICS OF DIFFERENT PHENOTYPES OF VRE[56]

The best studied vancomycin resistance determinant is the Van A operon .It is a system of genes packaged in a self-transferable plasmid containing a transposon closely related to Tn 1546. There are two open reading frame that code for transposase and resolvase; the remaining seven genes code for vancomycin resistance and accessory proteins.

The van R and van S genes are a two component which regulates sensitive pattern of enterococci to Vancomycin or Teicoplanin in the environment where as van H, van A and van X are required for vancomycin resistance. Van H and van A encodes for proteins - depsipeptide d-Ala-d-lactate rather than the normal peptide D-Ala-D-Ala .These depsipeptide, when linked to UDP–muramyl tripeptide, forms a pentapeptide precursor to which vancomycin and teicoplanin cannot bind. Van X

encodes a dipeptidase that depletes normal D-Ala-D-Ala dipeptide. van A ,van B and van D code for d-ala –d-Lac ,while van C and van E codes for d-ala-d-Ser.[56]



Schematic representation of the *vanA* (A), *vanB* (B), and *vanD* (C) operons. P_R and P_H are the promotors controlling the gene expression

Figure: 6

Van A phenotype:

The strain carrying Van A gene is transposon mediated, which display inducible high level resistance to both vancomycin (MIC higher than 64µg/ml) and Teicoplanin (MIC higher than 16µg/ml). Enterococci with Van A phenotype are most troublesome because these strains are able to transfer Van A resistance markers by a conjugation mechanism to other Enterococci and other gram-positive organisms including *Staphylococcus aureus.*[48]

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Van B phenotype

These strains have acquired inducible resistance to various concentration of vancomycin ranging MIC 8 to 64µg/ml but remain susceptible to Teicoplanin -MIC 1 µg/*ml.*[48]

Van C phenotype

Isolates of Van C genotype show intrinsic constitutive low level resistance to Vancomycin (MIC 8µg/ml and 32µg/ml) and are susceptible to Teicoplanin (MIC ≥1µg/ml). It is chromosomal in origin and is not transferred to other organisms by conjugation. It is usually seen in *E.casseliflavus* -C_1 type, *E.gallinarium* –C2 type[48]

Van D phenotype

It was first described in a New York Hospital in 1991 and is seen in *E.faecium*. These strains are inhibited by Vancomycin at 64µg/ml and Teicoplanin at 4 µg/ml. It is chromosome mediated and is not transferable to other Enterococci.[48]

Van E phenotype

It is seen in *E.faecalis* which is resistant to low level of Vancomycin (MIC 16µg/ml) and susceptible to Teicoplanin (MIC 0.5 µg/ml) [48]

Characteristic	Phenotype				
	Van A	Van B	Van C	Van D	Van E
Vancomycin MIC ($\mu g/ml$)	64-1000	4-1024	8-32	128	16
Telcoplanin MIC ($\mu g/ml$)	16-512	≤ 0.5	≤ 0.5	4	0.5
Most frequent Enterococcal species	E.faecium E.faecalis	E.faecium E. faecalis	E. gallinarum. E.casseliflavus	E.faecium	E. faecalis
Genetic determinant	Acquired	Acquired	Intrinsic	Acquired	Acquired
Transferable	Yes	Yes	No	No	No

Table : 1 Shows phenotypic characters of Van genes

VRE in the Community and hospital settings:

In the **United States**, epidemiology of VRE has been focused mainly in hospitals, but there was little evidence to suggest that transmission of VRE to healthy adults occurs to any significant extent in the community. As colonized patients leave the hospital environment, the possibility that transmission might occur in the community cannot be discounted.[47,48] According to the National Health Care safety Network in 2006-2007, *Enterococci* showed overall 33% resistant to Vancomycin.[54]

The situation in **Europe** is quite different from that in the United States, VRE have been isolated from sewage and various animal sources in Europe. It has been suggested that it could be due to the use of glycopeptide-containing animal feeds like Apovarvin, a growth promoter in some regions of Europe. These observations suggest a potential for VRE or the resistance genes of VRE to reach humans through the food chain or through contact with domesticated animals. Colonization of healthy individuals with VRE does not necessarily indicate a risk of infection with these organisms.[47,48]

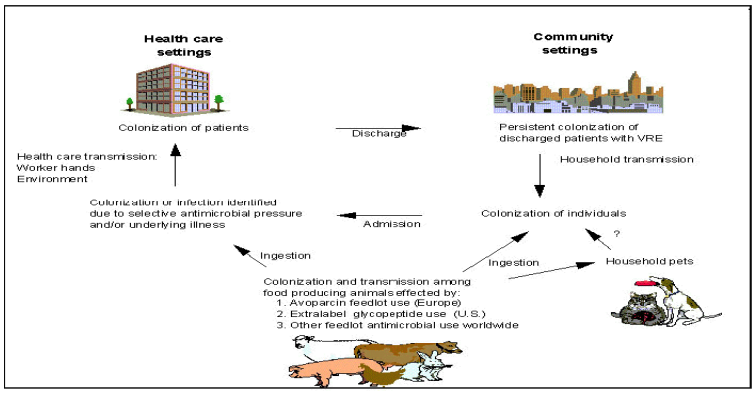


Figure : 7-Epidiemology of VRE in community and hospital settings *Courtesywww.cdc.gov*

Indian scenario of VRE:

Although the prevalence of VRE infections in India is much lower than in the Western world, it has been increasing in the past one decade. In India, Mathur et al reported VRE for the first time from New Delhi in 1999. Another study from New Delhi reported incidence of VRE as 1%, followed by 5.5% in Chandigarh and 23% in Mumbai[48]. The following table depicts the Indian scenario from 2003-2006.[54]

Table/Fig 3
VRE isolation: Indian Scenario

Year	Author	Number positive	Sample (no. positive)	Species isolated	Phenotype	MIC values (µg/ml)
2003	Mathur *et al*	5	Blood (3), urine (1), soft tissue (1)	*E. faecalis*	4 Van A, 1 Van B	256-512
2004	Taneja *et al*	8	Urine	*E. faecium* (5), *E. faecalis* (1), E.casseliflavus (1), E.pseudoavium (1)	Van B and Van C	8-32
2004	Karmarkar *et al*	12	Urine, blood, pus	*E. faecalis*, *E. faecium*	Van B	> 4
2005	Kapoor *et al*	4	Blood (in pediatric age group)	*E. faecalis* (2), *E. faecium* (2)	-	8
2006	Ghoshal *et al*	10	Blood,Tissue,Urine, CVP Tip	*E. faecium*	Van A	62-256

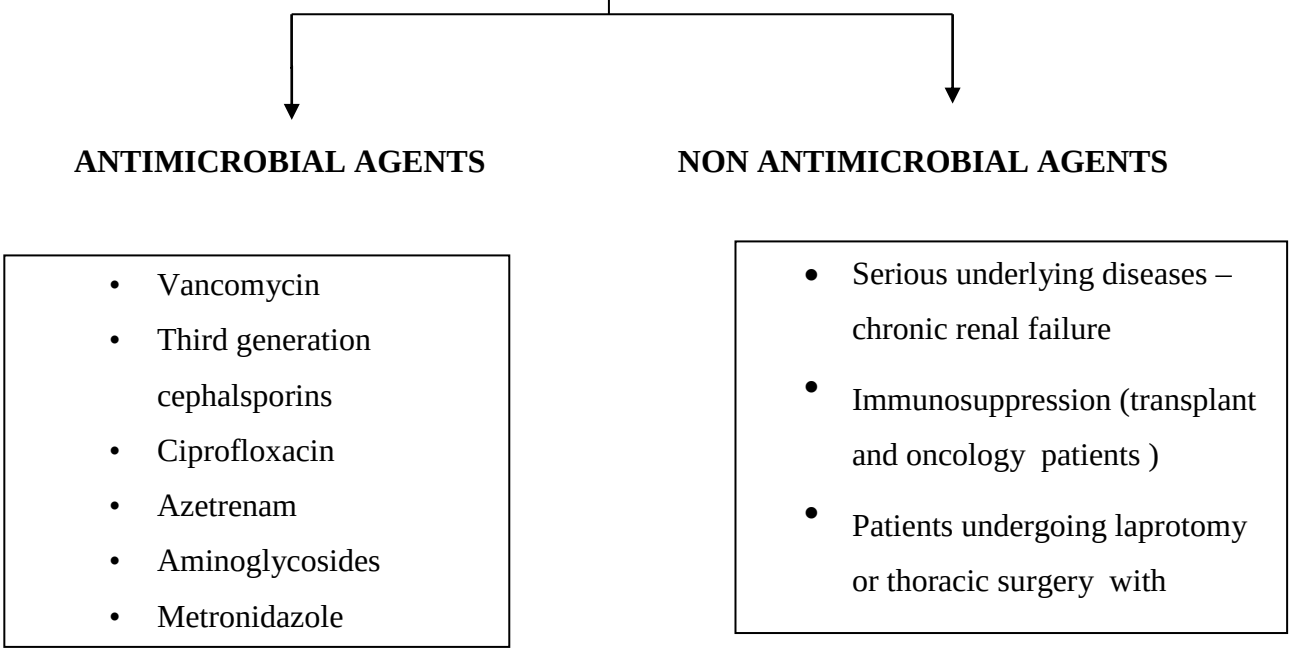
Table :2 Shows VRE scenario in India

Later from 2007- 2014, studies from different areas shows incidence of VRE strains.[2,57,58,59,12,60,61]

Year	Author	Place	% VRE
2007	Uma Chaudary	Chandigarh	2%
2010	Baragundi Mahesh	Bagalkot	7.5%
2012	Modi G B	Ahmedabad	4%
2013	Ira Praharaj	Puducherry	8.7%
2013	Sanal C	Mangalore KMC, Manipal	8.6%
2013	Sharama	Lucknow	3%
2014	Saroja Golia	Bangalore	5%

Table :3 shows VRE incidence i

Risk factors for VRE colonization [52]



Others factors:[47]

- Previous antimicrobial therapy.
- Exposure to contaminated medical equipment such as electronic thermometers.
- Proximity to a previously known VRE patient.
- Exposure to a nurse who was assigned on the same shift to another known patient

Colonization and Infection [47,52]

The ratio of colonized to infected patients may reach as high as 10:1 at hospitals, perirectal or rectal swab specimens from high-risk patients are screened for VRE colonization. VRE strains often cause intra-abdominal, urinary tract, bloodstream, surgical sites and vascular catheter sites infections. VRE infections tend to occur in more debilitated or seriously ill hospitalized patients. Mortality rates in

patients with VRE bacteremia may reach upto 60 to 70%. There is no evidence that VRE are more virulent than Vancomycin susceptible strains of the same Enterococcus species. It is not always easy to assess the clinical significance of VRE in routine cultures or to differentiate colonization from infection.

