A STUDY ON DETECTION OF HIGH LEVEL GENTAMICIN RESISTANCE AMONG ENTEROCOCCAL SPECIES AND ITS MOLECULAR CHARACTERIZATION IN A TERTIARY CARE CENTRE

DISSERTATION SUBMITTED FOR

BRANCH – IV - M.D. DEGREE

(MICROBIOLOGY)

MAY 2018

THE TAMILNADU

DR.M.G.R.MEDICALUNIVERSITY

CHENNAI, TAMILNADU

BONAFIDE CERTIFICATE

This is to certify that the dissertation entitled "**A STUDY ON DETECTION OF HIGH LEVEL GENTAMICIN RESISTANCE AMONG ENTEROCOCCAL SPECIES AND ITS MOLECULAR CHARACTERIZATION IN A TERTIARY CARE CENTRE "** submitted by **Dr.J. VIJAY ANAND** to the Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the requirement for the award of M.D degree Branch– IV (Microbiology) is a bonafide research work carried out by him under direct supervision & guidance.

DR.MARUTHUPANDIAN,M.S.,FICS;FAIS
DEAN
Madurai Medical College &
Govt. Rajaji Hospital,
Madurai.

DR.MANGALA ADISESH,M.D.,
Director
Institute of Microbiology,
Madurai Medical College,
Madurai.

CERTIFICATE FROM THE GUIDE

This is to certify that the dissertation **"A STUDY ON DETECTION OF HIGH LEVEL GENTAMICIN RESISTANCE AMONG ENTEROCOCCAL SPECIES AND ITS MOLECULAR CHARACTERIZATION IN A TERTIARY CARE CENTRE"** is a bonafide record of work done by **DR.J.VIJAY ANAND**, under my guidance and supervision in the Institute of Microbiology, Madurai Medical College, Madurai during the period of his Post graduate study of M.D. MICROBIOLOGY from 2015 – 2018.

Prof.Dr.S.RADHAKUMARI, M.D.,
Institute of Microbiology,
Madurai Medical College,
Madurai.

DECLARATION

I, **DR.J.VIJAY ANAND** declare that, I carried out this work on, **"A STUDY ON DETECTION OF HIGH LEVEL GENTAMICIN RESISTANCE AMONG ENTEROCOCCAL SPECIES AND ITS MOLECULAR CHARACTERIZATION IN A TERTIARY CARE CENTRE "** at the Institute of Microbiology, Madurai Medical College. I also declare that this bonafide work or a part of this work was not submitted by me or any others for any award, degree or diploma to any other University, Board, either in India or abroad.

This is submitted to The Tamilnadu Dr. M. G. R. Medical University, Chennai in partial fulfillment of the rules and regulations for the M.D. Degree examination in Microbiology.

Place : MADURAI **DR.J.VIJAY ANAND**

Date :

ACKNOWLEDGEMENT

I am grateful to the Dean**, DR.MARUTHUPANDIAN M.S., FICS., FAIS.,** Madurai Medical College and Government Rajaji Hospital, Madurai for permitting me to carry out this study.

I would like to express my deep sense of gratitude and sincere thanks to Professor **Dr.MANGALA ADISESH, M.D.,** Director, Institute of Microbiology, Madurai Medical College, for her constant help, guidance and encouragement given to me throughout this study.

I express my sincere thanks to **Prof. Dr. R.Vibhushanan, M.D., Prof. Dr. V. Dhanalakshmi, M.D., and Prof. Dr. S. Radhakumari, M.D.,** for their valuable suggestions and moral support given to me throughout the study.

I would like to express my sincere thanks to my guide **Prof. Dr. S. Radhakumari, M.D,** co-guides **Dr. C. Sugumari, and Dr. N. Rammurugan, M.D.,** their valuable suggestions and guidance given to me.

I would like to express my sincere thanks to all my Assistant professors **Dr.S.Maheshprabhu,M.D, Dr.N.Anuradha,M.D., Dr.J.Suria kumar, M.D., Dr.M.R.VasanthaPriyan, MD., Dr.D. Saradha, M.D., Dr. G. Manjula, M.D Dr.S.Nalayini, M.D.** for their valuable suggestions and guidance given to me.

I am thankful to all my colleagues **Dr. S. Meerah, Dr. B. Sree Bavai Malar, Dr. Sasireka, Dr. Minu George, Dr. S. Rajeswari, Dr. R. Vasanthi,**

and Dr. Tamilselvi for their moral support and cooperation rendered during the work.

I extend my thanks to all staff members, Institute of Microbiology for giving full cooperation and timely help in carrying out the laboratory studies.

I extend my thanks to my parents, My wife **Mrs. N. Rekha,** daughters **Srinidhi** and **Srinika** for their esteemed moral support rendered during the study.

CONTENTS

INTRODUCTION

Enterococci are the fast emerging organisms causing serious nosocomial outbreaks. This is mainly due to acquiring virulence factors and multiple drug resistance pattern by the enterococcal species. Enterococci were considered as organisms of low virulence previously but recently, Enterococci has the ability to acquire and share extrachromosomal elements encoding virulence traits or antibiotic resistance genes. This leads to their increased environmental survival and their nosocomial infection.

Enterococci are normal residents of oral cavity, gastrointestinal and biliary tracts and also seen in vagina and male urethra. Enterococci are considered as opportunistic pathogen.[49] They are the common cause of nosocomial urinary tract, blood stream, surgical sites, intra abdominal and pelvic regions, neonatal and central nervous system infections[86].

In the French literature in 1899[56] the term ente'rocoque was used. The "Enterococcus" was coined by Thiercelin to state bacteria in pairs and short chains seen in human stools. The organism was initially designated 'Micrococcus zymogenes' due to its fermentative properties.[56] The name Streptococcus faecalis was used to describe an organism of fecal origin by Andrews and Harder in 1906 which fermented lactose and mannitol but not raffinose. Second organism, Streptococcus faecium differed from the S.faecalis by the fermentation property which was described by Orla Jensen in 1919. In 1970 Kalina named S.faecalis and S.faecium as Enterococcus based

on the phenotypic characters and cellular arrangements. In 1984, based on DNA-rRNA hybridization studies and 16S rRNA[92] sequencing, Schleifer and Kilpper Balz allocated S.faecalis and S.faecium into a separate genus Enterococcus .

Enterococcus genus include the Enterococcal members previously classified with the group D streptococci.About 80-90% of human Enterococcal infections is caused by the most common isolate E.faecalis.**(Jett et al,1994[40];Jones et al , 2004[42]**

E. faecium second common among Enterococcal infections is isolated from 10-15% of infections. Other Enterococcal species are infrequently isolated from human infections like E.durans, E. gallinarom, E .casseliflavus.

General characters of Enterococci:

i) They are Gram positive cocci, usually appear oval in shape, arranged in pairs and short chains.

ii) All are facultatively anaerobic.

iii) Most of them react with the Lancefield group D antisera.

iv) All are non motile (except E. gallinarum and E. casseliflavus).

v) They are able to grow at 10°C and 45°C and at pH 9.6.

vi) Enterococci are usually alpha or gamma hemolytic on 5% sheep blood agar. Some produce beta hemolysis on human, horse and rabbit blood.

vii) They survive at the temperature of 60°C for 30 min.

viii) They are capable of growing in media containing 6.5% Nacl.

ix) They are capable of hydrolyzing esculin in the presence of 40% bile salts.

x) They are capable of producing a Leucine aminopeptidase (LAP) and a Pyrrolidonyl arylamidase (PYR) (except for E. cecorum, E. columbae, E. pallens and E. saccharolyticus).

- 3rd most common cause for nosocomial UTI[49]
- 2 nd or 3 rd most common cause for nosocomial bacteremia [49]
- 5% to 20% of infective endocarditis caused by Enterococci [49]
- Important feature of Enterococci are intrinsic resistance to various group of antobiotics
- Enterococci has the ability to transfer the resistant genes to other bacteria.

Virulence factors :

1. Cytolysin / hemolysin : It acts on human, rabbit, equine and bovine erythrocytes (but not sheep erythrocytes). The quorum sensing mechanism regulates expression of hemolysin [74]
2. Gelatinase : The gelatinase, a protease that has the capability of hydrolyzing gelatin, casein, collagen, hemoglobin and other peptides and gelE gene coding for gelatinase is involved in biofilm formation[74]

3. Aggregation substances(AS) : It is a surface bound, plasmid encoded protein that promotes clumping of the organisms to facilitate the plasmid exchange. Agg genes are involved in biofilm formation. Phagocytosis is significantly resisted by Enterococci expressing aggregation substances .

4. Extracellular surface protein (Esp) : It helps the organisms evade antibodies by its ability to be retracted away from the cell surface. Esp gene is involved in biofilm formation.

5. Extracellular superoxide: Most E.faecalis and some E.faecium isolates from bacteremia produce large amounts of extracellular superoxide that may enhance Enterococcal virulence in mixed flora abscesses with Bacteroides fragilis.

6. Lipoteichoic acid: It constitutes group D antigen of Enterococci and contributes to virulence by inducing the production of tumor necrosis factor (TNF) and interferon.

7. Coccolysin: 50-65% of E. faecalis strains produce coccolysin, an extracellular metallo endopeptidase, involves in virulence by inactivating endothelin.

8. Biofilms: The ability of Enterococci to cause device associated infections is enhanced by the production of biofilms.

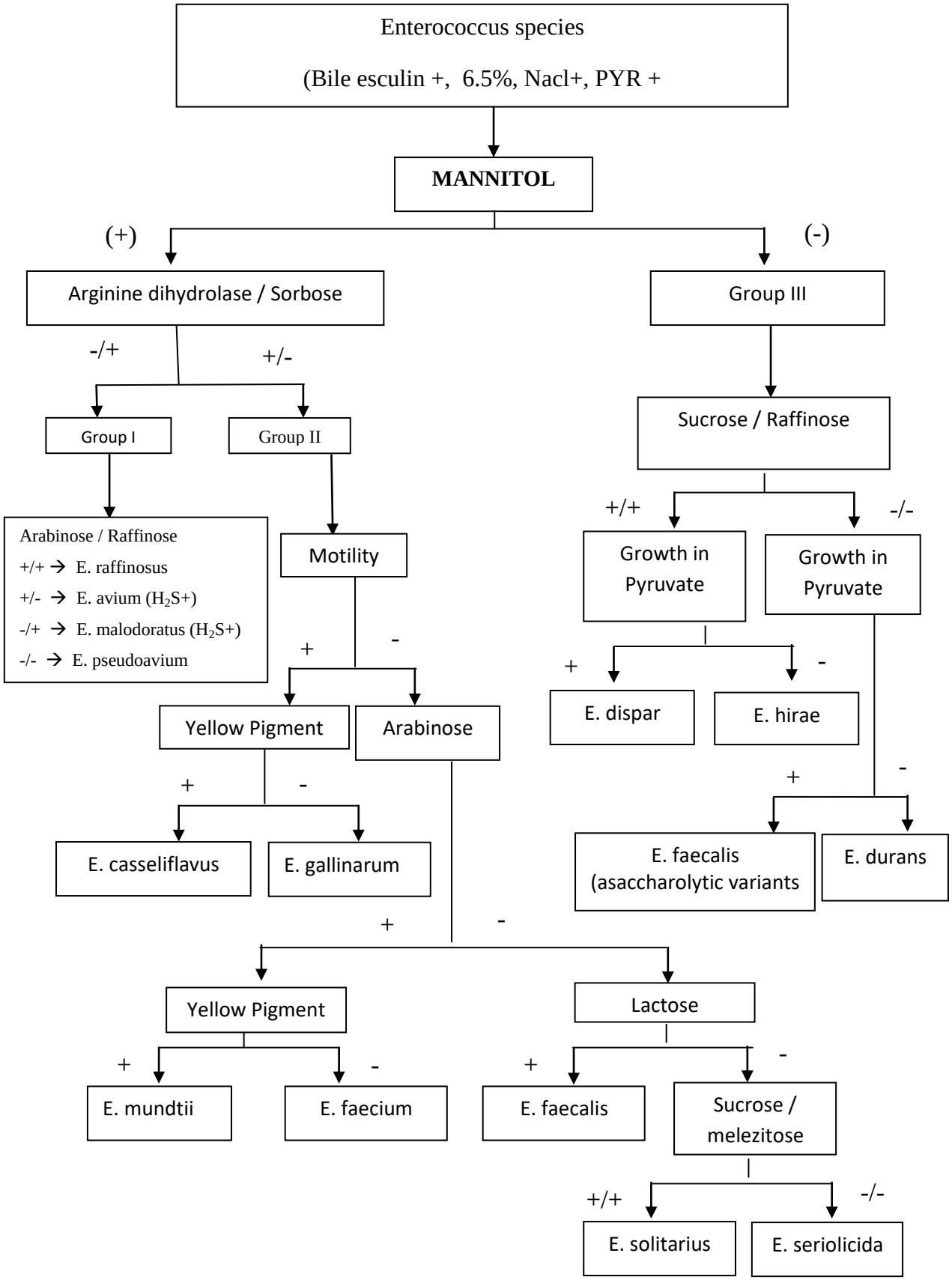
9. Cell wall carbohydrate and capsular polysaccharides

10. Gls24: It is a general stress protein that has important factor in virulence of E.faecalis in both mouse peritonitis and rat endocarditis models[96,97]

Identification of Enterococcal species :

Facklam and Collins[(25)] proposed physiological five groups to classify enterococcal species based on hydrolysis of arginine and acid production from mannitol and sorbose. Further speciation is based on acid production from sugars like arabinose, sorbitol, raffinose, sucrose, pyruvate, trehalose and motility and pigment production.

FACKLAM AND COLLIN FLOW CHART FOR IDENTIFICATION OF ENTEROCOCCUS SPECIES



Clinical presentations of Enterococcal diseases

Endocarditis and Bacteremia:

Endocarditis and bacteremia are the usual presentations of enterococcal disease. Enterococcus species are the third most common cause of nosocomial bacteremia. Sources of the bacteremia are usually the genitourinary and gastrointestinal tracts. Intravascular or urinary catheters associated infection by enterococcus are mainly responsible for nosocomial bacteremia. Intra abdominal, pelvic, biliary tract, wounds (includes burns patients) and bones infection by enterococcus have also been documented as sources of the bacteremia. E.faecium bacteremia have poor prognosis than E.faecalis because E.faecium are more resistant to antibiotics and are very difficult to treat.[(49)] Enterococci can affect both native and prosthetic valves and cause both community and nosocomial associated endocarditis. E.faecalis is being recovered more frequently than E. faecium or other Enterococcal species. Most patients are elderly patient with comorbidities. Procedures predisposing to Enterococcal endocarditis include cystoscopy, caesarean section, prostatectomy, transrectal prostatic biopsy, extracorporeal shock wave lithotripsy, colonoscopy, fiberoptic sigmoidoscopy transjugular intrahepatic portosystemic shunt (TIPS) and liver biopsy. Mortality ranges from 11% to 35% mainly due to heart failure or embolization[(56)].

Urinary tract infections :

Enterococcal UTIs are well recognised infection in hospitals and usually associated with indwelling catheters, instrumentation and abnormalities of the genitourinary tract. Enterococci are the third most common organism causing catheter associated UTI. Removal of the catheter may suffice to eradicate the presence of the organisms. Enterococci can also cause complicated UTIs with the development of pyelonephritis and perinephric abscesses and can lead to bacteremia.

Meningitis :

Enterococci are uncommon causes of meningitis accounting for about 0.3%-4% of meningitis. The most common species isolated is E.faecalis. Other species that can also cause meningitis. Presentation of meningitis may be Spontaneous and postoperative. The mortality rate approaches 20% and residual neurological sequelae may be seen. [(56)]

Intra-abdominal and pelvic infections :

Enterococci are commensals of the gastrointestinal and genitourinary tracts and commonly isolated from abdominal and pelvic infections, usually with Gram negative and anaerobic organisms. Enterococci are capable of causing spontaneous peritonitis and empyema in cirrhotic and chronic kidney failure patients. Enterococci may also cause peritonitis associated with chronic ambulatory peritoneal dialysis.

Neonatal infections :

Enterococci are part of the normal adult vaginal flora and can be acquired by neonates during delivery. The organisms have been associated with 6% of late onset sepsis, 9% of surgical site infections, 5% of pneumonia, 10% of bacteremia and 17% of UTIs in neonatal units. Enterococcal infections are usually hospital associated. Affected patients usually have a prolonged hospital stay, low birth weight, prior antibiotic therapy and several invasive procedures.

Skin, soft tissue and other infections :

Enterococci have been associated with skin, soft tissue and wound infection.They have also been isolated from bone in osteomyelitis.[56]

ANTIMICROBIAL RESISTANCE OF ENTEROCOCCI

Important feature of enterococci are that they are intrinsically resistant to various group of antibiotics like cephalosporins , clindamicin ,co-trimoxazole and low level resistant to aminoglycosides[12,13]. Due to this, it is very difficult to treat and eradicate enterococcal infection. Low-level resistance to all aminoglycosides is due to the decreased drug uptake in to the bacterial wall which is associated with the proteins involved in electron transport[38]. Enterococci has the ability to transfer the resistant genes to other bacteria.

Emergence of vancomycin resistant Enterococci in addition to the increasing incidence of high level aminoglycoside resistance presents a serious

challenge for clinicians treating the patients with infections due to Enterococci . It is well noted that resistance to glycopeptide antibiotics has been transferred between Enterococcus species and from Enterococci to other gram-positive bacteria including Staphylococci, Streptococci and Listeria by means of exchange of resistance genes by conjugation.

Optimal antimicrobial treatment for serious enterococcal infections requires combinations of a cell wall–active agent, such as a penicillin ,Ampicillin or a glycopeptide, with an aminoglycoside results in synergistic action which results in bactericidal activity against this organism. The recommended aminoglycosides for treating serious enterococcal infections are Gentamicin and Streptomycin. (**CLSI GUIDELINES 2017 AND Mandell Douglas, and Bennett"s principles and practice of Infectious Diseases)** Enterococci have acquired aminoglycoside resistance genes that mediate production of aminoglycoside-modifying enzymes, which in turn eliminate this synergistic bactericidal effect. Detection of these aminoglycoside-modifying enzymes has resulted in the traditional approach for predicting bactericidal synergism in enterococci.

High level aminoglycoside resistance :

All enterococci have low level intrinsic resistance to Aminoglycosides, with minimal inhibitory concentrations (MICs) ranging from 4 ug /mL to as high as 256 ug/mL. The MIC of gentamicin, the most commonly used aminoglycoside against enterococci, typically ranges from 6 to 48 mg/mL. High level Gentamicin resistance (HLGR) is defined by

the growth of organism above concentrations of 500µg/ml (MIC>500µg/ml) of Gentamicin.[12,13,49]

Enterococci is thought to produce their low-level resistance to all aminoglycosides by limiting drug uptake and it is associated with the proteins involved in electron transport. The addition of an agent that interferes with cell wall synthesis, such as ampicillin (or vancomycin), markedly increases uptake of the aminoglycoside, greatly enhance the killing of the Enterococcus . Intrinsic resistance in E. faecalis is attributed to an inability of the aminoglycoside to enter the cell as demonstrated in experiments by Moellering[70] and colleagues in the early 1970s. When enterococci were exposed to radiolabeled aminoglycoside with or without penicillin, higher intracellular aminoglycoside concentrations were achieved in the presence of the cell wall synthesis inhibitor. The combination of cell wall active antibiotics and aminoglycosides resulted in bactericidal activity (bactericidal synergism).

Enterococci have acquired aminoglycoside resistance genes that code various aminoglycoside-modifying enzymes, which result in very high resistance to aminoglycosides.

Enterococci are intrinsically resistant to aminoglycosides which do not cross the cell wall efficiently.The addition of cell wall acting antibiotic however sufficiently disorganizes the cell wall and then aminoglycosides gain access to the ribosomal target, thereby causing synergism[38].

But when Enterococci have acquired high resistance to aminoglycosides due to the presence of resistant determining genes that mediate production of

aminoglycoside modifying enzymes which eliminates synergistic bacteriacidal effect. Serious Enterococcal infections like endocarditis, meningitis and sepsis are usually treated with combination of two antibiotics that is one acts on cell wall synthesis like beta lactam or glycopeptides (pencillin, ampicillin or vancomycin) and an aminoglycoside which inhibits bacterial protein synthesis(i.e gentamycin or streptomycin).[11,12,13,92,49,88), These agents act synergistically to enhance killing of the bacteria, since increased uptake of aminoglycoside in to the cell occur after cell wall damage by the beta lactam agent or glycopeptide antibiotics.

All Enterococci have low level resistance to gentamicin (minimum inhibitory concentration 6ug to 48ug/ml) due to decreased uptake in to cell which invalidates use of the disc test with usual concentrations (10 ug disc)of gentamicin. High level gentamicin resistance (minimum inhibitory concentration usually above 500 ug /ml) testing with 120 ug disc is the correct method when an Enterococcal strain is tested for antibiotic susceptibility[12]. If the Enterococci has high level resistant to gentamicin, there is no synergism between cell wall acting antibiotic and gentamicin, then the combination therapy with the cell wall active agents will be ineffective. Therefore, it is important to detect the presence of high level gentamicin resistance in order to predict aminoglycoside synergy with cell wall acting antibiotics. Strains that show high level aminoglycoside resistance to gentamicin posses one or more aminoglycoside modifying enzymes. There are three types of aminoglycoside modifying enzymes.

They are

1. N-Acetyltransferases (AAC) – catalyzes acetyl CoA-dependent acetylation of an amino group
2. O-Adenyltransferases (ANT) – catalyzes ATP-dependent adenylation of hydroxyl group
3. O-Phosphotransferases (APH) – catalyzes ATP-dependent phosphorylation of a hydroxyl group[16]

These enzymes may make them resistant to one or more of a variety of other aminoglycosides . The genes coding for these enzymes are situated in the plasmid or transposon. In case of High level Gentamicin resistance in Enterococci,the enzymes responsible for high level resistance are N-Acetyltransferases (AAC) and O-Phosphotransferases (APH) .

Aminoglycoside[39]

Resistance gene	GM	Tobra	AK	Kana	Netil	Dibek	Strep	Arbek
aac(6')-Ie-aph(2")-Ia	R	R	R	R	R	R	S	S
aph(2")-Ib	R	R	S	R	R	R	S	S
aph(2")-Ic	R	R	S	R	S	S	S	S
aph(2")-Id	R	R	S	R	R	R	S	S
aph(3)-IIIa	S	S	R	R	S	S	S	S
aac(6)-Ii	S	R	S	R	R	NT	S	NT
ant(3)-Ia	S	S	S	S	S	S	R	S
ant(4)-Ia	S	R	R	R	S	NT	S	S
ant(6)-Ia	S	S	S	S	S	S	R	S

(NOTE. NT, not tested; R, resistant to synergism; S, susceptible)

The four genes responsible for high level gentamicin resistant are[39]

1. ***aac(6')-Ie-aph(2")-Ia,***
2. ***aph(2")-Ib,***
3. ***aph(2")-Ic,***
4. ***aph(2")-Id***

As stated in other reviews published in the number of literatures, enzymatic modification is the most important mechanism of aminoglycoside resistance in bacteria.

The presence of different aminoglycoside modyfing enzymes each one capable to introduce a change in the Gentamicin molecule. The capability of a bacterial isolate to both phosphorylate and acetylate aminoglycoside agents is usually justified by the presence of two different kind of enzymes, one belonging to the APH (phosphotransferases) group, and another belonging to the AAC (acetyltransferase-) group. The bifunctional enzyme AAC(6')-APH(2") (6-9), is peculiar because it posses both 6'-N-aminoglycoside acetyltransferase and 2"-O-aminoglycoside phosphotransferase activities which is present in the same polypeptide. This enzyme is exclusive of Gram-positive bacteria and is responsible for high level resistance to aminoglycosides in the genera Staphylococcus and Enterococcus.

The first reports on enzymatic resistance of Gram-positive bacteria to aminoglycosides was phosphorylation of kanamycin. In 1970s in France, and

later in other countries, strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* presented with a new pattern of aminoglycoside resistance. They were classified into two groups based on their resistance patterns: one group was resistant to tobramycin and kanamycin , but susceptible to gentamicin, and the other group was resistant to these three antibiotics. A 4'-O-nucleotidyltransferase could explain the first resistance pattern and whereas two activities (phosphotransferase and acetyltransferase) explained the second type of resistant. The phosphotransferase activity modifies the gentamicin components and the acetyltransferase inactivates other aminoglycoside antibiotics. Biochemical and genetic profile analysis demonstrated that both activities are present in the polypeptide, that are coded by a single gene. The N-terminal region of the polypeptide chain carries the acetyltransferase activity, and C-terminal region carries phosphotransferase activity. The acetyltransferase acetylates the 6' amino group of the aminoglycosides , so it classified as a 6'-N-aminoglycoside acetyltransferase (AAC(6')). The phosphotransferase activity phosphorylates the 2" hydroxyl group of the aminoglycoside hence classified as 2"-O-aminoglycoside phosphotransferase APH(2"). Current data suggest that *aac(6)-Ie-aph(2)* resistance gene results from fusion of two individual resistance determinants.

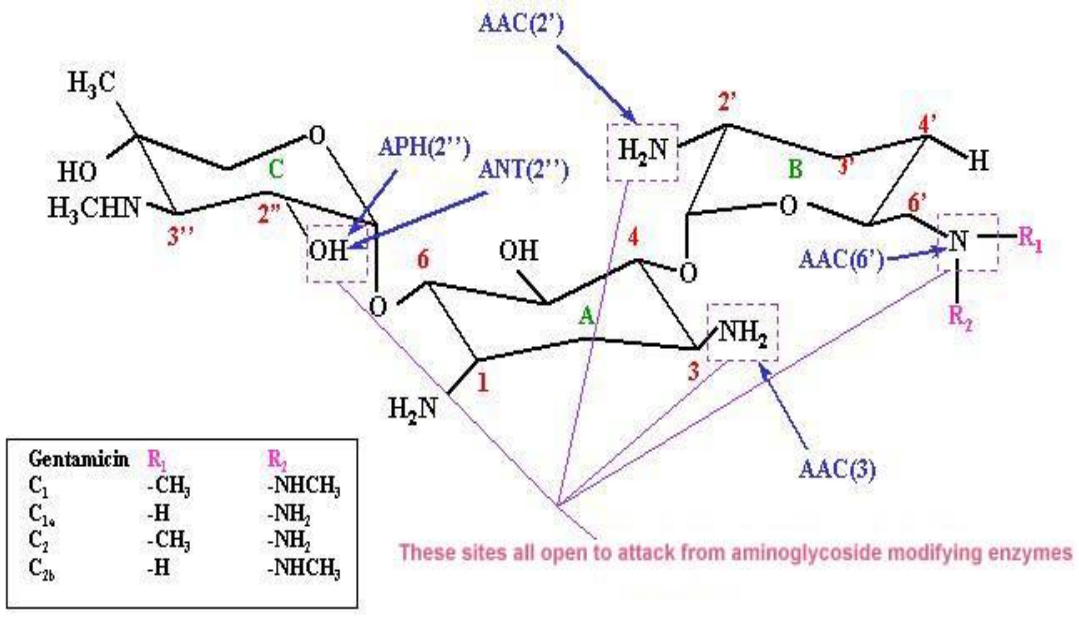
Enterococci that possess ***aac(6')-Ie-aph(2")-Ia*** are resistant to virtually all of the clinically available aminoglycosides, including gentamicin, tobramycin,amikacin, kanamycin, and netilmicin, but sensitive to streptomycin and arbekacin.[39]

Enterococci that possess ***aph(2")-Ib*** are resistant to gentamicin, tobramycin, kanamycin, and netilmicin, but sensitive to amikacin streptomycin and arbekacin. [39]

Enterococci that possess ***aph(2")-Ic*** are resistant to the following aminoglycosides, including gentamicin, tobramycin, kanamycin and sensitive to other aminoglycosides**.** [39]

Enterococci that possess ***aph(2")-Id*** are resistant to the following aminoglycosides, including gentamicin, tobramycin, kanamycin, and netilmicin, but not amikacin streptomycin and arbekacin. [39]

Gentamicin



Gentamicin	R_1	R_2
C_1	$-CH_3$	$-NHCH_3$
C_{1a}	$-H$	$-NH_2$
C_2	$-CH_3$	$-NH_2$
C_{2b}	$-H$	$-NHCH_3$

Modified from Antimicrob Agents Chemother 1999;43:727-37

Among the four gene that are responsible for high level gentamycin resistance in Enterococci , prevalence of aac(6')-Ie-aph(2")-Ia gene is 79%, aph (2")-Id 14%, aph(2")-Ib 5%, aph(2")-Ic 2% **(Joseph W.Chow et al 2000 clinical infectious diseases society of America).**

In another study at Mongolia china, prevalence of aac(6')-Ie-aph(2")-Ia gene is 89.3%, aph(2")-Id 10.7%, aph(2")-Ib not detected and aph(2")-Ic 7.1%.

In other study at Chennai prevalence of HLGR aac(6')-Ie-aph(2")-Ia gene is 38.7%. The other genes aph (2")-Id , aph (2")-Ib and aph(2")-Ic are not detected among isolated Enterococcal species.

Beta lactam antibiotics are not readily bactericidal for Enterococci hence the addition of an aminoglycoside achieves synergistic and bactericidal effect. Among the aminoglycosides, Gentamicin and Streptomycin are recommended for achieving this synergistic effect in clinical practice[49,56].The emergence of HLR to both aminoglycosides was reported in 1983 and has increased since then in both E. faecalis and E. faecium. High level Gentamicin resistance (HLGR) is defined by the growth of organism above concentrations of 500µg/ml (MIC>500µg/ml) of Gentamicin and High level Streptomycin resistance (HLSR) is defined by the growth of organism above concentrations of 2000µg/ml (MIC>2000µg/ml) of Streptomycin on brainheart infusion agar or 1000µg/ml (MIC>1000µg/ml) of Streptomycin on brainheart infusion broth.[11,13,49,56]

High level resistance to streptomycin can be due to mutations in the 30S ribosomal subunit and to the presence of a streptomycin 6' adenyl transferase.[27,28] Evaluation of HLGR in Enterococci has been the standard of care for treatment in endovascular (or) serious infection. **(Mandell Douglas &Benetts**).

Beta lactam resistance:

Initially beta lactam antibiotics were the choice of antibiotics used in the treatment of Enterococcal infections. Relative resistance to beta lactam drugs with minimal inhibitory concentration (MIC) of penicillin 10 to 100 times or more than of Streptococci is documented in Enterococci. Penicillins and carbapenems resistance usually found in clinical isolates of E.faecium and rare in E. faecalis. The mechanism of resistance in E.faecium involves mutations or overexpression of the PBP5 gene which decreases the affinity of its product to ampicillin. Beta lactam resistance in E. faecalis is also mediated by production of a beta lactamase enzyme.[56]

Eventhough Enterococci are normal flora in many sites in human it causes fatal infection among hospitalized patients. But only limited number of antibiotics are available to treat this infection .The best choice is the combination of cell wall acting agents and aminoglycosides (gentamicin or streotomycin).

Eventhough Enterococci is normal flora of many organ (GIT,ORAL,GENITAL etc) of human,it causes fatal infection like

endocarditis, meningitis,septicemia and treating the infection, only limited antibiotics are available. The best treatment for this fatal infection are the combination of cell wall acting agents & aminoglycosides. With this scenario the purpose of these study is to isolate the Enterococci in various clinical specimen,speciation of isolated Enterococci, antibiotic susceptibility testing according to the CLSI guidelines with particular reference to use high level gentamycin disc (120 ug), and prevalence of high level gentamicin resistance among the isolated Enterococci and detecting the frequency of the common gene responsible for high level gentamicin resistance by molecular technique. Important clinical aspect of ***aac(6')-Ie-aph(2")-Ia*** gene in addition to gentamicin it confers resistance to other commonly used aminoglycosides like amikacin, tobramycin, kanamycin, netilmycin, and dibekacin except streptomycin.[39]

AIMS AND OBJECTIVES

i) To isolate and speciate Enterococcus from various samples collected from Govt rajaji hospital,Madurai by standard micobiological culture method and confirmation by biochemical tests.

ii) To detect the pattern of antibiotic susceptibility among the isolated enterococcal species.

iii) To find out prevalence of High level gentamicin resistance among the isolated enterococcal species by phenotypic screening methods.(Disc diffusion method (gentamicin 120 ug), E –test and agar dilution)

iv) To find out antibiotic susceptibility pattern among the isolated high level gentamicin resistant Enterococci

v) Detecting the genes responsible for high level gentamicin resistance by molecular technique. (polymerase chain reaction) among the isolated High level gentamicin resistance(HLGR) Enterococci.

REVIEW OF LITERATURE

In 10th edition of the *Manual of Clinical Microbiology* by Murray[(59)]

Enterococcus is closely related to the "streptococci of fecal origin" or "enterococci". For a long time, Enterococcus were considered within the genus *Streptococcus,* After the introduction of molecular methods however, the enterococci have undergone considerable variation in taxonomy , splitting of the genus *Streptococcus*, and the creation of *Enterococcus* as a separate genus, in 1984 . *Streptococcus faecalis* and *Streptococcus faecium* were the first species that was transferred in to the new genus as *Enterococcus faecalis* and *Enterococcus faecium,* respectively. Subsequently, other earlier streptococcal species and subspecies were transferred in to the genus of *Enterococcus* . Since then, several new Enterococcus species have been described and included in the genus *Enterococcus* .

Phylogenetic analysis based on the comparison of the 16SrRNA gene sequences showed that members of the genus *Enterococcus* are closely related to the genera *Vagococcus, Tetragenococcus,* and *Carnobacterium* than to *Streptococcus* and *Lactococcus,* genera to which they are phenotypically associated .

The genus *Enterococcus* are gram-positive, catalase-negative cocci that occur singly or are in pairs or as short chains. Enterococcus cells are sometimes coccobacillary when smear are prepared from growth on solid medium but tend to be ovoid or chains when grown in liquid media. Growth on blood agar

media after 24 h incubation colonies are usually 1 mm to 2 mm in diameter .About 1/3rd of cultures of *E. faecalis* may be beta-hemolytic on agar containing rabbit, horse, or human blood and nonhemolytic on agar containing sheep blood. Sometimes cultures of *E. faecalis* and *Enterococcus durans* may be beta-hemolytic regardless of the type of blood used. Other species are usually alfa-hemolytic or nonhemolytic.