The extent to which VRE causes morbidity and mortality is often difficult to determine, because most affected patients have serious underlying diseases that cause substantial morbidity and death. [47]

Modes of Transmission

Transmission of VRE occurs in hospital through health care workers, whose hands become transiently contaminated with the organism while caring for affected patients. This concept is supported by the recovery of VRE and other resistant Enterococci from cultures of specimens from the hands of health care workers.[47]

Transmission of VRE may also occur through contaminated medical equipment, electronic thermometers, bedpans, stethoscopes, sphygmomanometers, bed rails, sinks, faucets and doorknobs, commodes, ECG wires, intravenous fluid pumps etc. Since Enterococci may remain viable for several days to weeks on dry surfaces and act as a source of infections, from which personnel may contaminate their hands or clothing. However, further studies are necessary to determine the extent to which these items contribute to the transmission of VRE. Recovery of VRE from animal sources in parts of Europe suggests that food-borne transmission may occur in certain geographic areas However, conclusive proof of food-borne transmission of VRE in Europe (or other areas) is not yet available.[47,48]

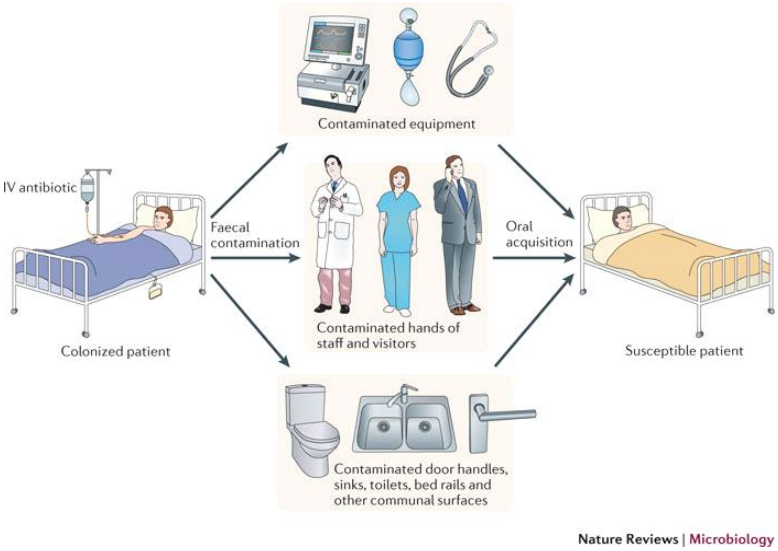


Figure:8 Modes of VRE transmission in the hospitals. Courtesy www.nature.com

Prevention and Control Measures [47,48,52]

Because of dramatic increase in Vancomycin resistance in Enterococci, the Subcommittee on Prevention and Control of Antimicrobial-Resistant Microorganisms in Hospitals of the CDC Hospital Infection Control Practices Advisory Committee (HICPAC) had several meetings in 1993 and 1994. In an effort to control the nosocomial transmission of VRE, HICPAC published recommendations in February 1995.These recommendations mainly focused on

- Prudent use of vancomycin
- Education of hospital staff
- Effective use of the microbiology laboratory
- Implementation of infection control measures (including the use of gloves and gowns and isolation or cohorting of patients, as appropriate to specific conditions)
- Surveillance cultures
- Attempts to eradicate gastrointestinal colonization.

TREATMENT OF INFECTIONS CAUSED BY VRE:

Vancomycin resistant Enterococci (VRE) have emerged as major nosocomial pathogen. VRE are typically resistant to multiple antimicrobials, Ampicillin and high level Aminoglycosides.[48,52] Primary therapeutic options for patients with VRE infections include Quinupristin/Dalfopristin, which is active against most strains of *E.faecium*. Linezolid is a glycopeptide which is active against both *E.faecium* and *E.faecalis*. It reaches adequate cerebrospinal fluid levels. There are reports of successful treatment of *E.faecium* meningitis with Quinupristin/Dalfopristin and Linezolid. Limited in vitro data indicated synergistic bactericidal activity when Quinupristin and Dalfopristin are combined with Linezolid, Doxycycline or Ampicillin.[47] Optimal treatment of endocarditis requires bactericidal antimicrobial therapy i.e. combination of Aminoglyciside and cell wall acting antibiotics.

Chloramphenicol is one of the few agents that retains its in vitro activity against many strains of multiple drug resistant *E.faecium*. However, this agent has been used with limited success in the treatment of VRE infections.[47]

Newer fluroquinolones like Clinafloxacin is the most active agent against Enterococci which can used for the treatment of Enterococcal infection.[47]

Detection of vancomycin resistance by screening methods.[52]

Screening methods have been introduced for detection of VRE as there is increase in rate of colonization with VRE strain and increasing concerns about enterococcal infection in patients with high risk factors.

Screening methods for VRE:

1. Agar screen method- In this method brain heart infusion agar with 6 μgm of vancomycin per ml is used. Spot inoculums of 10^5-10^6 CFU is done on plate and incubated at 35^0C for 24 hours. If growth occurs, it indicates resistance and no growth indicates susceptibility.

2. Entercoccal broth with bile esculin azide and sodium azide with 6 μgm of vancomycin

3. M –Enterococcus broth with sodium azide and triphenyl tetrazolium with 6 μgm of vancomycin.

4. Vancomycin E-strip method.

4. MATERIALS AND METHODS

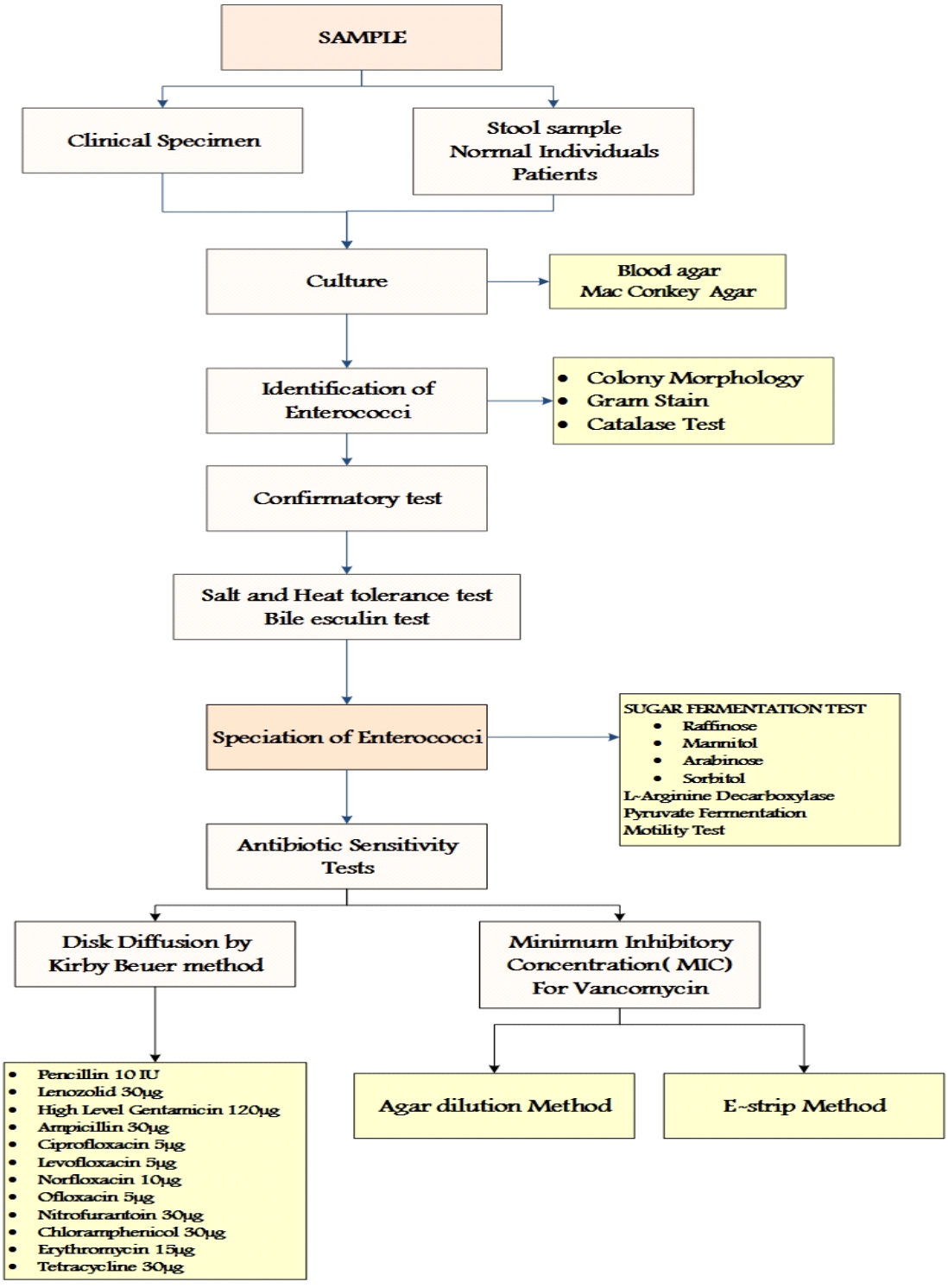
Present study was carried out in the department of Microbiology at Sri Devaraj Urs Medical College, Kolar over a period of one year 6 months, from January 2013-June 2014.

Methodology

100 Enterococci were isolated from various clinical samples of Hospitalized patients and Out Patients attending R L Jalappa Hospital, Kolar. The various clinical samples included Urine, Pus, blood, body fluids, endotracheal aspirations and sputum. 70 stool samples were also collected from hospitalized patients who had Enterococcal infection and 70 stool samples were collected from the healthy normal individuals.

Sample processing:

Specimens were processed by inoculating on to Mac Conkey agar and blood agar and incubated at 37^0C for 24 - 48 hours. Enterococci were identified by their typical colony morphology, arrangement in Gram stain, catalase test, Bile esculin test, and salt and heat tolerance tests. Speciation was done by subjecting the isolates to a battery of biochemical tests like sugar fermentation, motility, pyruvate fermentation, arginine dihydrolase test and reduction of tellurite on potassium tellurite blood agar. [13,15] [27] Antimicrobial susceptibility patterns were determined by performing Kirby-Bauer disc diffusion method and Minimum Inhibitory Concentration (MIC) values were identified by Agar dilution and E-strip methods.[62]



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*Stool samples were inoculated into Brain heart infusion broth and incubated at 37^0C for 18-24hours,[63] and then subcultured onto Mac Conkey agar and Blood agar plates and processed as per the flow chart as shown above.

Speciation was done using following reactions [14.15]

Table: 4

Reaction	E.feacalis	E.faecium	E.durans	E.avium
Arabinose fermentation	_	+	_	±
Mannitol fermentation	+	+	_	+
Pyruvate fermentation	+	_	_	_
Raffinose fermentation	_	±	_	±
Sorbitol fermentation	+	±	_	+
Arginine dihydrolase	+	+	+	_
Bile esculin hydrolsed	+	+	+	+

Antibiotic susceptibility[62]:

Antibiotic susceptibility testing of the isolates was done on Mueller Hinton agar using Kirby-Bauer disc diffusion method. Bacterial suspension was prepared by inoculating few isolated colonies of similar morphology into 4 – 5 ml of peptone water and incubated for 2 – 4 hours. The turbidity of the broth was adjusted to 0.5 McFarland turbidity standards and lawn culture was made on the surface of the medium using sterile cotton swabs. Antimicrobial discs were applied with the help of sterile forceps and the plates were incubated at 35^{o}C for 24 hours.

The zone of inhibition was measured and reported as susceptible, intermediate or resistant. Commercially obtained Hi-media discs were used. Antibiotic susceptibility testing was done with quality control strains by using *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 for appropriate antimicrobials. All the Enterococcal isolates were tested for antibiotic sensitivity

pattern against Ampicillin (l0µg), Penicillin (l0U), Ciprofloxacin (5µg), Vancomycin (30µg), Linezolid (30µg) Nitrofurantoin (300 µg), highlevel Gentamicin (120µg), Levoflaxacin (5µg), Ofloxacin(5µg), Norfloxacin(5µg), Tetracycline(30µg), Chloramphenicol(30µg) and Erythromycin(15µg) by Kirby Bauer disc diffusion method. The strength of discs used and their zone size interpretation was according to guidelines of CLSI.[62] Table :5

Antibiotics	**RESISTANT**	**INTERMEDIATE**	**SENSITIVE**
Vancomycin(l0µg) -Va	≤ 14 mm	15-16 mm	≥ 17 mm
Linezolid (30µg)-Lz	≤ 20 mm	21-22 mm	≥ 23 mm
Ampicillin(l0µg)-Amp	≤ 16 mm	_	≥ 17 mm
Penicillin (l0U)-P	≤ 14 mm	_	≥ 15 mm
High level gentamicin(120µg)	≤ 6 mm	7-9 mm	≥ 10 mm
Levofloxacin (5µg) -Le	≤ 13 mm	14-16 mm	≥ 17 mm
Ciprofloxacin(5µg)-Cip	≤ 15 mm	16-20 mm	≥ 21 mm
Norflaxacin (10µg)-Nx	≤ 12 mm	13-1 mm	≥ 17 mm
Nitrofurantoin(300µg)-Nf	≤ 14 mm	15-16 mm	≥ 17 mm
Tetracycline(30µg)-Te	≤ 14 mm	15-18 mm	≥ 19 mm
Erythromycin(15µg)-E	≤ 13 mm	14-22 mm	≥ 23 mm
Teicoplanin(30µg)-Tec	≤ 10 mm	11-13 mm	≥ 14 mm
Chloramphenicol(30µg)-C	≤ 12 mm	13-17 mm	≥ 18 mm

Minimum Inhibitory Concentration (MIC) by agar dilution method[64]

Procedure:

1) Antibiotic dilution.