Enterococci are facultative anaerobes with a homofermentative metabolism and produces l-lactic acid as the end product of glucose fermentation. They can ferment a wide range of carbohydrates to lactic acid, the enterococci are referred as lactic acid bacteria. Gas is not produced. They are able to grow at temperatures ranging from 10 to 45°C, and grow in broth containing 6.5% NaCl. Enterococci can hydrolyze esculin in the presence of bile salts (40%). They can hydrolyze leucine-b-naphthylamide by producing leucine aminopeptidase enzyme (LAP). Most enterococci, apart from *Enterococcus cecorum, Enterococcus columbae, Enterococcus pallens, Enterococcus saccharolyticus,* hydrolyze l-pyrrolidonyl-b-naphthylamide by producing pyrrolidonyl arylamidase enzymes (PYR) .

Antimicrobial resistance may be either intrinsic or acquired. Intrinsic resistance is due to the inherent or natural chromosomally encoded character that is present in all or most of the Enterococci. Specific mechanism of intrinsic resistance to some of the antimicrobial agents are typically associated with a particular Enterococcal species . In contrast, acquired resistance is more variable, resulting from mutations in the existing DNA or acquisition of new

genetic elements carried in plasmids or transposons. Intrinsic resistance in Enterococcal species involve several antimicrobial agents, particularly the aminoglycosides and the beta-lactams. Because of the intrinsic resistance to various antimicrobial agents against enterococci the recommended therapy for serious infections (i.e., endocarditis, meningitis, and other systemic infections), and in immunocompromised patients, includes a combination of drugs that acts on cell wall such as a beta-lactam (usually penicillin or ampicillin) or vancomycin, and an aminoglycoside (usually gentamicin or streptomycin). This type of combination can overcome the intrinsic resistance exhibited by the Enterococcal species, and a synergistic bactericidal effect is achieved. This is due to intracellular entry of the aminoglycoside is facilitated by the cell wall-active drugs.

In Jawetz, Melnick & Adelberg's Medical Microbiology the combinations of a cell wall–active agents like penicillin or ampicillin or vancomycin plus an aminoglycoside (streptomycin or gentamicin) is essential for severe Enterococcal infections, such as meningitis and endocarditis. Although Enterococci having intrinsic low-level resistance to aminoglycosides (MICs<500 μg/mL), they still effective because of the synergistic action when combined with a cell wall–active agents and an aminoglycoside.

Some Enterococci have high-level resistance to aminoglycosides (MICs >500 μg/mL) hence not susceptible to the synergism because of the expression of aminoglycoside-modifying enzymes,which inactivates Aminoglycosides even they entered into the cell.

The genes that code for these enzymes are usually located on plasmids or transposons. Resistance to the gentamicin predicts resistance to other aminoglycosides except streptomycin. (Susceptibility to the gentamicin does not predict susceptibility to other aminoglycosides.) Resistance to the streptomycin does not predict resistance to the other aminoglycosides.So, only the streptomycin or gentamicin is likely to have synergistic activity with a cell wall–active agents against Enterococci.

Enterococci that causes severe infections should have susceptibility testing for high-level aminoglycoside resistance(HLAR) and that MICs usually above 500 μg/mLfor gentamicin and above 1000 μg/mL for streptomycin in broth media in order to predict therapeutic efficacy.

In CLSI Clinical and Laboratory Standards Institute[12,13] for *Enterococcus* spp., aminoglycosides (except for high-level resistance testing), cephalosporins, trimethoprimsulfamethoxazole and clindamycin,may appear active *in vitro,* but they are not effective clinically, and isolates should not be reported as susceptible. Synergy between ampicillin, penicillin, or vancomycin and an aminoglycoside can be predicted for enterococci by testing the organism against high-level aminoglycoside (gentamicin and streptomycin)

The results of ampicillin susceptibility tests should be used to predict the activity of amoxicillin. Ampicillin susceptibility may be used to predict susceptibility to amoxicillin-clavulanate, ampicillin-sulbactam, piperacillin,and piperacillin-tazobactam among non beta-lactamase producing enterococci.

Ampicillin susceptibility can be used to predict imipenem susceptibility, providing the species is confirmed to be *E. faecalis.*

Enterococci susceptible to penicillin are predictably suscept ble to ampicillin, amoxicillin, ampicillin-sulbactam,amoxicillin-clavulanate, piperacillin, and piperacillin tazobactam for non beta-lactamase-producing Enterococci. However, Enterococci susceptible to ampicillin cannot be assumed to be susceptible to penicillin. If penicillin results are needed, testing of penicillin is required.

Combination therapy with ampicillin, penicillin, or vancomycin (for susceptible strains), plus an aminoglycoside is usually indicated for treatment of serious enterococcal infection unless high-level resistance (HLR) to both gentamicin (HLGR) and streptomycin (HLSR) is documented.The effect of such combinations are predicted in synergistic killing of the *Enterococcus.* Because other aminoglycosides antimicrobial activities against enterococci are not superior to gentamicin and streptomycin, they need not be tested. High level gentamycin resistance (MIC usually above 500 ug /ml) testing with high content disc that is 120 ug disc is the correct method when an Enterococcal strain is tested for antibiotic susceptibility testing by Kirby – Bauer disc diffusion method . In this method 6 mm is considered as Resistant, 7–9 mm considered as inconclusive and ≥ 10 mm is considered as Susceptible. Minimum inhibitory concentration (MIC) correlates with this disc diffusion method are Resistant > 500 μg/mL Susceptible ≤500 μg/mL.

Cong-Ran Li, Xin-Yi Yang, Ren-Hui Lou, Wei-Xin Zhang et al[19] the aminoglycoside antibiotics have been known for more than 60 years, since the discovery of streptomycin in 1944 .Aminoglycosides are polycationic broad-spectrum bactericidal antibiotics that are used to treat a number of bacterial infections. The primary target of aminoglycosides is the 30S small subunit of ribosomes . The use of aminoglycosides is restricted by the emergence of aminoglycoside-modifying enzymes (AMEs).

The AMEs are composed of three families aminoglycoside nucleotidyltransferase, aminoglycoside acetyltransferase (AAC), and aminoglycoside phosphotransferase(APH). Clinically, the bifunctional modifying enzyme AAC(6_)- APH(2_) is the most important AME in *Staphylococcus aureus* and *Enterococcus faecalis.* This enzyme is unique as it has two activities of two classes of AMEs, an N-terminal [AAC(6_)-Ie] and a C-terminal APH [(APH(2_)-Ia] and was believed to have resulted from fusion of the *aac* and *aph* genes . The two domains do not functionally interact but have intimate structural linkages that are important for its activity. The *aac(6_)-aph(2_)* genes can be present in both the R plasmids and chromosomes of aminoglycoside-resistant isolates, with locations on transposons, like Tn*4001* , Tn*4031* and Tn*5381.*

According to Esther Culebras , and Jose L.Martinez et al[24] the expression of the bifunctional aminoglycoside modyfing enzyme 6'-N-aminoglycoside acetyltransferase-2" -O-aminoglycoside phosphotransferase is the most important mechanism of high-level aminoglycoside resistance

(HLAR) in staphylococcus and *Enterococcus*. The enzyme is unique because it carries two different aminoglysoside-modifying activities located in different regions of the molecule. The gene *aac(6')-aph(2")* which encodes the synthesis of the enzyme is present in Tn*4100*-like transposons which are inserted both in R plasmids and the chromosomes of aminoglycoside-resistant isolates. The genetic structure of *aac(6')-aph(2")*-containing isolates indicates that their origin is not clonal, but plasmid conjugation together with multiple insertion events are in the basis of the rapid spread of aminoglycoside resistance among Gram-positive bacteria.

According to **Monolekha Bhattacharya,a Marta Toth,a Clyde A. Smith,b Sergei B. Vakulenkoa et al** the main mechanism of aminoglycoside resistance in Gram-positive and Gram-negative bacteria is due to the production of aminoglycoside modifying enzymes, aminoglycoside phosphotransferases (APHs), aminoglycoside acetyltransferases, (AAC) and aminoglycoside nucleotidyltransferases (ANT) These enzymes are capable to modify hydroxyl or amino groups on the drug which are important for its binding to the target site, the 30S subunit of the bacterial ribosome. APHs are capable of phosphorylating the 4, 6, 9, 3, 2, 3 and 7 position hydroxyl groups of various aminoglycosides and ATP is the major source of phosphate for these enzymes.

Studies showed that 2_-phosphotransferases [APH(2)], enzymes are widely distributed in staphylococcal and enterococcal isolates .They demonstrated that they can utilize GTP as a cosubstrate . Based on these

studies a new nomenclature for the APH(2_) enzymes was proposed, that reclassified the APH(2_)-Ia, -Ib, -Ic, and -Id enzymes as APH(2_)-Ia,-IIa, -IIIa, and –Iva enzymes, respectively. Both the APH(2_)-Ia and APH(2_)-IIIa utilize GTP only while APH(2_)-IIa and APH(2_)-IVa can utilize both ATP and GTP as a cofactor.

According to **Raffaele Zarrilli1, Marie-Francoise Tripodi** [(101)] High-level gentamicin resistance (MIC > 500 µg/ml) is mainly mediated by the aac(6)-Ie-aph(2)-Ia gene, that encodes the bifunctional enzyme AAC(6)-APH(2). In recent years, three more new aminoglycoside resistance genes [aph(2")-Ib, aph(2")-Ic and aph(2")-Id] were identified which mediate resistance to gentamicin have been detected in enterococci species. Other genes that confer resistance to various aminoglycosides but not to gentamicin, aph(3)-IIIa and ant(4)-Ia, that encode the aminoglycoside modyfing enzymes (AME) have also been identified. The types and distribution of resistance determining genes in enterococci species vary in different geographical area.

According to Joseph W. Chow[39] various aminoglycosides and their resistant gene pattern in enterococci as follows

	Aminoglycoside							
Resistance gene	GM	Tobra	AK	Kana	Netil	Dibe	Strep	Arbek
aac(6')-Ie-aph(2'')-Ia	R	R	R	R	R	R	S	S
aph(2'')-Ib	R	R	S	R	R	R	S	S
aph(2'')-Ic	R	R	S	R	S	S	S	S
aph(2'')-Id	R	R	S	R	R	R	S	S
aph(3)-IIIa	S	S	R	R	S	S	S	S
aac(6)-Ii	S	R	S	R	R	NT	S	NT
ant(3)-Ia	S	S	S	S	S	S	R	S
ant(4)-Ia	S	R	R	R	S	NT	S	S
ant(6)-Ia	S	S	S	S	S	S	R	S

(NOTE. NT, not tested; R, resistant to synergism; S, susceptible)

The gene responsible for high level gentamicin resistance are

1. ***aac(6')-Ie-aph(2")-Ia, 2. aph(2")-Ib, 3. aph(2")-Ic, 4. aph(2")-Id***

A study conducted by **Seema sood, Meenakshi Malhotra et al** [91]showed 42.9% of E.faecium and 40% of E.faecalis were the predominant species isolated in clinical specimen. In blood culture, E.faecium was the commonest isolate. In pus and urine samples ,E.faecalis was predominant .Other species such as E.avium, E.mundtii, E.durans, E.dispar, E.raffinosus and E.gallinarum were also isolated .In a study at New Delhi , E.faecium (66%)

was the most common species in blood samples .The next common species was E.faecalis(20%). A study at Chandigarh reported E.faecalis (55%) followed by E.casseliflavus (24%) and E.faecium (12%) from urinary tract infection.

YA marothi, H Agnihotri et al (2005)[(102)] described two types of antimicrobial resistance in Enterococci – Intrinsic and acquired resistance. Intrinsic resistance is chromosomally mediated and are species specific .It is present in all members of the species. Intrinsic antimicrobial resistance by enterococci was expressed to Penicillinase susceptible penicillin (low level), Penicillinase resistant penicillins, Cephalosporins, Aminoglycosides (low level), Lincosamides and Clindamycin (low level). Acquired resistance is mainly due to acquisition of new DNA or mutation in DNA. Examples of acquired resistance are resistance to Penicillin by beta lactamases, High Level Aminoglycoside Resistance,Vancomycin, Tetracycline, high level of Clindamycin , Chloramphenicol, and Fluoroquinolone resistance .

In DK Mendiratta, H Kaur et al 2008[(21)] study showed that high level resistance to both Gentamicin and Streptomycin was observed in Enterococcal isolates by means of both high content disc diffusion and agar dilution (Minimum inhibitory concentration) methods. E. faecium isolates (95.5%) showed higher high level aminoglycoside (HLGR) resistance than E. faecalis (37.5%). E.faecium (59.1%) showed higher combined resistance to both the aminoglycosides as compared to E. faecalis (7.8%). In E. faecium

High Level Gentamicin Resistance (HLGR) was higher (22.7%) than High Level Streptomycin Resistance (HLSR) (13.6%).

Baragundi MC, Sonth SB et al (2010)[5] study, out of 120 Enterococcal isolates from clinical sample 47.5% (57) are E. faecium , 44.16%(53) are E. faecalis, 4.16%(5) are E. mundti, 1.66%(2) are E. durans and 0.83%(1) are E. dispar. High level aminoglycoside resistance (HLAR) was seen in greater than 75% isolates for Gentamicin and in greater than 60% isolates for Streptomycin. Totally 47.18% isolates showed HLAR (both to HLGR+HLSR).

Srujana Mohanty et al, **Gupta et al**[93] study also showed such high level aminoglycoside resistance (HLAR). The treatment options are limited when there is loss of the synergism between aminoglycoside and the beta lactum drugs due to expression of aminoglycoside modifying enzymes by the enterococcal species, because the treatment of choice for serious enterococcal infections like endocarditis ,meningitis and bacteremia.

In the text book of **Bailey and Scott" diagnostic microbiology**[8] variety of enterococcal species have been isolated from various clinical speciemen of human infections. *E. faecalis* and *E. faecium* are the species most commonly encountered. Among the two species, *E. faecalis* is the most commonly encountered, and incidence of *E. faecium* infections is on the rise in many of the hospitals. Two additional species, *E. gallinarum* and *E. casseliflavus,* also isolated from the intestinal infections.

In the **Mandell Douglas, and Bennett"s principles and practice of Infectious Diseases**[56] High level gentamicin resistance were reported in 1979. Enterococcal species are intrinsic resistant to cephalosporins, clindamicin ,co-trimoxazole and low level resistant to aminoglycosides. The use of pencillin plus gentamicin therapy in patient with HLGR result in failure. There was steady increase in HLAR during 1980 to 1990 reaching 30%. Prevalence of HLSR was 40%. Rate of HLGR in E.faecalis exceeds 40% in Europe.

According to **Brian L. Hollenbeck and Louis B. Rice et al**[11] Intrinsic low level resistance in E. faecalis is attributed to an inability of the aminoglycoside to enter in to the cell as demonstrated in experiments by Moellering and colleagues in 1970. When enterococci were exposed to radiolabeled aminoglycoside with or without penicillin, higher intracellular aminoglycoside concentrations were reached in the presence of the cell wall acting antibiotics. The combination of cell wall active agents with aminoglycosides resulted in bactericidal activity (bactericidal synergism). These studies support the observations of improved clinical outcomes with aminoglycoside-penicillin combination therapy.

According to **Mitra Khani,Mahdie Fatollahzade,Hamid et al**[61] prevalence of isolates were 33 (24.1%) for *E. faecium* and 63 (46%) for *E. faecalis*. Eighty-nine percent of the isolates were high-level gentamicin resistant (HLGR), and 32.8% of *E. faecium* isolates and 67.2% of *E. faecalis* isolates carried *aac(6')-aph(2")*. The prevalence of *aph(3")-IIIa* among the *E. faecalis* and *E. faecium* isolates was 22.7% and 77.3%, respectively.

According to ***Haiying Niua, Hui Yub, Tangping Hua, Gailin Tiana et al***[35] the definition for high-level gentamicin resistance (HLGR) is MIC >500 µg/ml and high-level streptomycin resistance (HLSR) is MIC >µg2000 g/ml).

The primer used to detect the AME in resistant enterococci are as follows

aac(6')-Ie-aph(2'')-Ia

F-CAGGAATTTATCGAAAATGGTAGAAAAG

R-CACAATCGACTAAAGAGTACCAATC

aph(2'')-Ib

F-CTTGGACGCTGAGATATATGAGCAC

R-GTTTGTAGCAATTCAGAAACACCCT

aph(2'')-Ic

F-CCACAATGATAATGACTCAGTTCCC

R-CCACAGCTTCCGATAGCAAGAG

aph(2'')-Id

F-GTGGTTTTTACAGGAATGCCATC

R-CCCTCTTCATACCAATCCATATAACC

According to ***Haiying Niua, Hui Yub, Tangping Hua, Gailin Tiana et al***[35], the predominant species observed were *E. faecium* 53.8% and *E.faecalis* 28.2%. other species detected are *E. avium* 9.4%,*E. gallinarum* 5.1%, *E. casseliflavus* 2.6%, and *E.durans* 0.9%. In this study the prevalence of aminoglycoside modifying enzymes are 42.7% were High level gentamicin

resistance (HLGR) and 27.4% were High level streptomycin resistance (HLSR) 10.3% of the isolates were both HLGR and HLSR. The highest resistance was observed among *E. faecium*, followed by *E.faecalis* and *E. avium*.

Among the HLGR isolates 89.3% were carried *aac(6)-Ie-aph(2)-Ia*, 7.1% carried *aph(2'')-Ic*, 10.7% carried *aph(2'')-Id* and 25% carried *aph(3) IIIa*. but *aph(2'')-Ib* was not detected .

According to Niharika , Lall and Silpi et al[71] Detection of High Level Aminoglycoside Resistance (HLAR) in *Enterococcus* species can predict the loss of synergy between cell wall active antimicrobial agents and Aminoglycosides. the present study was undertaken to detect the incidence of High level Aminoglycoside Resistant (HLAR).

HLAR in Enterococcus species was detected by disk diffusion test using High level Streptomycin (HLS - 300 μg) disk and High level Gentamicin (HLG - 120 μg) disk and Agar dilution method as per CLSI guidelines.

High Level Aminoglycoside Resistance (HLAR) detected in 60.5% of the Enterococcus strains. Among the HLAR Enterococcus strains, 51.4% were E. faecalis and 48.6% were E. faecium. Only High level Gentamicin resistance (HLGR), only High level Streptomycin resistance (HLSR) and both HLGR and HLSR producing strains of E.faecalis were detected as 38.9%, 11.9% and 49.2% respectively among the HLAR positive E.faecalis . Similarly, among HLAR producing E.faecium strains, 60.7% produced only High level Gentamicin resistance (HLGR), 8.9% produced only High level

Streptomycin resistance (HLSR) and 30.4% produced both HLGR and HLSR.

According to CAROL A. SPIEGEL et al (15) A total of 104 strains from 93 patients were studied; 97 (93.3%) were E. faecalis and 7(6.7%) were Enterococcus faecium and studied for high-level resistance to gentamicin and streptomycin and they found 28.3% and 32.6% of the strains were high level resistant to gentamicin and streptomycin, respectively. No strain was resistant to both drugs.

According to Verma D, Sinha S, Ved Prakash et al 2013(100) In total 49% of Enterococci showed HLAR in their study set up out of which 4% were resistant to gentamicin only, 14% isolates were resistant to streptomycin only and 31% were resistant to both antibiotics.Such isolates may disseminate in health care facilities and therefore the routine screening of all enterococcal isolates with High strength Gentamicin is important.

According to Latika shah et al in 2012(51) E.faecalis (75%) was predominant isolate and E.faecium was about 23%. .In their study they reported high HLAR among isolated enterococci were 53% for gentamicin and 40 % for streptomycin with 8% VRE at their institute .

According to Chandrim Sengupta, Anusha Venkatesan, Sangamitra.V et al 2011- 12(17) study prevalence of enterococcal species from various clinical specimen were noted . The contribution of E. faecalis was 46.9% and E. faecium 33.3% ,E. durans 1.23%, E.avium 18.5%. In this study 29.62% of the enterococci showed HLAR. The HLAR among E. faecium

isolates was 29.62% which was a little lower than E. faecalis 34.21% . It was low in E.avium 12.5%.

According to Gülçin Baldır, Derya Öztürk Engin, Metin Küçükercan et al (30) they studied the prevalence of High level Aminoglycoside Resistance (HLAR) among the vancomycin resistant enterococci (VRE) strains, and found that high-level gentamicin resistance (HLGR) was 83% and high-level streptomycin resistance (HLSR) was 89%, and association of HLSR with HLGR was 78%. But in vancomycin sensitive enterococci (VSE) strains, HLGR was found to be 42% ,and HLSR was 48%. Both HLSR with HLGR was found to be 36%. HLAR in vancomycin resistant enterococci (VRE) strains was found to be higher as compared with vancomycin sensitive enterococci (VSE) strains ($p < 0.005$).

In the study of Verma D, Sinha S, Ved Prakash et al(100) ,they compared the high-level aminoglycoside resistance in the vancomycin resistant enterococci (VRE) strains and the vancomycin sensitive enterococci (VSE) strains.

In their study they screened for HLGR AND HLSR by disc diffusion method using 120 µg gentamicin and 300 µg of streptomycin disc. If disk diffusion result was inconclusive an agar screen test was performed on to Brain heart infusion Agar containing gentamycin 500µg/ml and streptomycin 1000µg/ml. Any growth on the plate indicates that the strain was resistant .. Vancomycin resistance were screened by using Vancomycin screening agar (VSA) containing Vancomycin at concentration of 6 µg/ml. Growth of >1

colony was interpreted as Resistant and confirmation of VRE was done by Agar dilution method.

They found that 49% of Enterococci showed HLAR and 12% were resistant to vancomycin with MIC≥ 32µg/ml. In comparision, High-level aminoglycoside resistance was 1.5 times more in vancomycin resistant enterococci (VRE) isolates than in vancomycin sensitive enterococci (VSE) isolates , this difference was statistically not significant.(p value>.05). High-level aminoglycoside resistance to both streptomycin and gentamicin (HLGR and HLSR) was more common in E. faecium than in E. faecalis strains.

According to the study by Sarika jain ,Aswani kumar et al(87) the prevalence of enterococcal bacteremia in hospitalized and outpatients in their study was 72% and 28%, respectively. Historically, the ratio of infection by E. faecalis to those due to the other Enterococcus species was approximately 10:1 but there has been a progressive decline in recent years. It is true in their study, E. faecium bacteremia was higher in prevalence than E. fecalis (53% and 33%, respectively). In this study, the prevalence of relatively high proportion of E. faecium was consistent with those reported in other Indian studies from various clinical samples (40 to71%). Many studies have also demonstrated that E. faecium is comparatively more resistant than E. faecalis.

In their study they demonstrated high prevalence of HLGR, HLSR, and HLAR (resistance to both gentamicin and streptomycin) among enterococci were 60%, 55% and 54%, respectively and the detection of HLAR in hospitalized patients (92%) was high when compared to community

acquired is also evident that is 8%. HLAR was more frequently observed in E. faecium isolates (71%) than other species.

In the study of Jyothi P, Metri BC, Peerapur BV et al(41) among the enterococcal isolates from the urinary tract infection , 63% were Enterococcus faecalis and 37 %were Enterococcus faecium. 49% of isolates showed a high level resistance to gentamicin and/or streptomycin.In this study, HLAR both HLGR and HLSR were significantly higher ($P < 0.05$) in E. faecium which contribute 56.7% and E. faecalis contributed 44.4%. In their observation that HLGR in enterococcal isolates from urinary tract infection were 14.2% and HLSR in enterococcal isolates from urinary tract infection were 11.4.%.

According to M. Emaneini, B. Khoramian, f. Jabalameli et al(63) the most prevalent species was E. faecalis (70.3%) followed by E. faecium (29.7%) which were isolated from burn wound infections. All isolates were screened for genes encoding resistance to aminoglycoside. The most prevalent aminoglycoside resistance gene found was aac(6')-Ie-aph(2'')-Ia and was, found in 96.2% of the isolates. The ant (4') gene was detected in 62.9% of the isolates.

According to V.S. Randhawa, L. Kapoor, V. Singh & G. Mehta et al(83) the distribution of high level gentamicin and high level streptomycin resistance among the enterococcal isolates were 68% and 43% respectively and the distribution of both HLGR + HLSR were 43%.

According to SR Moaddab and A Rafi et al(62) Out of enterococcus strains that were isolated , 45% were identified as E. faecalis, 43% as E.

faecium 7% , E. avium 3.5% as E. raffinosus 0.5% as E. durans . The percentages of high-level resistance to gentamicin and streptomycin were 16% and 15% for E. faecium in case of E. faecalis 13% and 17%,respectively.

MATERIALS AND METHODS

The present study was conducted in Government Rajaji Hospital, Madurai Medical College. The study period was from Sep 2016 to August 2017. Ethical committee clearance from the institution was obtained and informed written consent was received from the patients before collecting the specimens. A total of 396 clinical samples blood, pus wound swab,and urine were collected from the patients admitted in various wards and OP of Government Rajaji Hospital, Madurai.

Sample size and source :

Under aseptic precaution Urine, blood, pus and wound swab samples were collected from 396 patients attending in various wards of Govt. Rajaji Hospital during the study period(Sep-2016 to Aug 2017)

Inclusion criteria

1. All age groups and both sexs are included
2. The patients attending outpatient department and in the wards with symptoms of Urinary tract infection, Sepsis, Wound infection, Meningitis and lower respiratory tract infection were included in this study.

Exclusion criteria

Sputum and stool sample were excluded in this study.

Study type

Prospective study

Study period

Sep2016 – August 2017

Study population

396 samples were collected from the patients attended at various wards and op in GRH attached to Madurai Medical College. Madurai.

Study centre

Institute of Microbiology, Madurai Medical College. Madurai.

Collection of specimens:

i) Collection of blood samples:

As per the standard guidelines, blood samples were collected by sterile aseptic precaution. After wearing sterile gloves the skin over the venepuncture site was disinfected with 70% alcohol followed by 1% iodine for 1 minute and allowed to dry. Then 5 ml of blood was withdrawn and the blood was inoculated into the 50ml of brain heart infusion broth. (BHI) and the samples were transported to the microbiological laboratory immediately for processing.[57]

ii) Collection of urine samples :

The female patients were instructed to hold the labia apart and the male patients were asked to retract the foreskin of the penis, then washing the genitalia with soap and water. After several ml of urine have been passed, midstream clean catched urine was collected into a sterile, leak proof, wide mouthed ,screw capped container. Then the collected urine samples were transported to the laboratory within 30 minutes of collection. If there was a delay of more than two hours, the specimen were refrigerated at 4°C.[57]

iii) Collection of wound swab samples :

Two sterile cotton swabs were used to collect the samples from the wound sites of the patients. One was used for direct smear examination and the another swab were used for culture. The swabs were transported in sterile test tubes to the microbiologocal laboratory.[57]

iv) Collection of pus samples :

The wound sites were decontaminated with 70% ethyl or isopropyl alcohol, then washed with sterile saline. After drying using a sterile syringe and needle, pus was aspirated and transported in a sterile container to the microbiologocal laboratory.[57]

Processing of Samples :

The collected pus samples were properly labelled and registered with Name, age, sex and IP/OP no. of the patient, date and time of collection and

time of receiving the clinical samples. The clinical data of the patients were also registered. Then the samples were transported to the microbiology laboratory and processed immediately.

Blood samples :

Inoculated Brain heart infusion (BHI) broths were incubated at 37°C for 18-24 hrs. After that, the broths were examined for turbidity and subcultured onto the Nutrient agar plate, MacConkey agar plate and Blood agar plate for the isolation of organisms.

Urine samples :

Wet mount and gram staining were performed from uncentrifuged urine and the urine samples were inoculated on to the Cystine lactose electrolyte deficient medium (CLED) with the calibrated loop. Before inoculation, urine was mixed thoroughly. The calibrated loop was inserted vertically into the urine container. The centre of the CLED plate surface was touched with the loop and the inoculation was spread across the diameter of the entire plate. Without flaming the loop was drawn across the entire plate, and crossing numerous times in order to produce isolated colonies. The plates were then incubated at 37°C for 18-24 hours. A colony count of $>10^5$ CFU/ml was indicative of significanct bacteriuria

Wound swab and pus samples :

Direct Gram stain was done from wound swab and pus samples.Then the samples were inoculated on to the Nutrient agar plate, MacConkey agar plate and Blood agar plate for the isolation of organisms.

Culture identification :

After 24 hrs of incubation at 37°C, plates were examined for the presence of growth and the organisms were identified as follows,

Identification of Enterococcus species:

1. Nutrient agar plate : Tiny, opaque colonies
2. MacConkey agar plate : Tiny, magenta pink colonies
3. Blood Agar plate : alpha / beta / non hemolytic colonies
4. Gram staining : Gram positive cocci in pairs and short chains.
5. Catalase test : Negative
6. Bile esculin test : hydrolyze esculin in the presence of 40% bile - blackening of the medium.
7. 0.04% Tellurite agar produces black coloured colonies.
8. Salt tolerance test : Growth in 6.5% sodium chloride (Nacl) broth.
9. Heat tolerance test : Growth were detected before and after heating at a temperature of 60° C for 30 minutes in a water bath.
10. Mannitol motility medium : Non motile, ferment mannitol by producing acid only.

11. Arginine dihydrolase test – Hydrolyses arginine – produces deep purple colouration after initial colour change to yellow.

12. Carbohydrate (1%) fermentation test – differs in various species.

Species identification of Enterococcus

Enterococcal species were identified by biochemical tests such as acid produced from sugars like arabinose, mannitol, pyruvate, Sucrose,and Sorbose, Raffinose, Sorbitol and NH3 produced from arginine, esculin hydrolysis in the presence of 40% bile, ability to grow in 6.5% Nacl broth, presence of Pyrrolidonyl peptidase enzyme (PYRase) and Leucine aminopeptidase enzymes (LAP), motility and pigment production. Facklam and Collin flow chart for identification of Enterococcus species was followed.

Identification Tests

1. Gram staining :

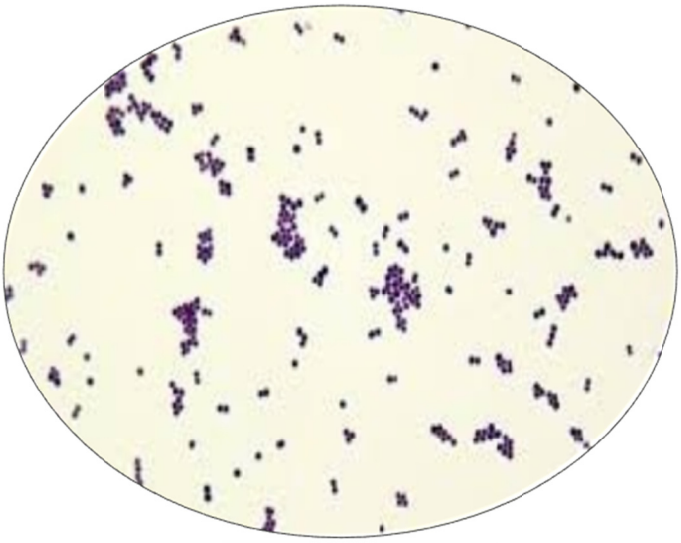
Gram positive elongated cocci arranged in pairs and short chains were identified presumably as Enterococci.

2.Catalase test (Tube method)

Procedure:

2ml of 3% hydrogen peroxide (H_2O_2) was taken in a clean test tube and few identical colonies of the test organism were taken from the nutrient agar

GRAM STAIN MORPHOLOGY



GRAM STAIN MORPHOLOGY

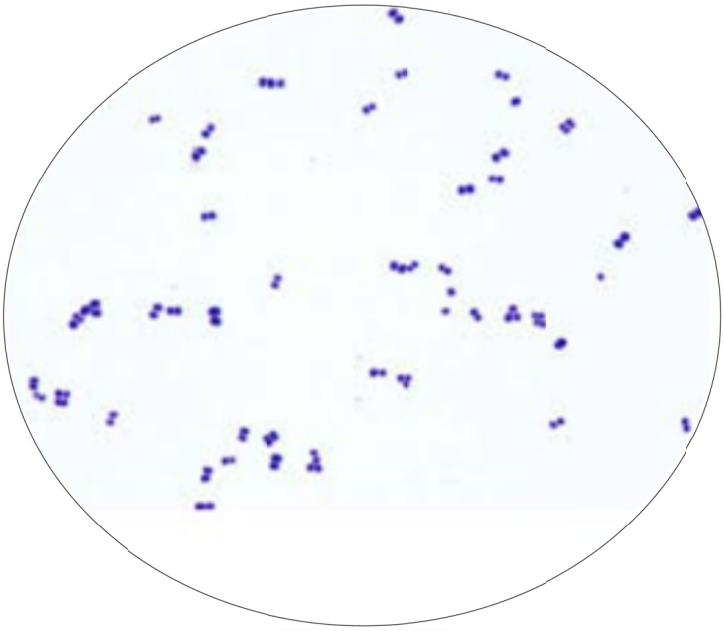


plate (NAP) with a sterile glass rod or wooden stick and introduced into the test tube that contains H_2O_2.[57]

Interpretation :

Catalase producing organisms split hydrogen peroxide into water and oxygen and produce brisk effervescence due to the release of gaseous oxygen. Organisms that were not producing catalase will not produce brisk effervescence. Enterococci were catalase negative.