2) Preparation of inoculums.

Antibiotic dilution.

Preparation of stock solution

The weight of antibiotic in mg required is calculated by the following formula

1000/P x V x C =W

Where P=Potency of preparation in relation to base

V=Volume (ml) required

C=Final concentration of solution in multiplies of 1000

W=weight of antibiotic to be dissolved in V

Stock solutions l, ll and lll

Stock solution I–(10,000mg/ml)-

20 ml solution was prepared by mixing 222.22 of powder base whose potency is 900mg per gram, with sterile distilled water

$$W = \frac{1000}{900} \times 20 \times 10 = 222.22$$

So, 222.22 mg of Vancomycin was dissolved in 20ml of distilled water. Stock solution can be kept at 8^0C for one week and pure drug in deep freezer at -20^0C.

Stock solution –ll (1000mg/ml)-

1ml of solution from the stock solution –l was transferred to 9 ml of distilled water (stock solution-ll) which gives a concentration of 1000mg/ml.

Stock solution –lll (100mg/ml)-

0.1ml of stock solution –l was transferred to 9.9 ml of distilled water (stock solution lll) which gives a concentration of 100mg**.**

Preparation of different dilution/concentrations of vancomycin was done as shown in the diagram below [65]

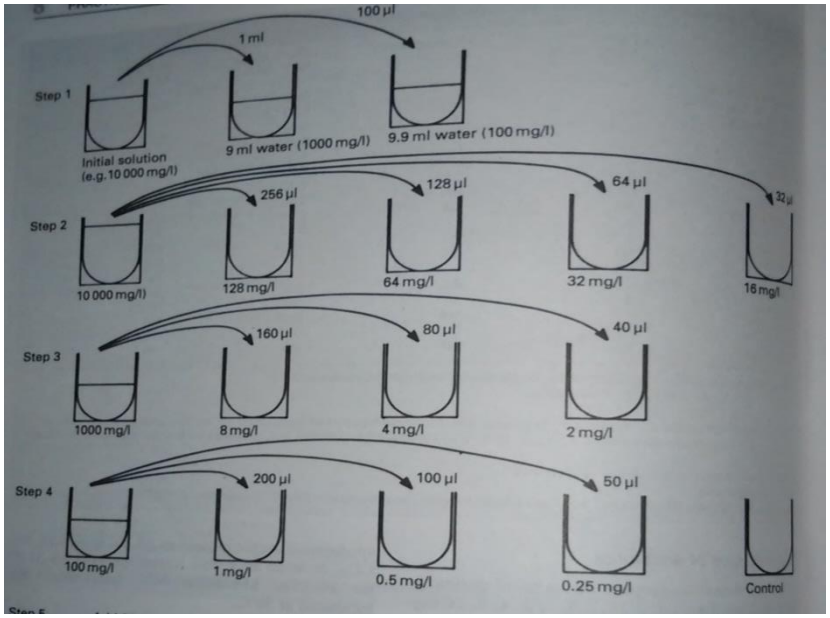


Figure:9

Preparation of different dilutions / concentrations of agar plates [128mg/l - 0.25mg/l]

Sterile Mueller Hinton agar was prepared and cooled to 45 to 50^0C, 20ml of Mueller Hinton agar was added to every dilution of test tube, mixed and poured into respective Petri dishes. All the plates along with control plate were incubated at 35^0C for 18hours to check for sterility.

Preparation of inoculums:

5-6 colonies of Enterococci were inoculated into 3ml of peptone water; incubated for 3 hours, and then turbidity was adjusted to 0.5 McFarland standards.

10µl of diluted test culture was inoculated as spot inoculum on to agar dilution plates prepared along with ATCC Vancomycin susceptible Enterococci as control and incubated for18-20 hours at 37^0C.[8]

Reading and interpretation:

- Control quadrant was examined for adequate growth. If growth was poor or absent (e.g. a few isolated colonies or a faint haze), test was taken as uninterpretable.
- Quadrants containing various concentrations of Vancomycin were examined for absence or presence of growth indicating sensitivity or resistance.[8]

MINIMAL INHIBITORY CONCENTRATION BY VANCOMYCIN E STRIP

2-3 colonies of test strain was suspended in 3ml of sterile peptone water and incubated at 37^0C for four hours .The turbidity was adjusted to 0.5 McFarland standards and inoculated onto Mueller-Hinton agar. The plate was allowed to dry for 3-5 minutes. Vancomycin E-strips (Hi-media) was placed at the center of the plate and incubated overnight at 37^0 C. Intersection of elliptical zone of inhibition with the E-strip was recorded and interpreted as below according to 2013 CSLI guidelines. **[63]**

	RESISTANT	**INTERMEDIATE**	**SENSITIVE**
VANCOMYCIN	≥ 32µg/ml	8-16µg/ml	≤4µg/ml

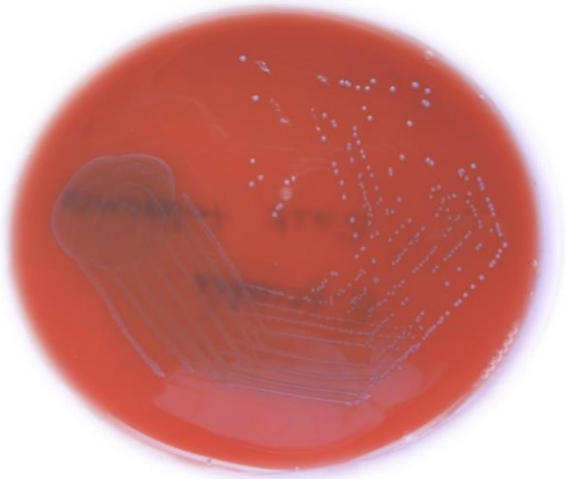


Figure: 9

Small, circular, greyish, colonies seen on Blood Agar plate

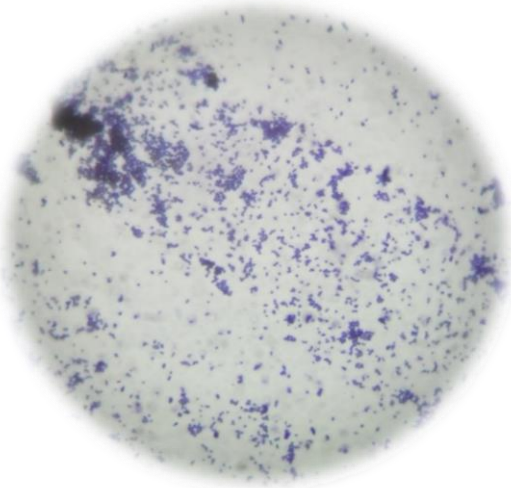


Figure: 10 Grams staining showing –Gram positive cocci arranged in pairs

Figure:11

Colonies were seen on blood agar plate after test strain inoculate into 6.5% Nacl broth for 24hrs indicating – Salt tolerance and Heat tolerance test

Figure: 12 showing identification of Genus Enterococci by Mannitol fermentation, Bile esculin tets and Arginine dihydrolase test with positive and negative controls

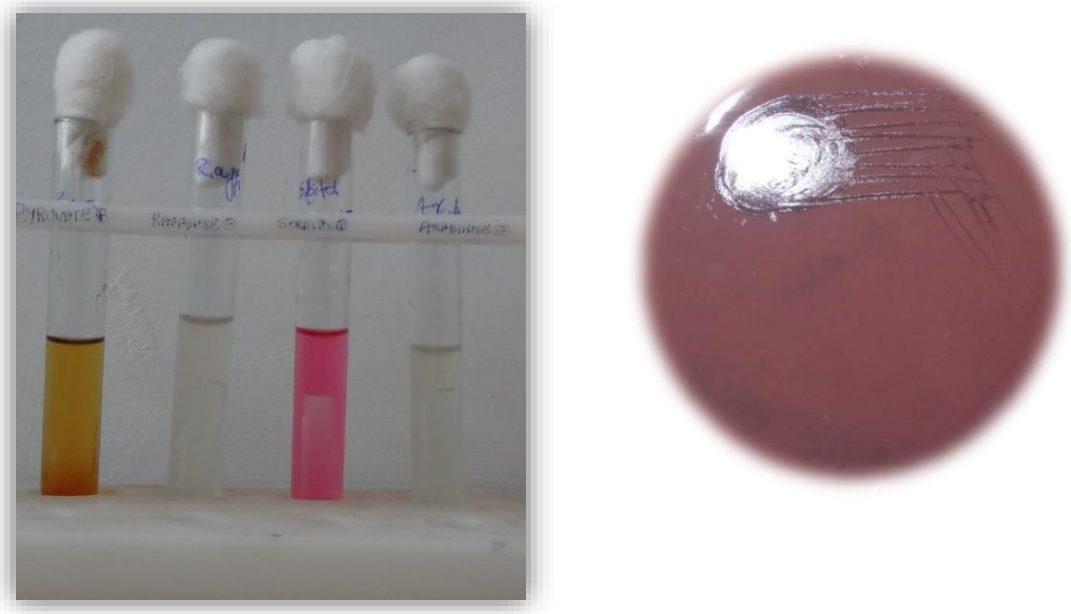
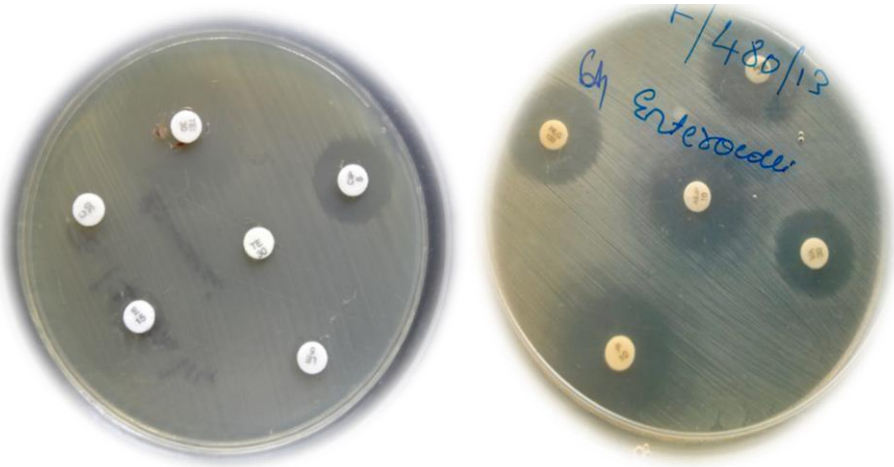


Figure: 13 Biochemial tests showing identification reaction for E.faecalis-Pyruvate and sorbitol fermented, Arabinose and Raffinose notfermented, black **color** colonies seen on potassium tellurite blood agar

Figure: 14

Biochemical tests showing identification Reaction for *E.faecium*---Pyruvate and sorbitol not fermented , Arabinose and Raffinose fermented

Figure 15 Antibiotic susceptibility test by Kirby Bauer method



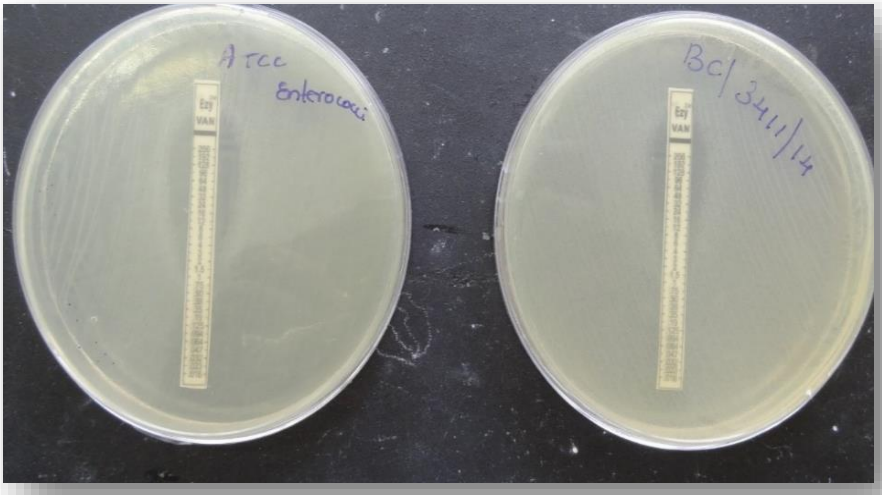
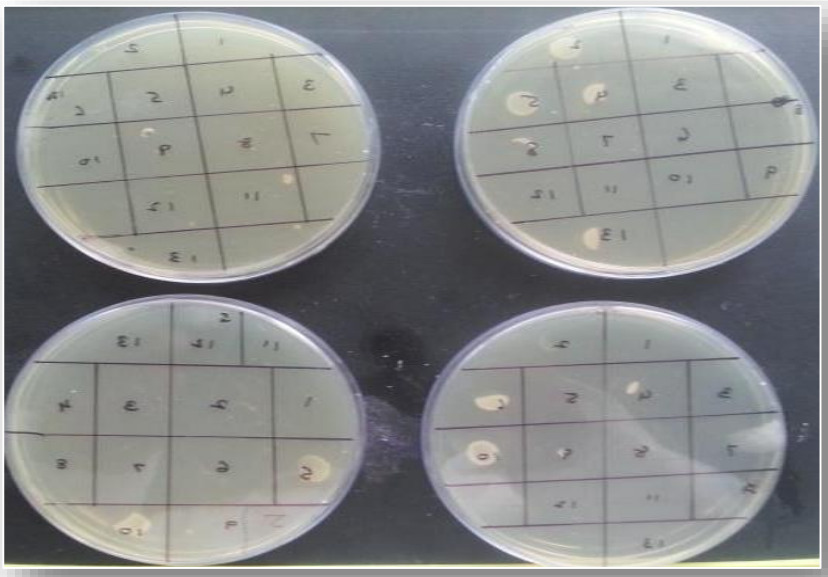