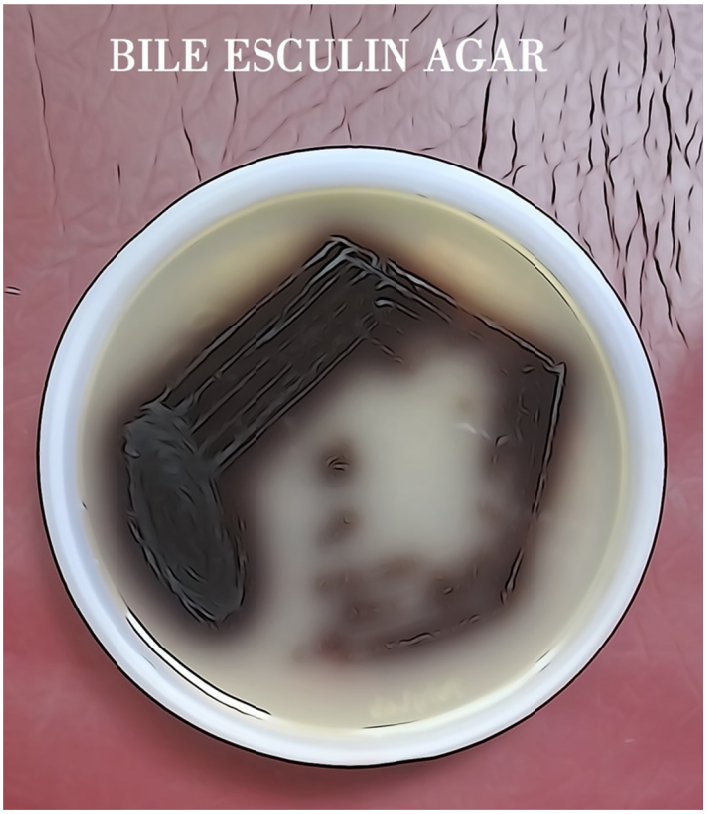
3. Bile Esculin Test :

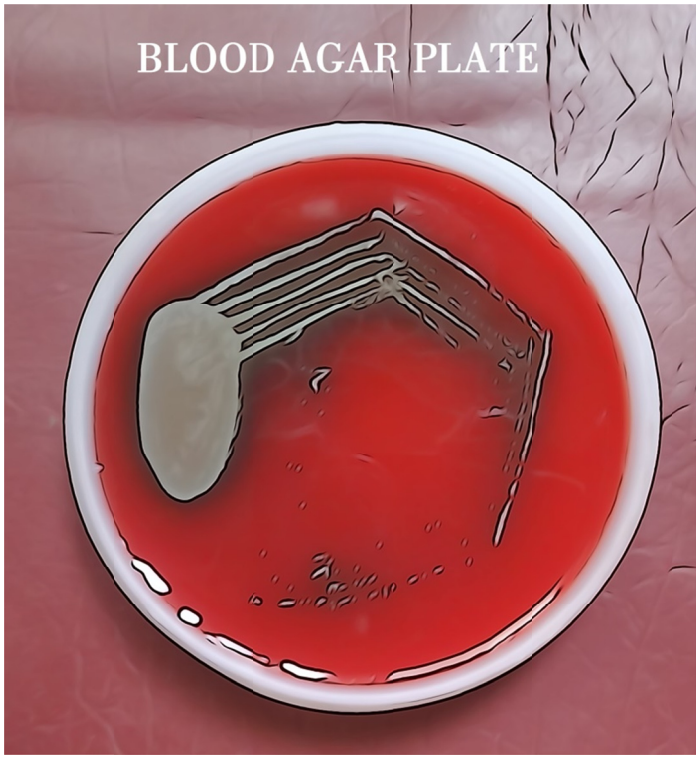
Procedure:

2-3 identical colonies of test organism were taken from the culture plate with a sterile inoculating wire or loop and streaked on the surface of the bile esculin agar plate. The plates were incubated at 35°C for 24-48 hours[57,49]

Interpretation :

Diffuse blackening of the bile esculin medium or production of black coloured haloes around the colonies was considered positive bile esculin test. In the presence of 40% bile, esculin, a glycoside ,which is hydrolysed in to glucose and esculetine.The esculetine reacts with ferric ions present in the medium to form a black diffusible complex. Enterococci are bile esculin positive.


BILE ESCULIN AGAR


BLOOD AGAR PLATE

4. Heat tolerance test :

Procedure :

Test organism from 18-24 hour culture was inoculated into brain heart infusion (BHI) broth and incubated at 60°C for 30 minutes in a water bath. The broth was subcultured on to the blood agar plate (BAP) and MacConkey agar plate before heating and after heating. ATCC strain of E.faecalis 29212 is used as positive control.[57]

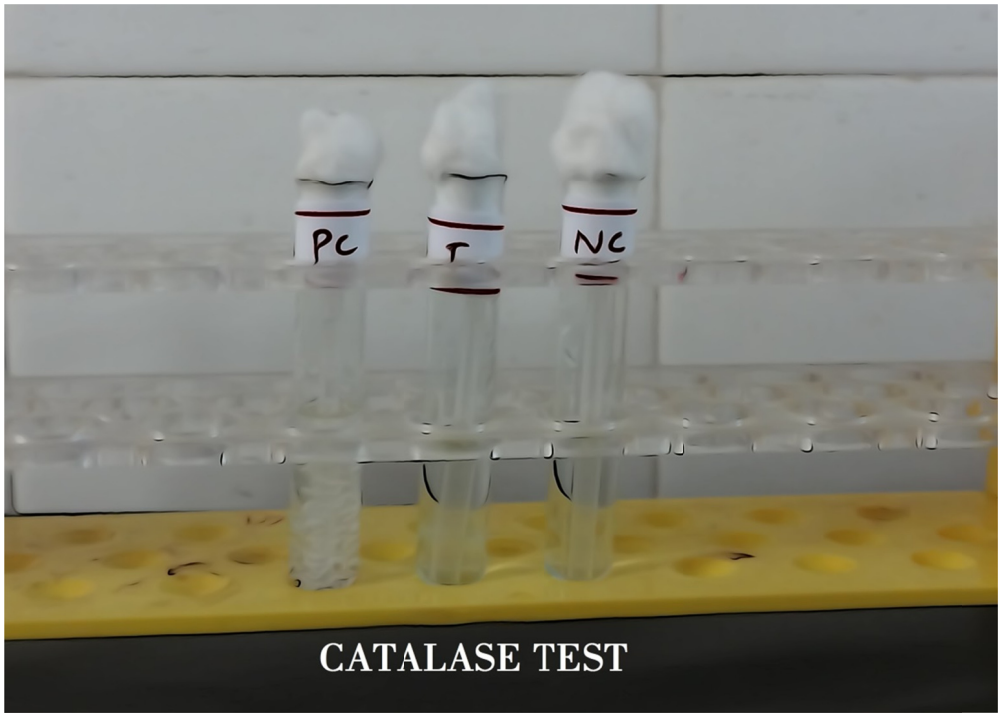
Interpretation :

The ATCC positive control strain showed growth in the culture plate which was steaked both before and after heating the broth at 60°C for 30 minutes. If the test organism culture plate shows growth in before and after heating were taken as positive heat tolerant test.

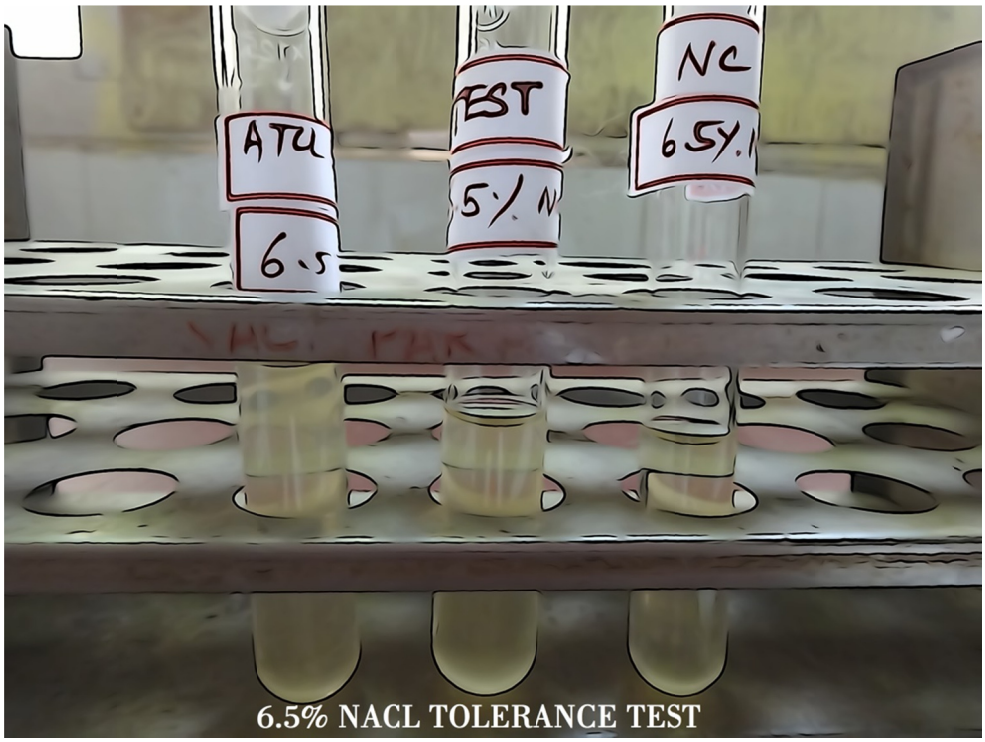
5.**Salt tolerance test :**

Procedure :

2 to 3 identical colonies from an 18-24 hours old culture of suspected enterococcal isolates were inoculated in to the Nutrient broth with 6.5% sodium chloride (6.5% Nacl). The Nutrient broth contains bromocresol purple as the Ph indicators. The tubes were incubated at 35 – 37°C for 48 hours with positive control strain of Enterococci.[8]



CATALASE TEST



6.5% NACL TOLERANCE TEST

Interpretation :

Turbid appearance of the broth with or without a color change from purple to yellow is considered as positive reaction. All the enterococcal isolates were salt tolerant .

6.Arginine dihydrolysis Test : Procedure: The isolated enterococcal colonies (2-3 identical) were inoculated in the Moeller's decarboxylase broth containing arginine and in the control tube containing Moeller's decarboxylase base broth without arginine. and both the tubes were overlaid with the sterile mineral oil. The tubes were incubated at 37°C for four days and were examined at 24, 48, 72 and 96 hours.[47]

Interpretation :

Development of deep purple colour after an initial change of yellow colour indicates positive reaction and the persistant yellow colour indicates negative reaction. Enterococcus faecalis and Enterococcus faecium were arginine dihydrolysis test positive.

7.Mannitol Motility Test :

Procedure :

The mannitol motility medium (MMM) was inoculated with the test organism by stabbing with positive and negative control. The tubes were incubated at 37°C for 24 hours.The Positive control used was Escherichia coli ATCC strain 25922 and negative control used was Staphylococcus aureus ATCC strain25923.[8]

MANNITOL MOTILITY MEDIUM

Interpretation:

Motility of test organism was indicated by a diffuse growth extending out from the line of inoculation in the Mannitol motility medium (MMM). Non motile organisms showed growth only at the site of inoculation. In the Mannitol motility medium, Mannitol fermenting organisms changed the colour of the medium from red to yellow due to acid production. Enterococcus casseliflavus and Enterococcus gallinorum were mannitol fermenting and motile Enterococcus faecalis and Enterococcus faecium were mannitol fermenting and nonmotile.

8.Carbohydrate Fermentation test :

Procedure :

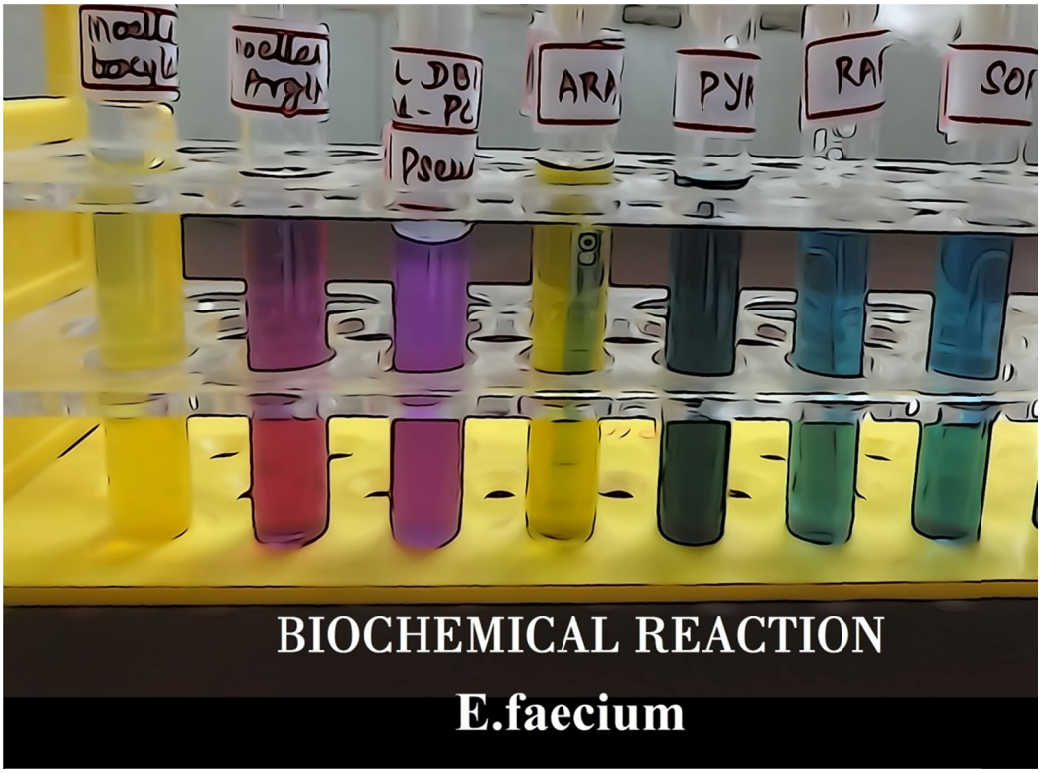
The isolated enterococci were inoculated into the carbohydrate fermentation media for speciation. The carbohydrate fermentation media contain 1% carbohydrate such as pyruvate, arabinose, sorbitol, sucrose and raffinose in separate tubes. The tubes were incubated at 37°C for 24 hours and Bromothymol blue is used as ph indicator.

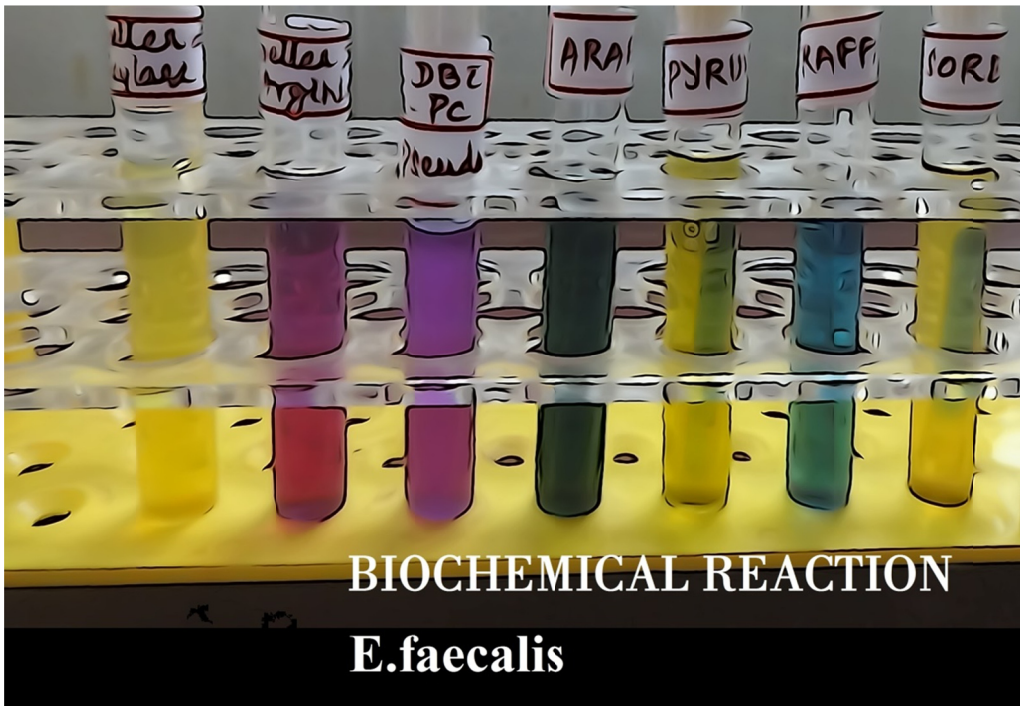
Interpretation :

Acid production from carbohydrate was indicated by the colour change from blue to yellow.

Antibiogram by Kirby – Bauer disc diffusion method :

As per the Clinical laboratory standards Institute (CLSI) guidelines (CLSI document M02&M07) ,the antibiotic susceptibility testing was done by


BIOCHEMICAL REACTION
E.faecium


BIOCHEMICAL REACTION
E.faecalis

using the Kirby – Bauer disc diffusion method. Mueller Hinton agar plate (MH plate) was used for antibiotic susceptibility testing. A sterile swab was dipped in the 0.5 Mcfarland standard adjusted inoculums. The excess fluid was squeezed out and it was streaked on the surface of the agar plate three times, turning the agar plate at 60° each time to produce a lawn culture. Then antibiotic discs were placed within 15 minutes of inoculation in the lawn culture. The inoculated plates were incubated aerobically at 37°C for 16 – 18hours. Zone size was interpreted under reflected light except for Vancomycin (transmitted light was used). The interpretation as susceptible, intermediate and resistant was done as per the CLSI guidelines.

Zone size (mm) S- Sensitive, I – Intermediate, R – Resistant

Antimicrobial Drug	**S(sensitive)**	**I(intermediate**	**R(resistant)**
Ampicillin 10µg	≥17	-	≤16
Ciprofloxacin 5µg	≥21	16-20	≤15
Doxycycline 30µg	≥16	13-15	≤12
Vancomycin 30µg	≥17	15-16	≤14
Teicoplanin 30µg	≥14	11-13	≤10
High level gentamicin (HLG)120µg	≥10	7-9	≤6
High level streptomycin (HLS)300µg	≥10	7-9	≤6
Linezolid 30µg	≥23	21-22	≤20
Nitrofurantoin 300µg For urine sample	≥17	15-16	≤14

Screening test for high level gentamicin resistance (HLGR)[12,13]

The enterococcal isolates were screened for high level resistance to gentamicin and streptomycin by using the high content gentamicin disc (HLG) – 120 µg and high content streptomycin disc(HLS) 300 µg on Mueller Hinton agar plate by disc diffusion method as per the guidelines. Bacterial suspension equal to 0.5 McFarland standard was used and incubated at 37°C for 16- 18 hours. The test also included E. faecalis ATCC 29212 as negative control and E. faecalis ATCC 51299 as positive control.

Interpretation :

A Zone size of 6 mm is considered Resistant, 7–9 mm considered as Inconclusive and ≥ 10 mm Susceptible.

Those isolated enterococci that were resistant to High content Gentamicin disc (120µg) and those that were inconclusive, they were further screened by the E- TEST (HLGR).concentration ranges from 0.064µg to 1024 µg and agar dilution method.

In the E- TEST (HLGR strip HIMEDIA) , Enterococcal isolates were inoculated as for disc diffusion and with the help of forceps the E- test strip was placed over the agar surface and incubated aerobically at 35^0-37^0 c for 16–18 hours.Minimum inhibitory concentration (MIC) was determined by the point of intersection of the inhibition ellipse with the E-test stip edge.[58]

In agar dilution method, brain heart infusion (BHI) agar containing 500 µg/ml of Gentamicin was used for the screening of high level gentamicin

resistance (HLGR). In this method Spot inoculation of 10µl of 0.5 McFarland standard bacterial suspension was done on to the agar surface along with the E. faecalis ATCC 29212 as negative control and E. faecalis ATCC 51299 as positive control. The plates were incubated for 16-18hrs aerobically at 37°C. Growth more than 1 colony indicates presumptive high level gentamicin resistance (HLGR).[12,13]

Molecular characterization of high level gentamicin resistant (HLGR) producing Enterococci by polymerase chain reaction:

Requirements :

PureFast® Bacterial DNA minispin purification kit [Kit contains Lysozyme, Lysozyme digestion buffer, Proteinase-K, Binding buffer, Wash Buffer-1, Wash Buffer-2, Spin columns with collection tube and elution buffer. HELINI 2X ReDdye PCR Master Mix, Agarose gel electrophoresis consumables and aac-6-le-aph-Ia, aph Ib, aph Ic and aph Id Primers are from HELINI Biomolecules, Chennai, India.

2X Master Mix:

It contains 2U of Taq DNA polymerase, 10X Taq reaction buffer, 2mM MgCl2, 1µl of 10mM dNTPs mix and RedDye PCR additives.

Agarose gel electrophoresis:

Agarose, 50X TAE buffer, 6X gel loading buffer and Ethidium bromide are from HELINI Biomolecules, Chennai.

PCR:

HELINI Ready to use aac-(6')-le-aph-(2") Ia gene Primer mix - 5µl/reaction

PCR Product size: 500bp

HELINI Ready to use aph-(2") Ib gene Primer mix-5µl/reaction

PCR Product size: 500bp

HELINI Ready to use aph-(2") Ic gene Primer mix-5µl/reaction

PCR Product size: 475bp

HELINI Ready to use aph-(2") Id gene Primer mix-5µl/reaction

PCR Product size: 385bp

Bacterial DNA Purification

1ml of overnight culture were centrifuged at 6000rpm for 5min and Supernatant were discarded. Pellet was suspended in 0.2ml PBS. 180µl of Lysozyme digestion buffer and 20µl of Lysozyme [10mg/ml] were added and incubated at 37C for 15min. 400µl of Binding buffer, 5µl of internal control template and 20µl of Proteinase K were added and mixed well by inverting several times. Then this was incubated at 56ºC for 15min.after that 300µl of Ethanol were added and mixed well. Then it was transferred into the PureFast® spin column. Centrifuged for 1 min and Discarded the flow-through and placed the column back into the same collection tube.

500µl Wash buffer-1was added to the PureFast® spin column. Centrifuged for 30-60 seconds and discarded the flow-through placed the column back into the same collection tube. Then 500µl Wash buffer-2 was added to the PureFast® spin column. Centrifuged for 30-60 seconds and discarded the flow-through and the column back was placed into the same collection tube and centrifuged for an additional 1 min. This step was essential to avoid residual ethanol.

The PureFast® spin column was transferred into a fresh 1.5 ml micro-centrifuge tube and 100µl of Elution Buffer was added to the center of PureFast® spin column membrane and Incubated for 1 min at room temperature and centrifuged for 2 min. The column was discarded and the purified DNA was stored at -20°C. The Quantity of extracted DNA was checked by loading in 1% agarose gel and 5µl of extracted DNA was used for PCR amplification.

PCR Procedure:

1. Reactions set up as follows;

Components	**Quantity**
HELINI RedDye PCR Master mix	10µl
HELINI Ready to use - gene primer mix	5µl
Purified Bacterial DNA	5µl
Total volume	20µl

2. Mixed gently and spin down briefly.

3. Placed into PCR machine and programmed as follows

Initial Denaturation: 94ºC for 5 min

Denaturation: 94ºC for 30sec

Annealing: 58ºC for 30sec 35 cycles

Extension: 72ºC for 30sec

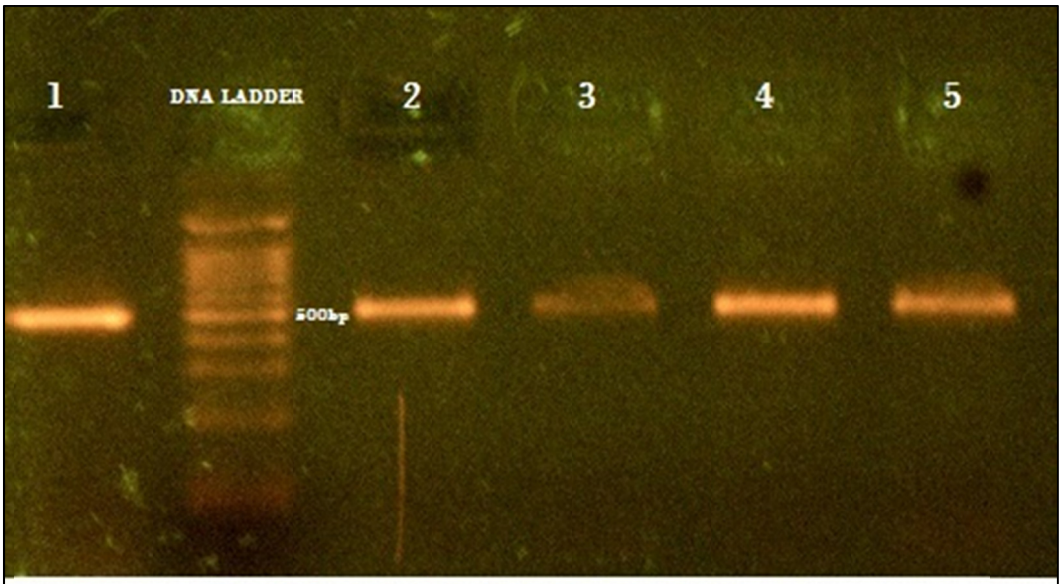
Final extension: 72º C for 5 min

Agarose gel electrophoresis:

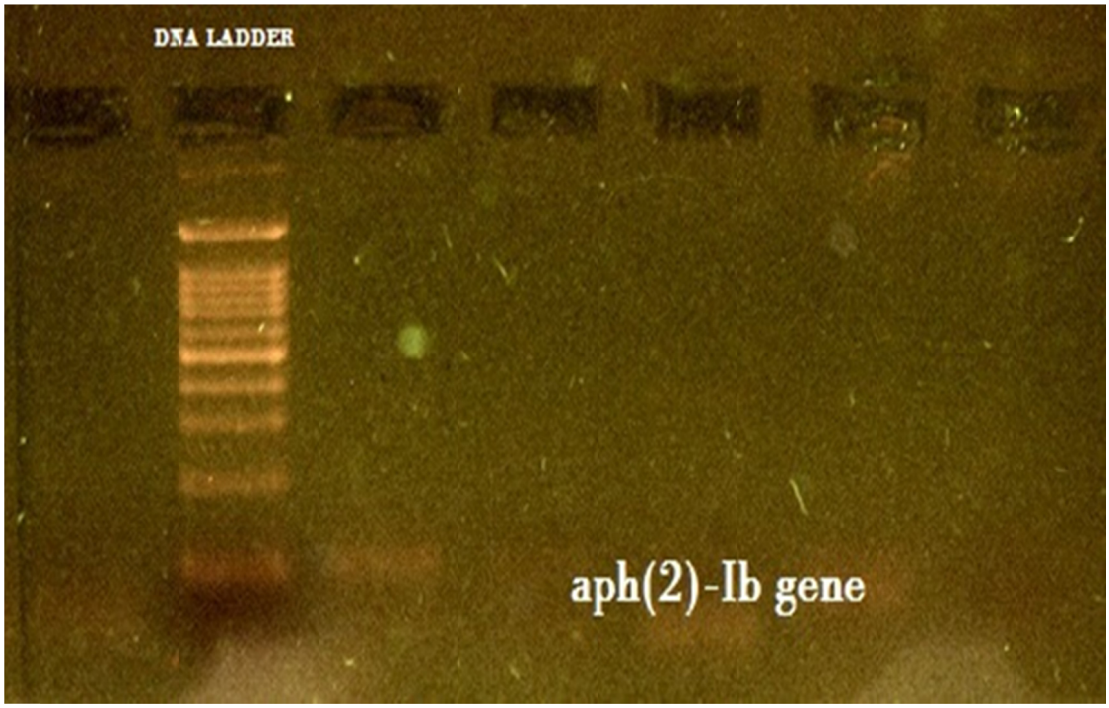
2% agarose was prepared (2gm agarose in 100ml of 1X TAE buffer and melted using microoven) .When the agarose gel temperature was around 60ºC, 5µl of Ethidium bromide was added. Agarose solution was poured slowly into the gel platform and the gel was kept set undisturbed till the agarose solidifies. Poured 1XTAE buffer into submarine gel tank. The gel platform was placed into the tank. Maintained the tank buffer level 0.5cm above than the gel. PCR Samples are loaded after mixed with gel loading dye along with 10µl HELINI 100bp DNA Ladder. [100bp, 200bp, 300bp, 400bp, 500bp, 600bp, 700bp, 800bp, 900bp,1000bp and 1500bp]. Electrophoresis was done at 50V till the dye reaches three fourth distance of the gel. Gel was viewed in UV Transilluminator and the bands pattern was observed.

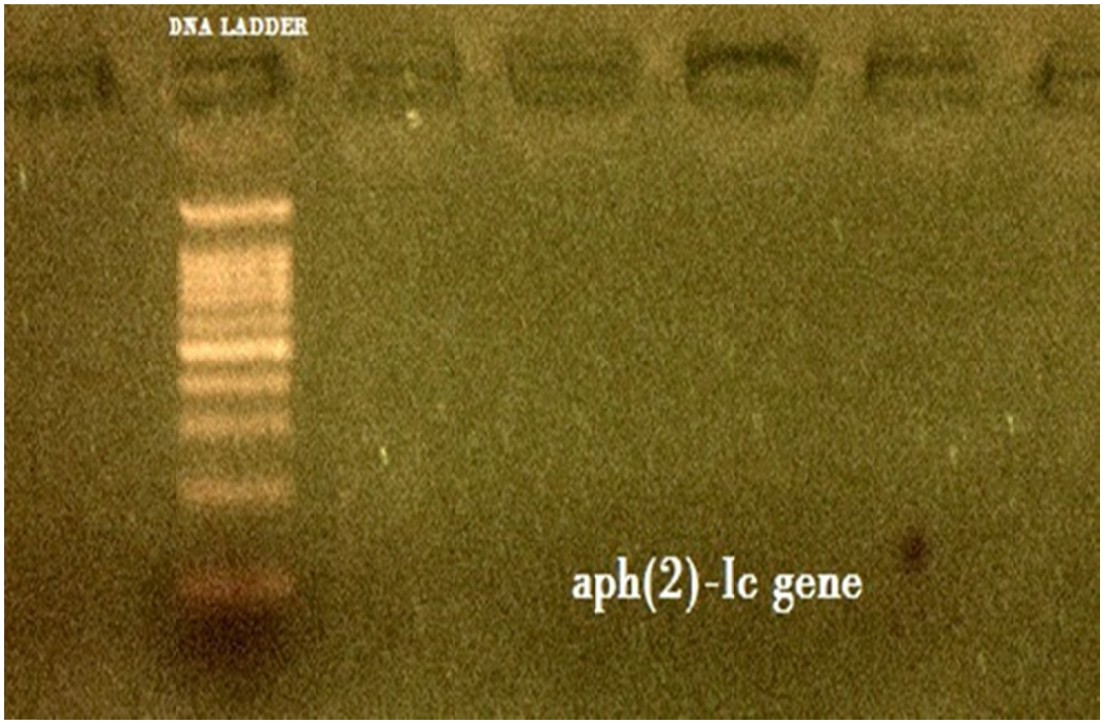
INTERPRETATION:

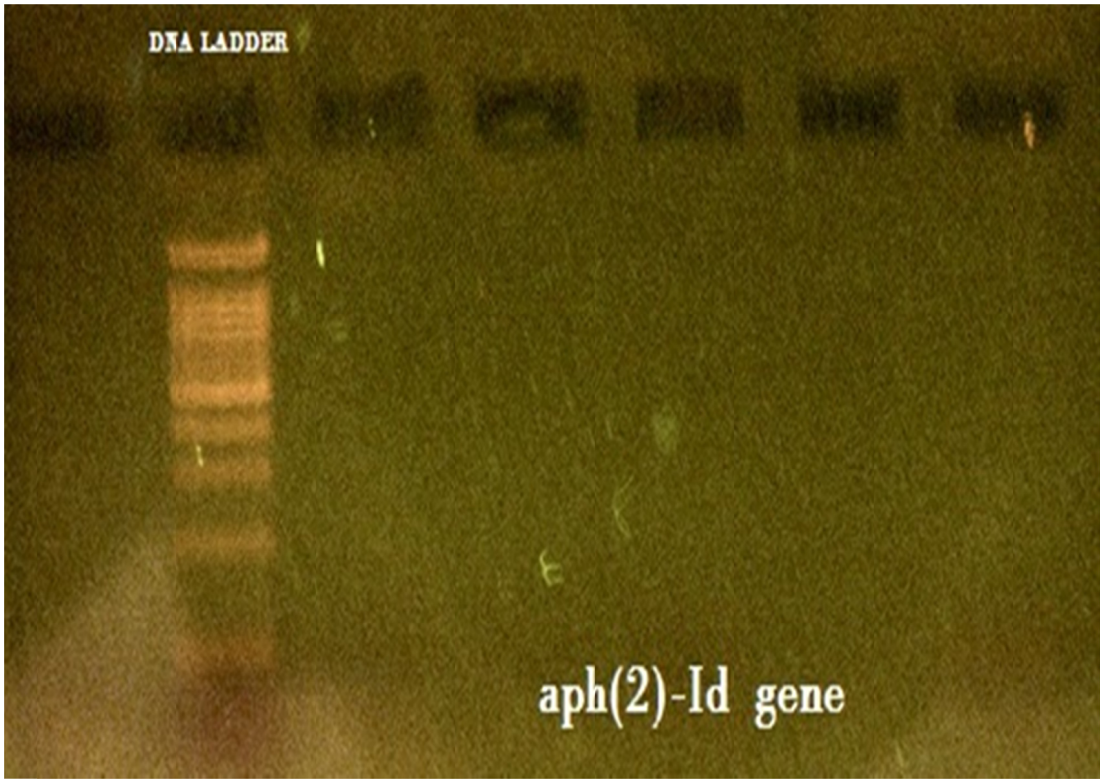
The presence of ***aac(6')-Ie-aph(2")-Ia*** gene was indicated by the amplification of 500 bp PCR product.



(aac(6)-Ie-aph(2)-Ia GENE




DNA LADDER
aph(2)-Ic gene


DNA LADDER
aph(2)-Id gene

The presence of ***aph(2”)-Ib*** gene was indicated by the amplification of 500 bp PCR product .

The presence of ***aph(2”)-Ic*** gene was indicated by the amplification of 475 bp PCR product.

The presence of ***aph(2”)-Id*** gene was indicated by the amplification of 385 bp PCR product .

RESULTS

Urine, blood, pus and wound swab specimen were collected from 396 patients who were admitted at Govt. Rajaji Hospital. Both sexes of all age groups were included. Among 396 specimen, 368 showed growth and 28 specimen showed no growth. Out of 396 samples, 159 were from urine, 103 were from pus, 74 were from blood and 60 were from wound swab. Among 368 samples, 104 were enterococci, 121 were other gram positive cocci and 143 were gram negative bacilli.