Figure 16: Minimum inhibitory concentration for Vancomycin by E-Strip showing value of 2µg/ml for ATCC strain of *Enterococcus faecalis and* 32µg/ml value in test strain

Figure: 17 MIC for vancomycin by agar dilution method



5.RESULTS

Table VI: Age wise distribution of patients with Enterococcal infection

Age	Number	%
0-20	16	16
21-40	32	32
41-60	32	32
61-80	20	20

Table 6. Showed enterococcal infection seen in the age group between 21--60 years followed by 61-80 years comprising 32% and 20% respectively.

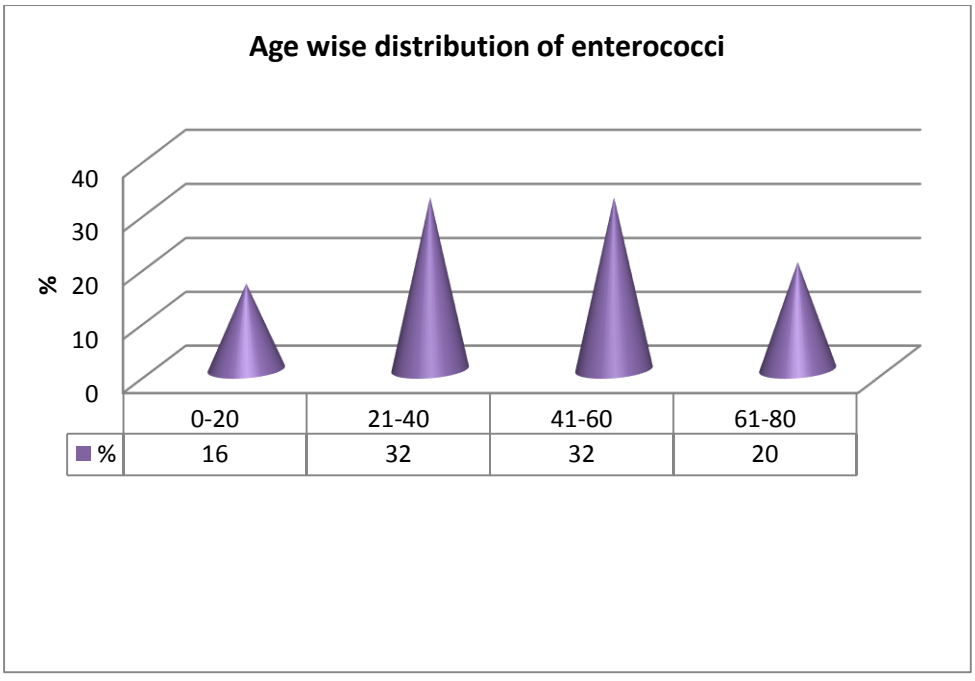


Table VII: Gender wise distribution of Enterococci isolates

Gender	n=100	%
Males	58	58
Females	42	42

Table 7 shows Enterococci infection was seen more in male patients than females

Distribution of Enterococci infections in relation to sex

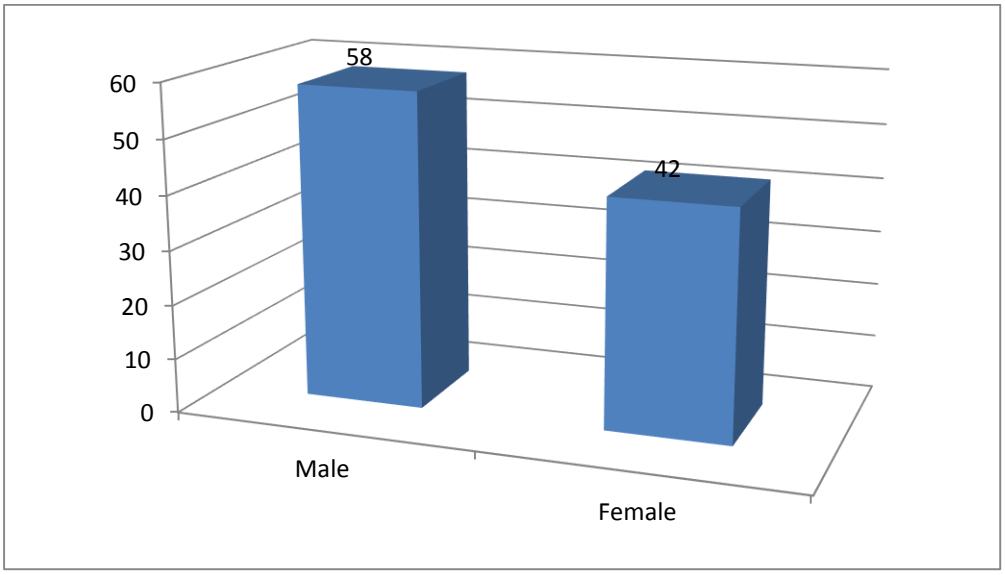


TABLE VIII: Species distribution of Enterococci

Total no Enterococci isolated	**No of *E.faecalis***	**No of *E.faecium***
100	55(55%)	45(45%)

Table 8 100 isolates of *Enterococci were* isolated from various clinical samples 55 % were *E. faecalis* and 45% were *E. faecium.*

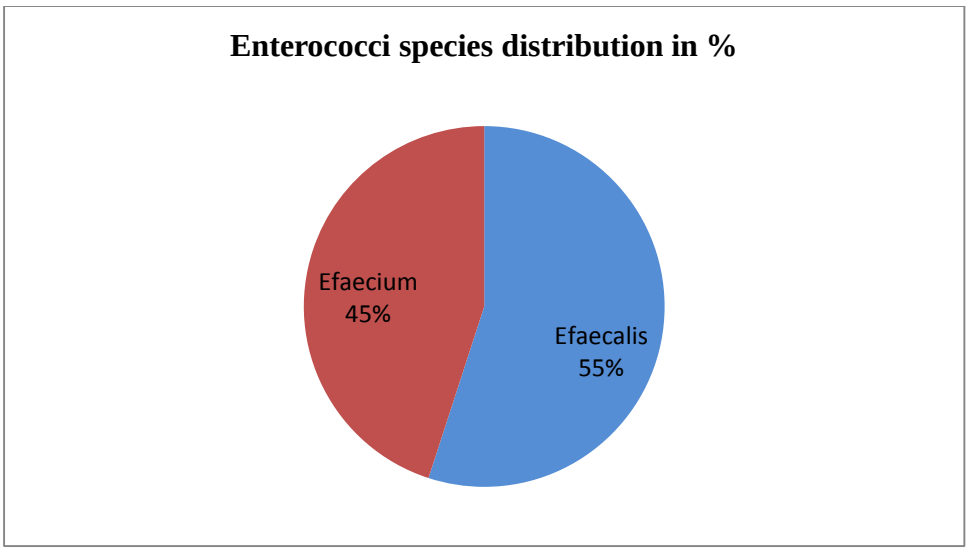
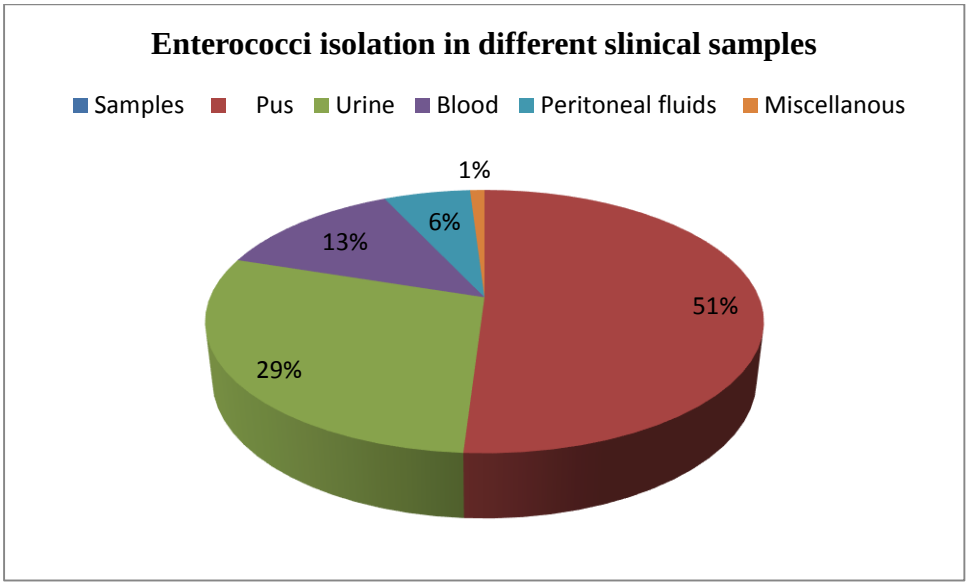


Table IX: Enterococci species Isolation from different clinical samples

Samples n=100	n=100	*E.faecium*	*E.faecalis*
Pus	51	29%	71%
Urine	29	59%	41%
Blood	13	62%	38%
Peritoneal fluids	06	100%	-
*Miscellaneous	01	-	100%

*Semen sample

Table9:shows enterococci isolation from various clinical samples where pus accounting for 50% followed by urine (29%) and blood(13%).Among 51 pus sample ,71 % were E feacalis and 29%were *Efaecium* ,in urinary isolates 59 % were *E.faecium* and 41% was *E.faecalis* Similarly among 13 blood isolates 62% were E.faecium and 38% were E.faecalis.



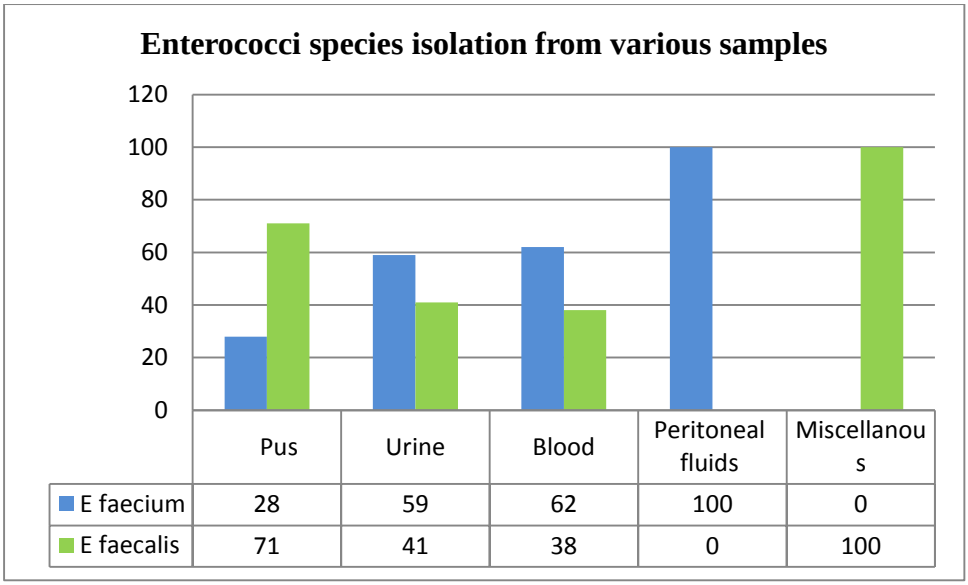


Table: Clinical conditions associated with Enterococcal infection

Clinical condition	**% of isolation**
Post op wound	26
Urinary tract infection (UTI)	14
Wound infection	13
Bacteremia/sepsis	13
Catheter associated UTI	11
Diabetic foot	6
Ileal perforation	4
Cellulitis, furunculitis	4
Device associated (JJ feeding ,VP shunt)	2
Others*	6

*Periodontal abscess, prostatitis, renal calculi, endocarditis, osteomyelitis, nephritic syndrome, CSOM,

Table 10: shows clinical condition associated with enterococci infection. Maximum infection was seen with Post op wound infection (26%) followed by UTI (14%), bacteremia and wound infection (13%) and catheter associated UTI (11%)

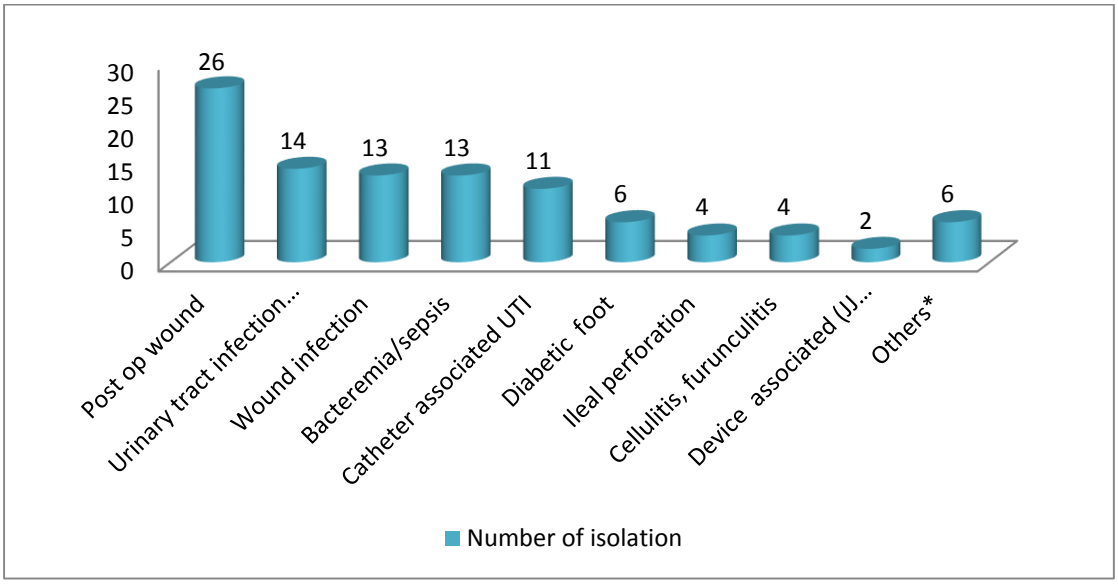
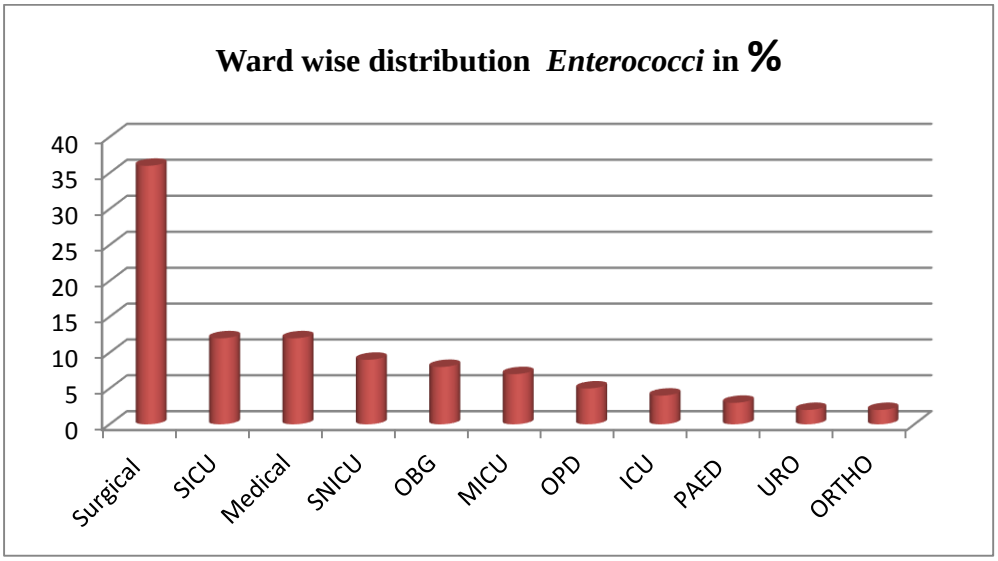
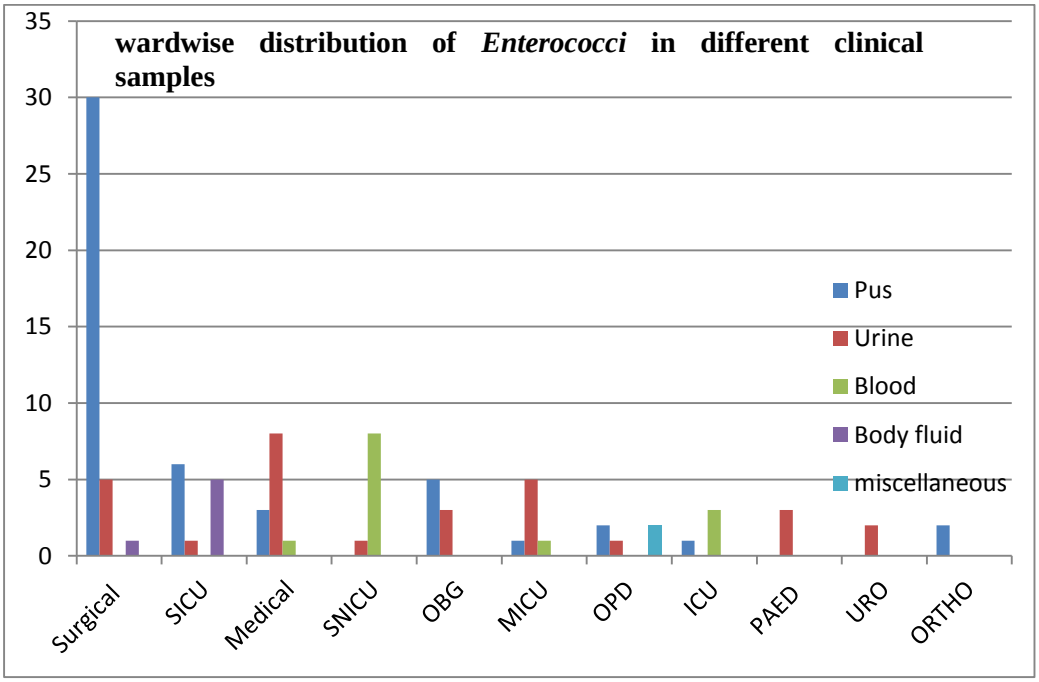


TABLE XI: ward wise distribution of enterococci in various clinical specimens

Ward	**Pus**	**Urine**	**Blood**	**Body fluid**	**miscellaneous**	**Total%**
Surgical	30	5	-	1	-	36
SICU	06	1	-	5	-	12
Medical	03	8	1	-	-	12
SNICU	-	1	8	-	-	09
OBG	05	3	-	-	-	08
MICU	01	5	1	-	-	07
OPD	02	1		-	2	05
ICU	o1	-	3	-	-	04
PAED	-	3	-	-	-	03
URO	-	2	-	-	-	02
ORTHO	02	-	-	-	-	02
Total %	**50**	**29**	**13**	**6**	**2**	**100**

Table showed ward-wise distribution of enterococci, 36% of enterococci were isolated from surgical ward followed by SICU and medical ward accounting for 12%.


Ward wise distribution Enterococci in %
40
35
30
25
20
15
10
5
0
Surgical
SICU
Medical
SNICU
OBG
MICU
OPD
ICU
PAED
URO
ORTHO


wardwise distribution of Enterococci in different clinical samples
35
30
25
20
15
10
5
0
Pus
Urine
Blood
Body fluid
miscellaneous
Surgical
SICU
Medical
SNICU
OBG
MICU
OPD
ICU
PAED
URO
ORTHO

XII: Organisms isolated along with enterococci in various specimens

Organism	**Pus**	**Urine**	**Fluid**	**Blood**	**Total**
E.coli	15	1	2	-	18
Klebsiella spp	6	-	-	-	6
Enterobacter	3	-	-	-	3
MRSA	3	-	-	1	4
Pseudomonas	2	-	-	-	2
Candida	2				2
MSSA	2	1	-	1	4
Providencia spp	-	1	-		1
Citrobacter spp	1	-	-	-	1
Streptococci spp					2
Total	**34**	**3**	**2**	**2**	**43**

Table 12. shows 43% polymicrobiol infection of enterococci where 42 % E coli was isolated followed by Klebsiella spp 14%, Enterobacter 7%, MRSA& MSSA -9%, 5% candida and pseudomonas 5%

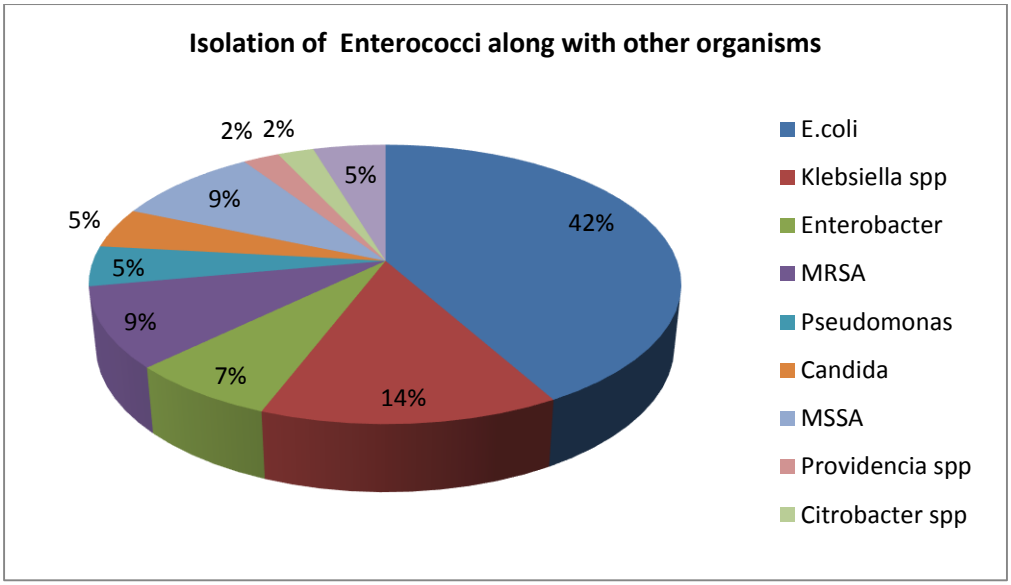


Table XIII: Shows the antibiotic resistance pattern of Enterococci species by disc diffusion

Antibiotics	**E faecium n=45**	**E faecalis n=55**
Penicillin	36(80%)	11(28%)
Ampicillin	36(80%)	15(38%)
High level gentamicin	27(60%)	32(58%)
levofloxacin	37(82%)	37(68%)
Ciprofloxacin	39(87%)	41(74%)
Vancomycin	0%	0%
Teicoplanin	0%	0%
Chloramphenicol	18%	18%
Erythromycin	34(76%)	39(71%)
Tetracycline	37(82%)	42(76%)
Linezolid	0%	0%

Table 13; shows the antibiotic resistance of Enterococci species *E.faecium* showed more resistance than *E faecalis*.