Table -I and Chart I : Specimen wise distribution cases (n=396)

Site	Number of specimen
Urine	**159 (40.15%)**
Pus	**103 (26.01%)**
Blood	**74 (18.6%)**
Wound swab	**60 (15.15%)**

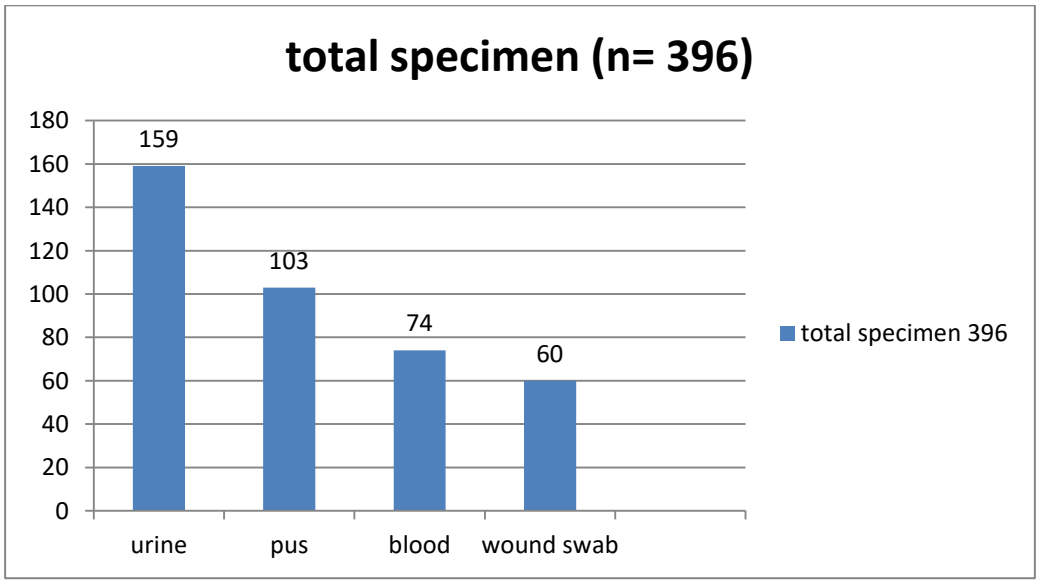
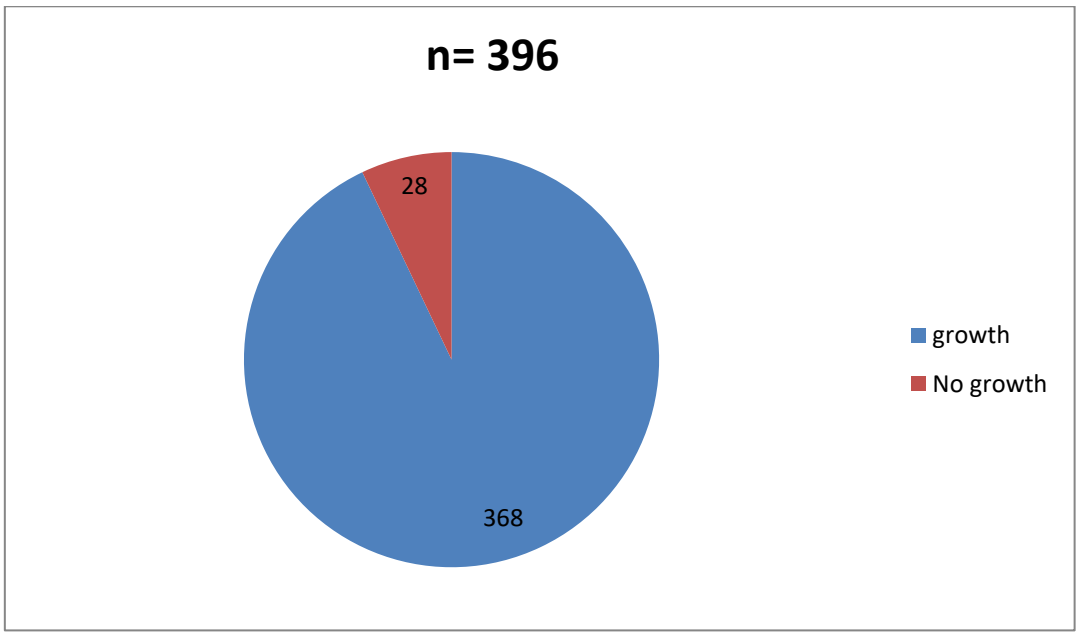


Table I and Chart I shows out of 396 samples 159 (40.15%) were urine,103(26.01%) from pus, 74(18.6%) from blood and 60(15.15%) from wound swab.It was found maximum number of samples were from urine(40.15%).

Table II and Chart II: GROWTH Vs NO GROWTH FROM SPECIMEN:

Total specimen	Showed growth	Showed no growth
396	**368 (92.29%)**	**28 (7.07%)**

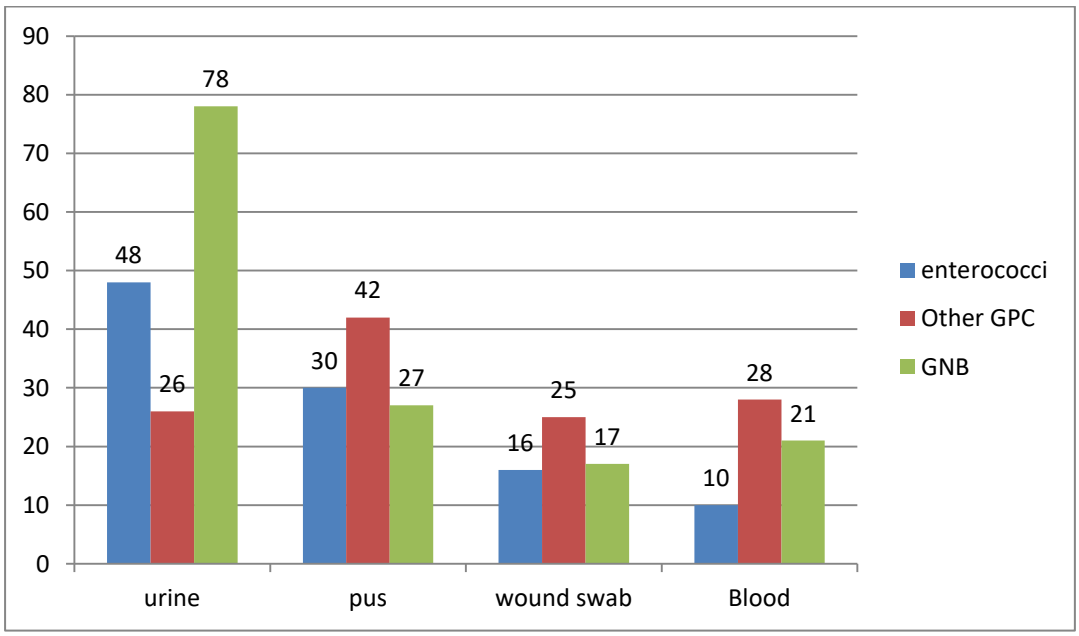


From the table II &Chart II, out of 396 samples, 368 (92.29%) showed growth and 28(7.07%) showed no growth.

Table III & Chart III: Specimen wise isolation of Organisms (n=368)

SPECIMEN	ENTEROCOCCI	OTHER GPC	GNB	TOTAL
URINE	48(31.57%)	26(17.1%)	78(51.3%)	152
sPUS	30(30.3%)	42((42.42%)	27(27.27%)	99
BLOOD	10(16.9%)	28(47.45%)	21(35.59%)	59
WOUND SWAB	16(27.53%)	25(43.1%)	17(29.31%)	58
TOTAL	104((28.2%)	121(32.88%)	143(38.85%)	368

Chart III : Specimen wise isolation of Organisms (n=368)



From the Table III Chart III, it was found that, Out of 152 urine specimen which showed growth, 48 (31.57%) were enterococci, 26 (17.1%) were other gram positive cocci and 78 (51.3%) were gram negative bacilli.

Out of 59 blood specimen which showed growth, 10(16.9%) were enterococci, 28(47.45%) were other gram positive cocci,21(35.59%) were gram negative bacilli.

Out of 99 pus specimen which showed growth, 30 (30.3%) were enterococci, 42 (42.42%) were other gram positive cocci, and 27 (27.27%) were gram negative bacilli.

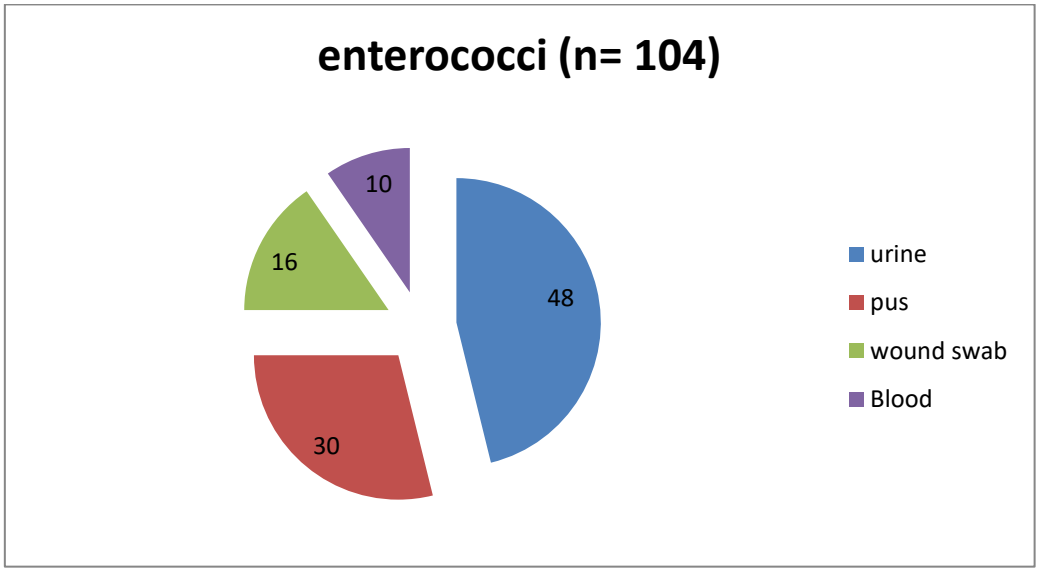
Out of 58 wound swab specimen which showed growth, 16 (27.53%) were enterococci, 25 (43.1%) were other gram positive cocci and 7 (29.3%) were gram negative bacilli.

Out of total 368 specimen showed growth, 104(28.26%) were enterococci,121(32.88%) were other gram positive cocci, 143 (38.85%) were gram negative bacilli.

Table IV : Specimen wise isolation of Enterococci (n=104)

SPECIMEN	NO OF ENTROCOCCAL ISOLATES	PERCENTAGE
URINE	48	46.15%
PUS	30	28.84%
WOUND SWAB	16	15.38%
BLOOD	10	9.6%

Chart IV : Specimen wise isolation of Enterococci (n=104)

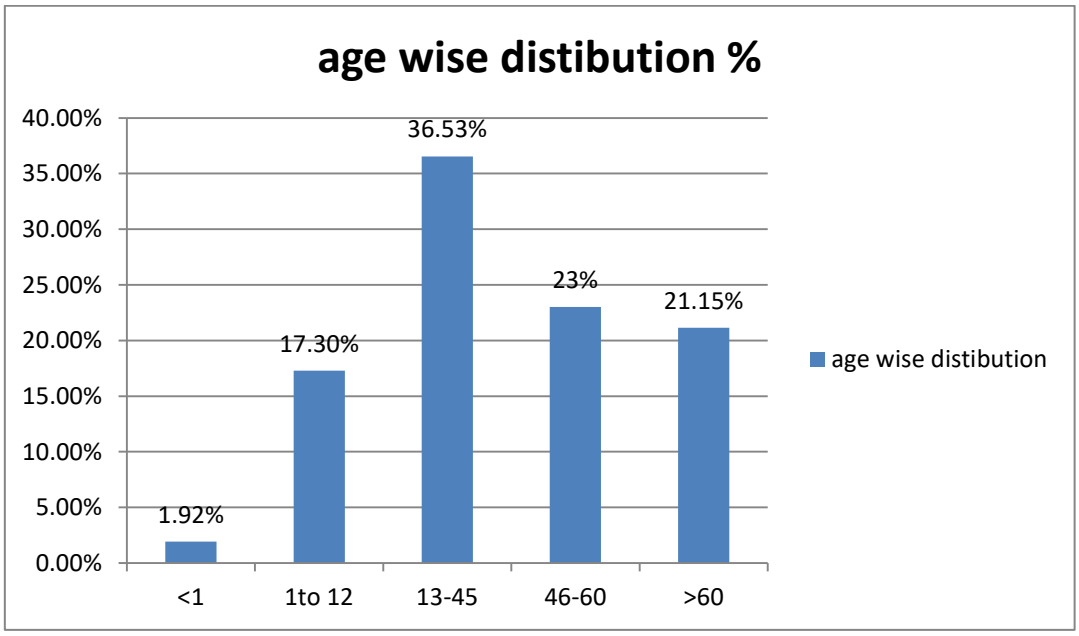


From the Table IV & Chart IV, it was found that, Out of 104 enterococcal isolates from various samples, 48(46.15%) were isolated from urine samples, 10 (9.6%) from blood samples,30(28.84%)from pus samples and 16(15.38%) from wound swab samples. From the above table, it was observed that Enterococci were isolated more from urine sample (46.15%) followed by pus sample (28.84%).

Table V : Age wise distribution of Enterococcal isolates (n=104)

AGE IN YEARS	NO OF PATIENTS	PERCENTAGE
< 1	2	1.92%
1 -12	18	17.3%
13 – 45	38	36.53%
46 – 60	24	23%
>60	22	21.15%

Chart V : Age wise distribution of Enterococcal isolates (n=104)

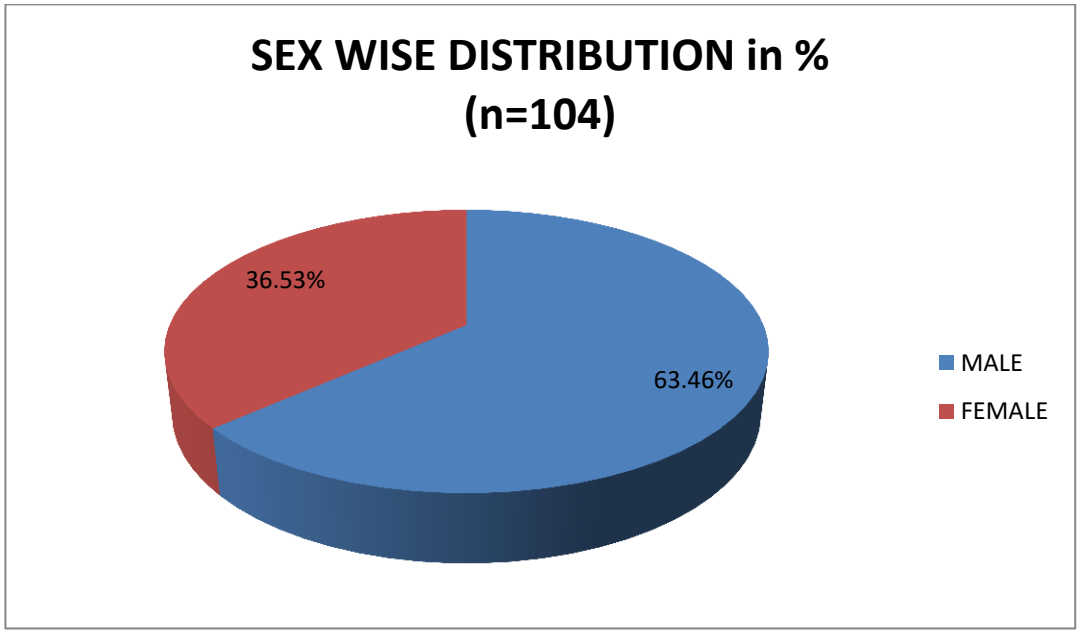


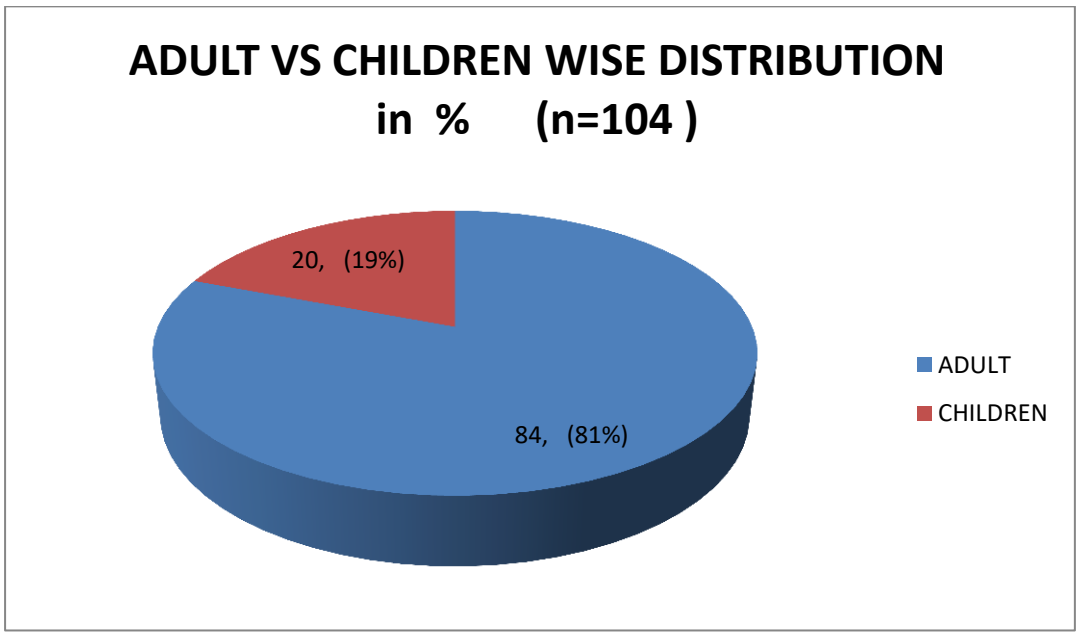
From the Table V & Chart V, it was found that the analysis of age wise distribution of isolated Enterococcal species from various clinical specimen showed predominance of enterococcal infection in the age group of 13-45yrs that is 38 cases (36.53%).