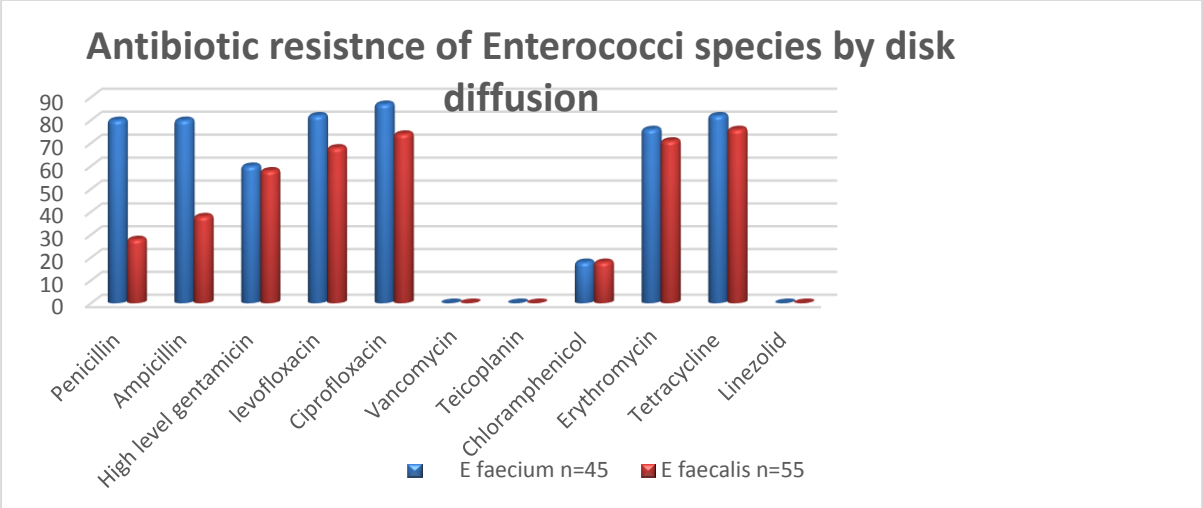


Table XIV: Shows the antibiotic resistance pattern of Enterococci species from urinary Isolates

Urinary isolates	**E faecium n=17**	**E faecalis n=12**
Nitrofurantoin	1(6%)	1(2)%
Norfloxacin	14(82%)	11(92%)
Oflaxacin	15(88)	11(92%)

Table 14: Shows less resistance to Nitrofurantoin in both species and more resistance to Norfloxacin and Oflaxacin

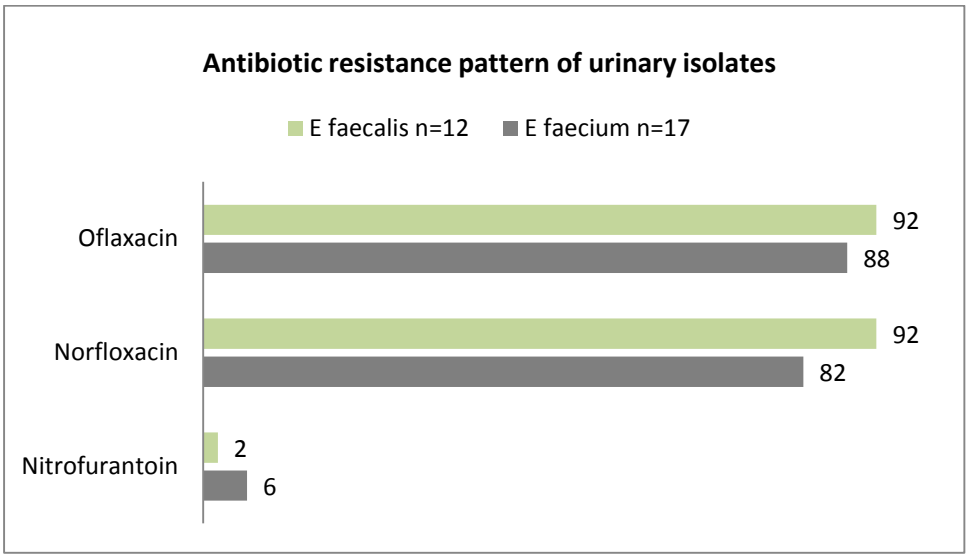


Table XV; MIC of Enterococci species using vancomycin E strips and agar dilution

Method	≤4µg/ml	8-16 µg/ml	≥32µg/ml
E-strip	99	0	01
Agar dilution	99	0	01

Table 10: The results of Minimum inhibitory concentration (MIC) are presented in table MIC for vancomycin by E strip and agar dilution method showed 99 out of 100 isolates were sensitive and 1strain of E faecium showed resistance accounting for 1%. Further confirmation was done by **Vitek 2 system** which showed vancomycin -MIC-32µg/ml and Teicoplanin MIC -32µg/ml.

Figure18: Flow chart shows: Isolation of enterococci species from gastrointestinal tract (GIT) of normal healthy individuals and hospitalized patients with enterococcal infection

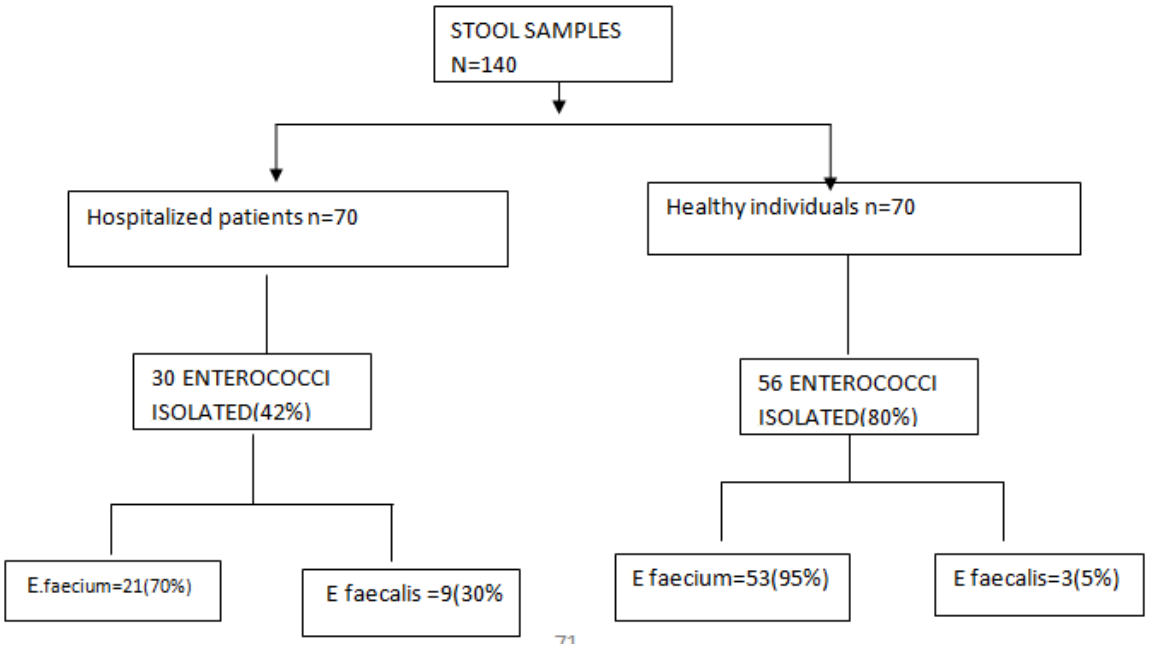


Figure: 18 In healthy individuals and hospitalized patients, *E.faecium* was more commonly isolated than *E. faecalis.*

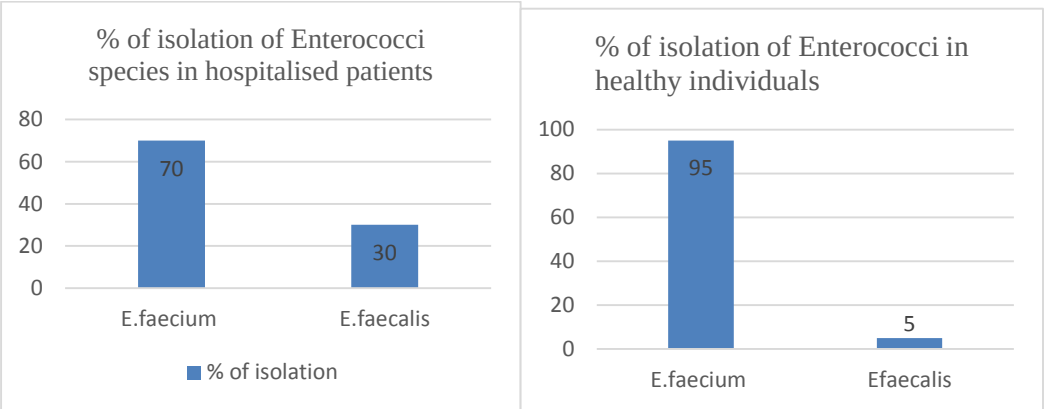


Table XVI: Antibiotic resistance pattern of *E faecium* isolated from clinical specimens and stool samples of hospitalized and healthy individuals

Antibiotics	Isolates from Clinical specimens n=45	Isolates from the stool samples of Hospitalized patients n=21	Isolates from the stool samples of healthy individuals n=53
Penicillin	36(80%)	13(62%)	27(51%)
Ampicillin	36(80%)	16(76%)	29(55%)
Highlevel gentamicin	27(60%)	15(71%)	23(43%)
levofloxacin	37(82%)	16(76%)	32(60%)
Ciprofloxacin	39(87%)	17(80%)	36(68%)
Vancomycin	1(2.2%)	0%	0
Teicoplanin	0%	0%	0
Chloramphenicol	18%	8(38%)	6(11%)
Erythromycin	34(76%)	16(76%)	37(70%)
Tetracycline	37(82%)	18(85%)	31(58%)
Linezolid	0%	0%	0
Norfloxacin(u)*	14(82%)	18(85%)	41(77%)
Oflaxacin(u)*	15(88)	16(76%)	36(68%)
Nitrofurantoin(u)*	1(6%)	-	-

* U=urinary isolates

Tables 16 Clinical isolates of E.feacium shows more resistance to antibiotic than the E.faecium isolated from stool samples of the patients and healthy individuals.

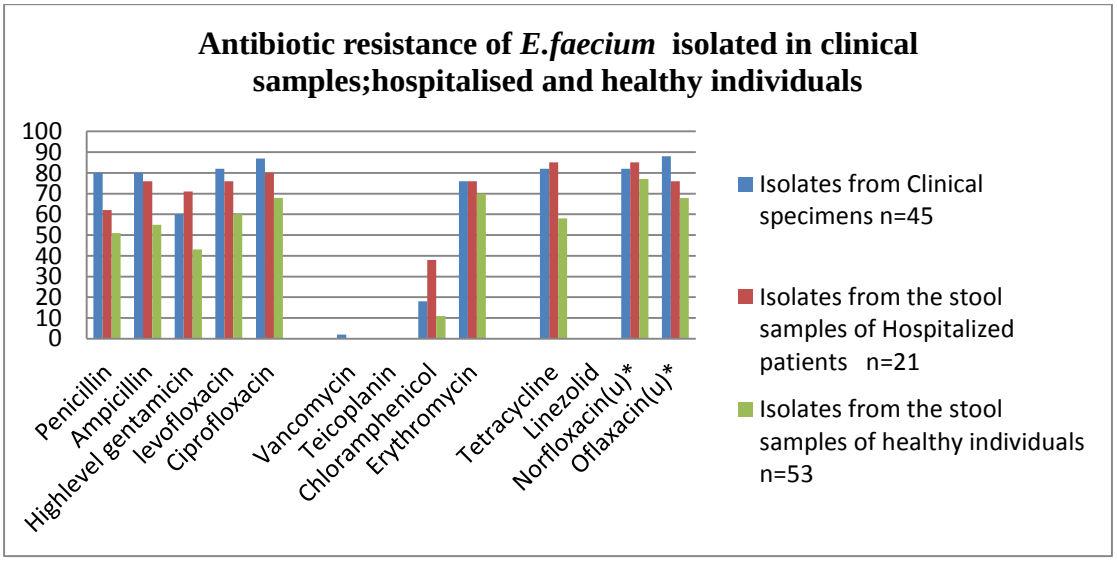


Table XVII shows percentage of antibiotic resistance pattern of *E. faecalis* isolated in clinical specimens, hospitalized and healthy individuals

Antibiotics	**E faecalis Isolated from Clinical n=55**	**Isolates from stool samples of hospitalized patients n=9**	**Isolates from stool samples of healthy individuals. n=3**
Penicillin	11(28%)	3(33%)	0
Ampicillin	15(38%)	4(44%)	0
High level gentamicin	32(58%)	6(67%)	1(33%)
levofloxacin	37(68%)	1(11%)	1(33%)
Ciprofloxacin	41(74%)	1(11%)	0
Vancomycin	0%	0	0
Teicoplanin	0%	0	0
Chloramphenicol	18%	2(22%)	0
Erythromycin	39(71%)	7(77%)	1(33%)
Tetracycline	42(76%)	7(77%)	0
Linezolid	0%	0	0
Norfloxacin(u)*	11(92%)	7(78%)	1(33%)
Oflaxacin(u)*	11(92%)	7(78%)	1(33%)
Nitrofurantoin(u)*	1(2)%	-	-

Table XVIII: Minimum inhibitory concentration for vancomycin in normal healthy individuals and hospitalized patients using E- strip and by agar dilution

Enterococci	**Number of VRE by Agar dilution**	**Number of VRE by E-strip**
Healthy individuals n=56	0	0
Hospitalized patients n=30	0	0

Table 17: Shows there were no VRE seen in hospitalized patients with enterococcal infection and healthy individuals and minimum inhibitory concentration was ≤4µg/ml by both agar dilution and E-strip methods.

6.Discussion

Enterococci are the normal commensals of the human intestine. Sites less often colonized are oral cavity, genitourinary tract and perianal area.[65] Enterococci were previously considered as low grade pathogens but recently it gained importance because of changing trends in infections caused by different species of *Enterococci* and emergence of multidrug resistant strains. Most of the laboratories identify the organism only up to genus level.[16] Enterococci identification upto species level in the clinical microbiology is important because some of the Enteroccoci species have intrinsic resistance to several antibiotics like Beta-lactams (cephlosporins, low level aminoglycosides), and species like *E.gallinarium* and *E.classeliflavus* show intrinsic resistant to vancomycin.[16] So correct speciation can help to predict antibiotic resistance pattern and treatment.

The present study was undertaken to speciate and detect antibiotic susceptibility pattern of enterococci isolated from different clinical specimens. Stool samples were also processed from hospitalized patients and healthy individuals to look for colonization, antibiotic susceptibility pattern and the prevalence of Vancomycin resistant Enterococci.

Over a period of 1 year 7 month from January 2013 to July 2014, we isolated 100 enterococci from various clinical samples of patients attending R L Jalappa Hospital attached to Sri Devaraj Urs Medical College. Most of the Enterococcal infection was seen in the age group 21-40 years and 41-60 years each accounting for 32% respectively. This was followed by age group 61-80 years (20%) and 0-20years (16%) which included 6 newborns and 3 infants. Thus most of the patients in our study with

Enterococcal infections belonged to 3rd to 6th decade of life. Studies from Pondicherry,[38] Jaipur [65] and Gujarat [66] showed higher infection rate in the 21-40 years age group however study by Modi et al from Ahmadabad, showed Enterococcal infection more in 61-75 years age group.[58] This indicates that all the age groups are at risk of acquiring Enterococcal infections.

Of the 100 Enterococci isolate, we found a slight preponderance in male patients over female patients. Males accounted for 58% whereas female patients accounted for 42% [Male: Female ratio was 1.38:1]. These findings were in concordance with Patel et al from Gujarat and Revati Sharma et al from Navi Mumbai.[67]. However in some studies, there was preponderance of female patient over male patient. [1,65] This shows that there was no statistically significant difference in gender wise distribution of Enterococcal infections.

Most of the enterococci were isolated from pus (50%) followed by urine (29%), blood (13%) and peritoneal fluid (6%). This is contrary to other studies where the maximum numbers of isolates were from urine.[38,61] A study from Bangalore by Sreeja et al found higher rate of isolation from pus samples which was similar to our study.[68]

Enterococci are the second most common cause of nosocomial urinary tract and wound infections and third most common cause of nosocomial bacteremia.[2,5,6] In our study most of Enterococci isolation was from with post-operative wound infections (26%) followed by Urinary Tract Infection (12%) and bacteremia (13%). Modi et al from Gujarat showed most common clinical condition associated with enterococcal infection was septicemia whereas study done by Palaniswamy et al from Tamilnadu, showed UTI was the most common condition. This shows that isolation of Enterococci from clinical conditions with varies from hospitals to hospitals.

Among 100 isolates of enterococci, 43% of isolates were associated with other microorganisms indicating polymicrobial infection. The organisms isolated along with Enterococci were *Escherichia coli* (42%) followed by, *Klebsiella spp* (14%), *MSSA* (9%), *MRSA*(9%),*Enterobacter spp* (7%), *Pseudomonas* (5%) and *Candida*(5%).Patel et al from Gujarat and Chaudary U et al from Haryana also showed polymicrobial infection in their studies varying from 15%-17%.In a study from Tamilnadu reported, polymicrobial infection as 46.5% where *E coli* accounted for 12.5% followed by *klebsiella* 9% and *candida* 8%.[69] Several studies suggest that enterococci can act synergistically with other intestinal bacteria to enhance the occurrence of infection.[2]

Wardwise distribution of *Enterococci* isolated from various clinical specimens showed that maximum number of isolation was from General surgical ward (36%) followed by Surgical Intensive Care Unit (12%) and medical ward (12%) Study from Ahmedabad[58] showed maximum isolation from medical ward (38%) followed by surgical ward (27%) and pediatric ward (12%), whereas in our study least Enterococcal isolation was from pediatric ward (3%). Another study by Deepa et al from Pondicherry showed maximum isolation was from OBG ward (67%) followed by medical ward (20%) and least isolation was from surgical ward (1.8%).[38]

It was reported previously that, *E.faecium* and *E.faecalis* were the only two species prevalent in India; however in recent studies, other species of Enterococci like *E.gallinarum, E.casseliflavus, E.durans, E.hirae, E.mundtii and E.raffinosus* were also isolated from clinical samples.[2,57]Various studies conducted from central and south India found that E.faecalis was the predominant species isolated followed by *E.faecium* but studies carried out in North India have showed Enterococcal infections caused by *E.faecium* was more common than *E faecalis.*[12,1] In our study *E.faecalis*

(55%) was more common than *E.faecium* (45%) and no other species have been isolated, which was similar to the studies conducted from Bangalore, Chennai and Davangere.[68,70,71]

Multidrug resistant *Enterococci* are being increasingly reported from all over the world. Most of the *Enterococci* isolated in our study also showed multidrug resistance. *E. faecium* was found to be more resistant to antimicrobial agents than *E.faecalis*. It could be due to change in patient population in the hospital, the antimicrobial use, coupled with the greater antibiotic resistant nature of *E.faecium* which might have conferred a greater selective survival advantage as compared to *E.faecalis*.[57]

E.faecium showed 80% resistance to Penicillin and Ampicillin whereas *E.faecalis* showed 28% and 38% respectively. This is could be attributable to either beta lactamase production or low affinity to penicillin –binding proteins. Studies in Bangalore,[68] Chennai,[70] and Davangere[71] showed 40-50% resistance to Ampicillin and Penicillin whereas results of Mahesh et al from Bagalkot and Mathur et al from Delhi showed more than 70% resistance to Ampicillin.[57,72]

Resistance to aminoglycoside is often associated with multi drug resistance and is due to various aminoglycoside modifying enzymes. It makes the combination therapy of aminoglycoside and beta-lactam antibiotics ineffective in treating serious Enterococcal infection like endocarditis. Our study revealed that both *E.faecalis* and *E.faecium* were resistant to High Level Gentamicin (60%); similar findings were also reported in the study conducted from New Delhi, Belgaum and Bangalore [1,8,68]

High resistance to Fluroqinolones was seen in our study, which was 70% in both *E.faecalis* and *E.faecium* but Patel et al from Gujarat reported low resistance to ciprofloxacin (6%) and Levofloxacin (19%).[66]

Nitrofurantoin is a urinary antibiotic for treating UTI .It is both a bacteriostatic and a bactericidal agent and has been used for many years. It is effective against both species of Enterococci including VRE strains as the mutants showing resistances are very rare.[2]Our study showed urinary isolates of *Enterococci* were less resistant to Nitrofurantoin: 6% in *E.faecium* and 2% in *E.faecalis* which correlates with findings from Delhi.[8] However Preethi et al from Jaipur showed 88% resistant to nitrofurantoin.[65]

All enterococci isolates showed more than 70% resistance to Erythromycin and tetracycline which correlates with results of Mahesh et al from Bagalkot and Mathur et al (85%). Seema et al and Preethi et al showed about 47% resistances to tetracycline whereas Saroja et al from Bangalore showed 62% tetracycline resistance in *E.facium* and 55% in *E.faecalis*.

Our study showed that enterococci were least resistant to chloramphenicol (18%), whereas study by Seema et al showed 32% resistance. Study from Manipal reported 37.2% resistance to Chloramphenicol in *E.faecium* and 45.2% in *E.faecalis.*[12]

All enterococci isolates in our study were 100% sensitive to Vancomycin, Linezolid and Teicoplanin by disk diffusion method. But one strain isolated from term baby with severe sepsis showed resistance to vancomycin with MIC of 32µg/ml by both Agar dilution and E-strip methods..Hence there are chances of getting false sensitivity results with disk diffusion method which leads to inappropriate selection of Vancomycin as a therapeutic option leading to treatment failure. Further confirmation

of the Vancomycin Resistant Enterococci [VRE] strain was done by Vitek 2 system (Biomeruix) which showed MIC value of 32μg/ml for both vancomycin and Teiecoplanin and sensitive to Linezolid. VRE prevalence in our region is about 1% which is very less compared to other studies which showed of VRE prevalence ranging from 10-24%.[73,74]Linezolid is the drug of choice for treating infection caused by VRE strains, however lately there are reports of Enterococci developing resistance to Linezolid limiting the therapeutic options.[10]

Asymptomatic fecal carriage of VRE if present in the community, individuals admitted to hospital and subjected to the selective pressure of antibiotics on the normal gut flora may act as the source of hospital outbreaks. The rapid emergence of resistance in enterococci and the increasing incidence of colonization and infection with VRE have become health care issues that have caused serious concern to physicians and health authorities alike. Our study found that 80% of normal healthy individuals were colonized with enterococci whereas colonization of hospitalized patients with *Enterococcal* infection was 42%. *E.faecium* was found in 70% of hospitalized patients and 95% of healthy individuals. There was no prevalence of vancomycin resistance in both the groups i.e hospitalized patients and normal healthy individuals. Priyanka et al from Bihar, showed majority of the isolates from the fecal samples were *E.faecalis* 44.4% followed by *E.gallinarium* 34.9%, *E.faecium* 17.7% and 1.5% each of *E.raffinosus* and *E.dispar.* Among these isolates, 22.7% of *E.faecium* and 10.7% of *E.faecalis* was found to be VRE.[75]

Study from Netherland showed enterococci colonization in 49% of the hospitalized patients and 80% of the people living in the community. *E faecium* was found in 43% of hospitalized patients and 32% in the community. Prevalence of VRE in hospitalized patients and community was 2%.[76] According to another study from

Czech Republic, prevalence of VRE in GIT was 1.9% in the hospitalized and 0.4% in the community subjects[77].

Antibiotic susceptibility of *E.faecium* isolated from hospitalized patients with Enterococcal infections showed more than 70% resistance to Ampicillin, HLG, fluoroquinoles, Erythromycin and Tetracycline, 62% resistance to penicillin and 38% resistance to Chloramphenicol. All isolates showed 100% susceptibility to Vancomycin, Linezolid and Teicoplanin.

Antibiotic sensitivity of *E.faecium* isolated from healthy individuals showed more than 50% resistance to Penicillin, Ampicillin, and tetracycline, around 70% resistance to Erythromycin, fluroquinolones except Levofloxacin (60%) and least resistance to Chloramphenicol (11%). High level Gentamicin resistance was 43%. All isolates showed 100% susceptibility to Vancomycin, Linezolid and Teicoplanin.

We conclude that Enterococcal strains with high rate of resistance to multiple drugs are not only prevalent in the clinical environment but also in the gastrointestinal tract of the colonized patients and the healthy individuals. Though the study showed there was no prevalence of fecal carriage of VRE, hospital should keep in mind to implement Screening surveillance to detect fecal VRE carriage in the hospitalized and in the normal population as these colonized people contaminate themselves as well as environment.

7.Summary

Over a period of 1 year 7 month from January 2013 to July 2014, 100 enterococci were isolated from various clinical samples of patients attending R L Jalappa Hospital attached to Sri Devaraj Urs Medical College.

Most of the Enterococcal infection was seen in the age group between 21-40 years and 41-60 years each accounting for 32% respectively in our study. Thus most of the patients with Enterococcal infection were belonged to 3rd to 6th decade of life with male to female ratio of 1.38:1. Males accounted for 58% of the patients whereas female patients accounted for 42% and found that there was no statistically significant difference in gender wise distribution of Enterococcal infections.

Maximum numbers of Enterococci were isolated from pus sample and most of these isolates were from post-operative wound infection (26%) followed by UTI (12%) and bacteremia (13%). The highest rate of Enterococcal isolation was from surgical ward (36%). We found Escherichia coli was most common isolate along with Enterococci in 43% of polymicrobial infection.

We isolated two species of Enterococci; E. faecalis (55%) being the predominant species followed by E. faecium(45%). *E.faecium* showed 80% resistance to Penicillin and Ampicillin whereas *E.faecalis* showed 28% and 38% respectively. Both *E.faecalis* and *E.faecium* showed resistant to High Level Gentamicin (60%). Urinary isolates of Enterococci were least resistance to Nitrofurantoin. All enterococci isolates in our study were 100% sensitive to Vancomycin, Linezolid and Teicoplanin by disk diffusion method. But one strain of E. faecium isolated from blood sample of a term baby with severe sepsis, showed MIC of 32µg/ml for Vancomycin both by Agar dilution and E-strip methods. Hence there are chances of getting false sensitivity results with disk diffusion method which leads to

inappropriate selection of Vancomycin as a therapeutic option leading to treatment failure.

We processed 70 stool samples each from healthy individuals and patients and found Enterococcal colonization in 80% of healthy individuals and 42% of hospitalized patients. *E.faecium* was the common isolate found in 70% of hospitalized patients and 95% of healthy individuals without any VRE carriage among them.

8. Conclusion:

Enterococci identification upto species level in the clinical microbiology is important because some of the Enteroccoci species have intrinsic resistance to several antibiotics like Beta-lactams (cephlosporins, low level aminoglycosides) and species like *E.gallinarium* and *E.classeliflavus* show intrinsic resistant to vancomycin. So correct speciation can help to predict antibiotic resistance pattern and treatment.

The use of appropriate methods for the detection of antibiotic resistance among these organisms should be stressed upon in routine laboratory practice. So effective detection of vancomycin resistance in laboratory helps in reducing the morbidity and mortality due to VRE. Early detection of patients colonized or infected with VRE is an essential component of any hospital Programme, so that effective therapy and infections control measures can be initiated.

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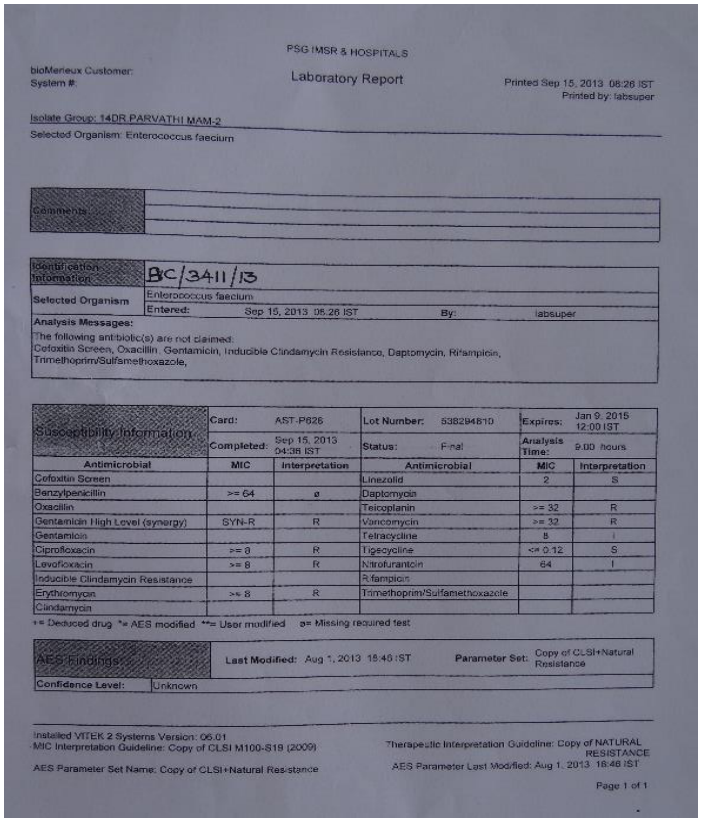
Annexure I – Report of vancomycin strain by Vitek 2 system



PSG IMSR & HOSPITALS

bioMerieux Customer:
System #:

Laboratory Report

Printed Sep 15, 2013 08:26 IST
Printed by: labsuper

Isolate Group: 14DR PARVATHI MAM-2
Selected Organism: Enterococcus faecium

Comments:

Identification Information: BC/3411/13
Selected Organism: Enterococcus faecium
Entered: Sep 15, 2013 08:26 IST By: labsuper

Analysis Messages:
The following antibiotic(s) are not claimed:
Cefoxitin Screen, Oxacillin, Gentamicin, Inducible Clindamycin Resistance, Daptomycin, Rifampicin, Trimethoprim/Sulfamethoxazole,

Susceptibility Information
Card: AST-P626 Lot Number: 53829481D Expires: Jan 9, 2015 12:00 IST
Completed: Sep 15, 2013 04:38 IST Status: Final Analysis Time: 9.00 hours

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Cefoxitin Screen			Linezolid	2	S
Benzylpenicillin	>= 64	ø	Daptomycin		
Oxacillin			Teicoplanin	>= 32	R
Gentamicin High Level (synergy)	SYN-R	R	Vancomycin	>= 32	R
Gentamicin			Tetracycline	8	I
Ciprofloxacin	>= 8	R	Tigecycline	<= 0.12	S
Levofloxacin	>= 8	R	Nitrofurantoin	64	I
Inducible Clindamycin Resistance			Rifampicin		
Erythromycin	>= 8	R	Trimethoprim/Sulfamethoxazole		
Clindamycin					

+= Deduced drug *= AES modified **= User modified ø= Missing required test

AES Findings: Last Modified: Aug 1, 2013 18:46 IST Parameter Set: Copy of CLSI+Natural Resistance
Confidence Level: Unknown

Installed VITEK 2 Systems Version: 06.01
MIC Interpretation Guideline: Copy of CLSI M100-S19 (2009)
AES Parameter Set Name: Copy of CLSI+Natural Resistance
Therapeutic Interpretation Guideline: Copy of NATURAL RESISTANCE
AES Parameter Last Modified: Aug 1, 2013 18:46 IST

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I. MacConkey agar (Commercially available from Himedia)

Ingredients:	Gram/litre:
Peptic digest of animal tissue	17
Agar	15
Lactose	10
Sodium chloride	5
Protease peptone	3
Bile salts	1.5
Neutral red	0.03
PH (at 25^{o}C)	7.1 $\pm$ 0.2

51.53 grams of the dehydrated medium was added to 1 liter of distilled water and boiled till the powder dissolved. This was autoclaved at 15 lbs pressure

(121°C) for 15 minutes and then poured into sterile 90 mm petri plates upto a thickness of about 4mm.

II. Blood agar

Nutrient agar was prepared from commercially available medium from Hi-media.

Ingredients:	Gram/litre:
Peptic digest of animal tissue	5
Sodium citrate	5
Beef extract	1.5
Yeast extract	1.5
Agar	15
Final pH (at 25°C)	7.4 $\pm$ 0.2

28 grams of the medium was suspended in 1000 ml of distilled water. This was then boiled until the powder dissolved completely and then sterilized by autoclaving at 15 lbs. pressure (121°C) for 15 minutes. This preparation was cooled to 50°C and sterile sheep blood was added aseptically upto a concentration of 10%. This was then poured into sterile petri plates.

III. Mueller – Hinton agar (Commercially available from Hi media)

Ingredients:	Gram/litre:
Beef, infusion from	300
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17

Final pH (at 25°C)	7.3 ± 0.1

38 grams of the medium was suspended in 1000 ml of distilled water and heated to dissolve the medium completely. It was sterilized by autoclaving at 121°C for 15 minutes and then poured into sterile petri plates.

IV. Decarboxylase Tests (commercially available from Hi media)

Moeller's decarboxylase broth base was prepared from commercially available dehydrated medium as follows:

Ingredients:	Grams/litre:
Peptic digest of animal tissue	5
Beef extract	5
Dextrose	0.5
Pyridoxal	0.005
Final pH (at 25°C)	6.0 ± 0.2

10.52 grams of medium was dissolved in 1000 ml distilled water. Then 10 gram of L-Lysine, L-Arginine or L-Ornithine was added. The medium was dissolved completely with heat when necessary and dispensed in 5 ml amounts in test tubes and sterilized by autoclaving at 15 lbs. pressure (121°C) for 10 minutes.

V. Mannitol Motility medium (Commercially available from Hi media)

Ingredients per 1000 ml:

Peptic digest of animal tissue	20
Mannitol	2
Potassium nitrate	1
Phenol red	0.04

Agar	3
Final pH (at 25°C)	7.6 ± 0.2

26.04 grams of media was dissolved in 1000 ml of distilled water and was gently heat to dissolve the medium completely. Then it was dispensed into test tubes and sterilized by autoclaving at 15 lbs (121°C) for 15 minutes. This was followed by cooling the tubed medium in upright position.

VI. Bile Esculin Agar (commercially available from Hi media)

Ingredients:	Grams/litre:
Peptic digest of animal tissue	5
Beef extract	3
Ox gall	40
Esculin	1
Ferric citrate	0.5
Agar	15
Final pH (at 25°C)	6.6 ± 0.2

64.5 grams of medium was added to 1000 ml distilled water and boiled till it dissolved completely. It was sterilized by autoclaving at 15 lbs. pressure (121C) for 15 minutes and poured into test tubes and allowed to cool in slanting position.

VII. Sugar Fermentation media:

Peptone- 1 gram

Sodium chloride – 0.5 gram

Sugar (glucose/ lactose/ sucrose/mannitol/sorbitol/arabinose) - 1 gram

Andrade's indicator (0.005%) - 1 ml

Distilled water- 100 ml

PH-7.4-7.5

VIII. Pyruvate fermentation media:

Ingredients:	Grams/litre:
Tryptone	10gms
Yeast extracts	5gms
Dipotassium phosphate	5gms
Sodium chloride	5gms
Sodium pyruvate	10gms
Bromothymol blue	Ethanolic 4mg/ml
Agar	10gms
D W	1litre
PH -7.1-7.4	

Medium is dispensed in small tubes and sterilized by autoclaving at 120^{o}c for 20 minutes. Allowed to cool .Test strains were inoculated into medium and incubated at 37^{0}C for 2 days .Change in color from green to yellow indicate positive test.

PROFORMA

Name :

Lab No.:

Age & Sex: Ip No./Op No.:

Address : Ward :

DOA : Ref Dr:

Presenting Complaints:

History of present illness:

Past History & Treatment :

General Physical Examination :

Systemic examination :

Laboratory data

Blood routine	Urine routine
TC	Albumin
DC	Sugar
CRP	Microscopy

Others :

Microbiology Investigations :

Sample :

Colony morphology on Blood agar :

And Mac Conkey agar:

Gram's stain from colony:

Catalase test :

Bile Esculin hydrolysis test :

Salt tolerance (6.5% NaCl) test:

Heat tolerance 60^{0} C for 30 minutes:

Speciation :

Motility by hanging drop method:

Arginine dihydrolase test

Potassium tellurite (0.04%) reduction :

Sugar fermentation tests :

Mannitol

Sorbitol

Arabinose

Raffinose

Pyruvate fermentation test

Antibiotic susceptibility test by Kirby Bauer disc diffusion method

MIC of Vancomycin by Agar dilution method:

MIC (μg/ml) →

Proforma for healthy individuals

NAME:

AGE/SEX:

ADDRESS:

Not suffering from Signs and symptoms of disease

Consent for collecting stool samples

Later sample processed