Table VI : Sex wise and adult -children distribution among Enterococcal species (n= 104)

	ADULT	CHILDREN	TOTAL
MALE	51	15	66(63.46%)
FEMALE	33	5	38(36.53%)
TOTAL	84(80.76%)	20(19.2%)	104

Chart VI : Sex wise and adult -children distributionamong Enterococcal species (n= 104)



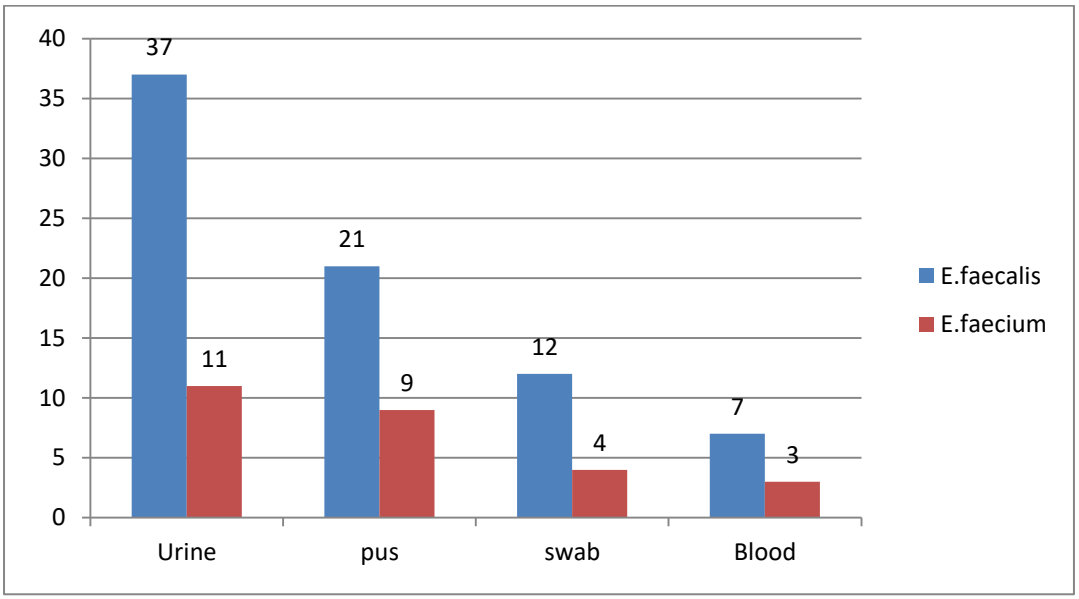


From the Table VI & Chart VI, it was found that, Out of 104 enterococcal isolates, 66 (63.43%) were isolated from male patients and 38(36.53%) were isolated from female patients. Out of 104 enterococcal isolates 84 (80.76%) of adults and 20 (19.23%) of children were affected by Enterococcal infection.

Table VII :Distribution of Enterococcal species among the specimen

SPECIMEN	E.faecalis	E.faecium	TOTAL
URINE	37 (77.1%)	11 (22.9%)	48
PUS	21 (70%)	9 (30%)	30
WOUNDSWAB	12 (75%)	4 (25%)	16
BLOOD	7 (70%)	3 (30%)	10
TOTAL	77 (74.03%)	27 (25.96%)	104

Chart VII : Distribution of Enterococcal species among the specimen



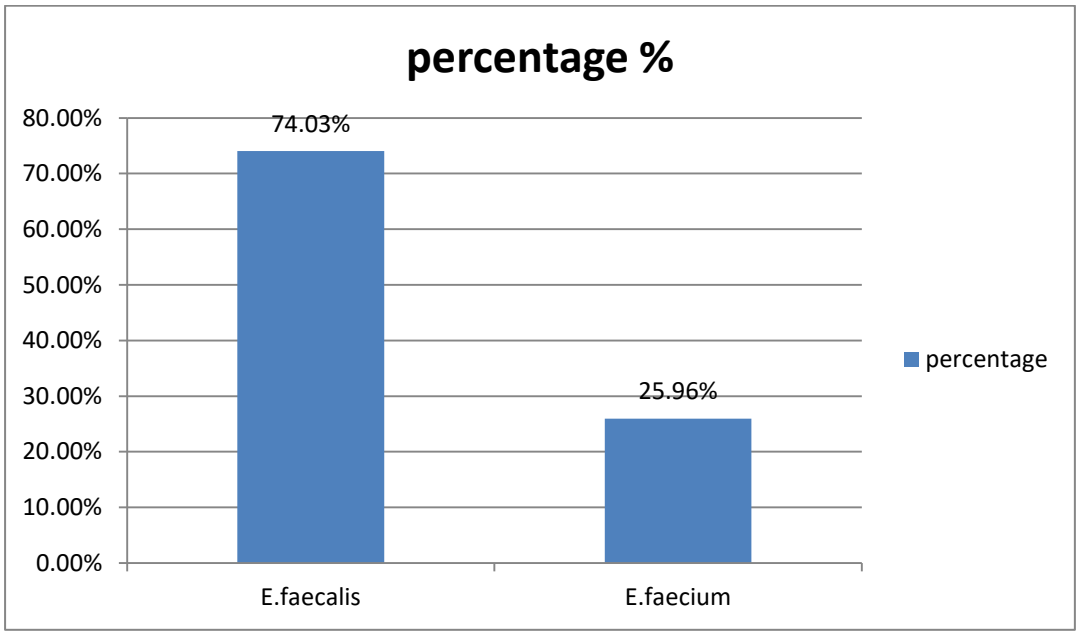
From the Table VII & Chart VII, it was found that, In the urine samples ,out of 48 enterococcal isolates 37 (77%) were E.faecalis,11 (22.9%) were E.faecium. In the pus samples ,out of 30 enterococcal isolates 21(70%) were E.faecalis, 9(30%) were E.faecium. In the blood samples ,out of 10 enterococcal isolates 7(70%) were E.faecalis, 3(30.%) were E.faecium In the wound swab samples ,out of 16 enterococcal isolates 12 (75%) were E.faecalis

4 (25%) were E.faecium..No other enterococcal species were isolated from the various specimen collected during the study period

Table VIII : Species wise distribution of isolated Enterococci (n=104)

Species	Number
E.faecalis	77(74.03%)
E.faecium	27(25.96%)

Chart VIII: Species wise distribution of isolated Enterococci (n=104)



From the TableVIII &ChartVIII, it was found that, E.faecalis 77 (74.03%) was the predominant species followed by E.faecium, 27 (25.96%).

Table IX: Antibiotic susceptibility pattern of Enterococcus species by Kirby Bauer disc diffusion method

ENTERO COCCUS SPECIES	TOTAL ISOLATES	PEN		AMPI		CIP		DOXY		HLS		HLG		TEICO		VANCO		LINE	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
E.faecalis	77	56 72.7%	21 27.2%	57 74%	20 25.9%	53 68.8%	24 31.1%	30 38.9%	47 61.1%	57 74.0%	20 25.9%	29 37.6%	48 62.3%	75 97.4%	2 2.6%	73 94.8%	4 5.19%	75 97.4%	2 2.6%
E.faecium	27	17 62.9%	10 37.0%	19 70.0%	8 29.6%	17 62.9%	10 37.0%	11 40.7%	16 59.25%	19 70.3%	8 29.6%	9 33.3%	18 66.6%	25 92.6%	2 7.4%	23 85.18%	4 14.8%	25 92.5%	2 7.5%

Antibiotic susceptibility pattern of isolated Enterococcus species from the various clinical specimen .

Chart IX a & b : Antibiotic susceptibility pattern of Enterococcus species by Kirby Bauer disc diffusion method Chart IX (a):

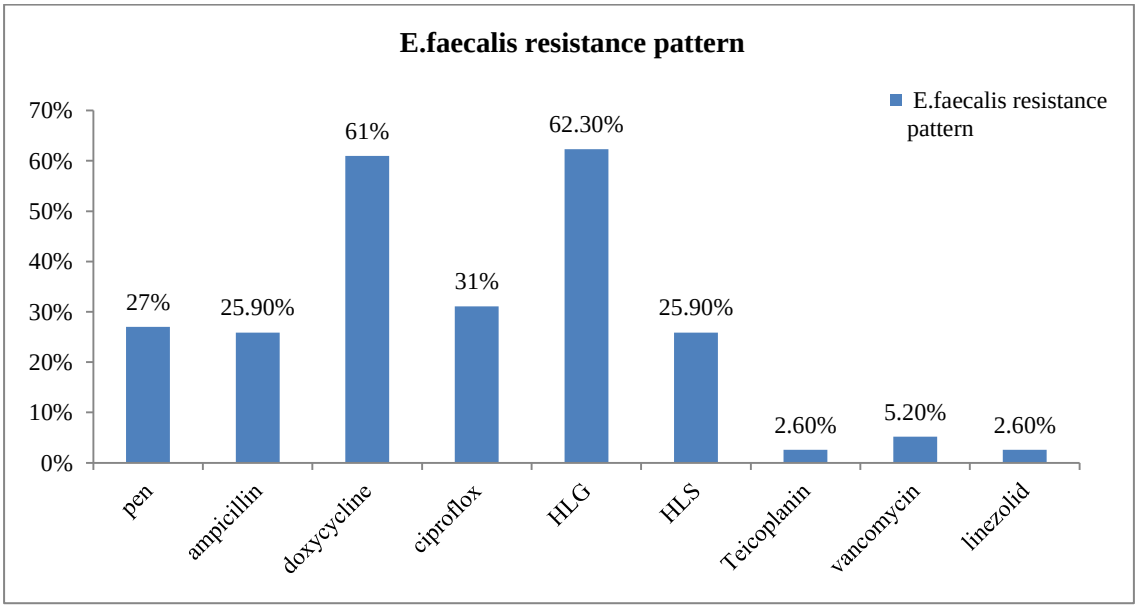
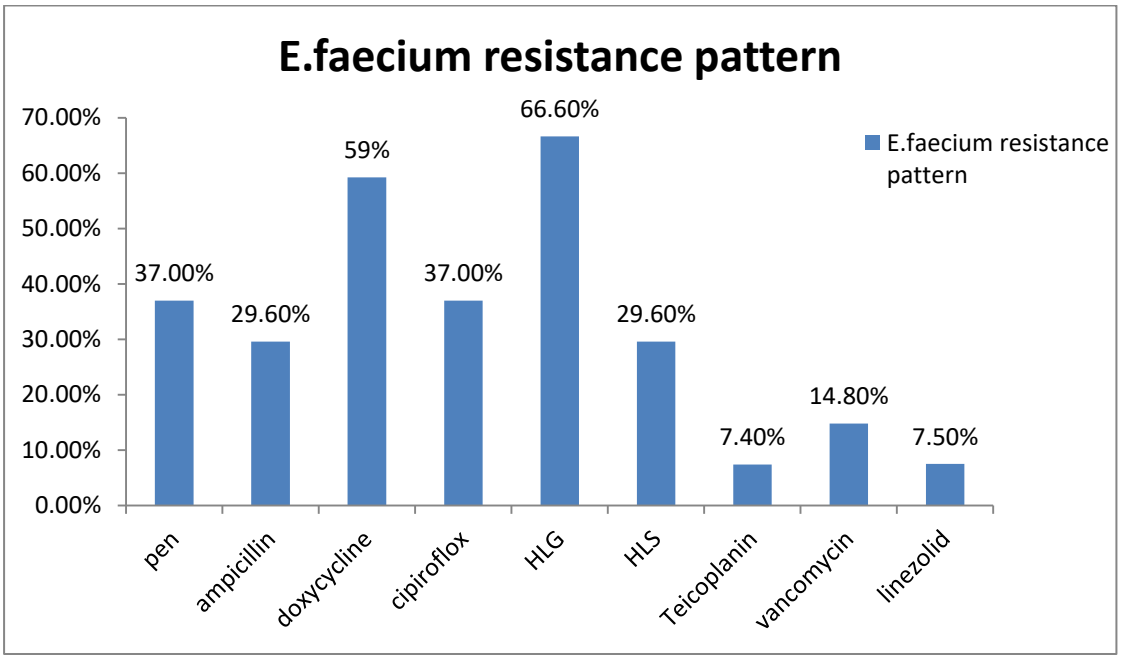


Chart IX (b)



From the Table IX & Chart IX a & b, it was found that, most of the E. faecalis were sensitive to ampicillin (74%), teicoplanin (97.4%) and vancomycin (94.8%). They were resistant to ciprofloxacin (31.1%) and

doxycycline (61%). Among the 77 isolated E .faecalis 48 (62.3%) were high level gentamicin resistant and 20 (25.9%) were resistant to high level streptomycin.

Among the 27 isolates of E .faecium, resistance pattern of various antimicrobial agents were as follows ampicillin 8 (29.6%), ciprofloxacin 10(37%), doxycycline 16(59.25%) , high level gentamicin resistance 18(66.6%) and to high level streptomycin resistance 8(29.6%). But E.faecium are sensitive to teicoplanin 25(92.6%) and vancomycin 23 (85.18%). E.faecium showed more antimicrobial resistance pattern than E.faecalis. Vancomycin resistance among the isolated E.faecalis and E.faecium were 5.19% & 14.8% respectively.

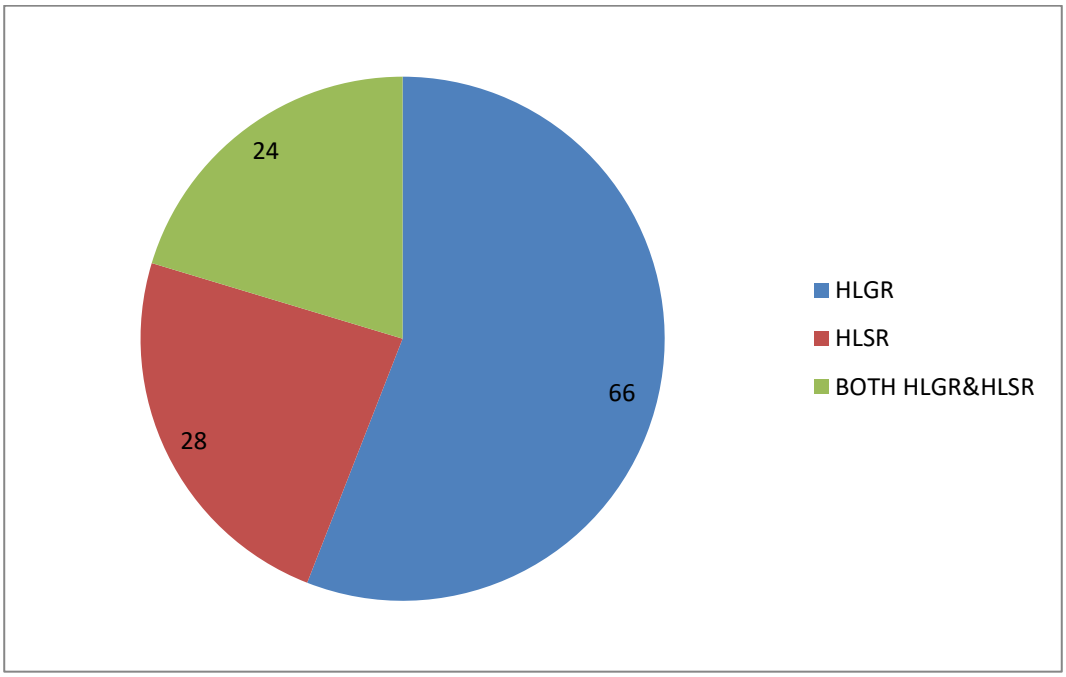
High level gentamicin resistance among the isolated 104 Enterococcal speciecies were 66 (E .faecalis -48, E .faecium,-18) 63.46%. High level streptomycin resistance among the isolated 104 Enterococcal speciecies were (E.faecalis-20,E .faecium,-8)26.9%. 62.3% of E .faecalis and 66.6% of E.faecium were high level gentamicin resistant.

Among the vancomycin resistant E.faecalis isolates that is out of 4, three isolates showed HLGR and 1 showed both HLGR and HLSR.Among the vancomycin resistant E.faecium that is out of 4, all showed HLGR and 2 showed HLSR and both HLGR & HLSR are 2. All the high level gentamicin resistant isolates were sensitive to teicoplanin and linezolid.

Table X : High level aminoglycoside resistance (HLAR) among E. faecalis and E. faecium isolates by disc diffusion method.

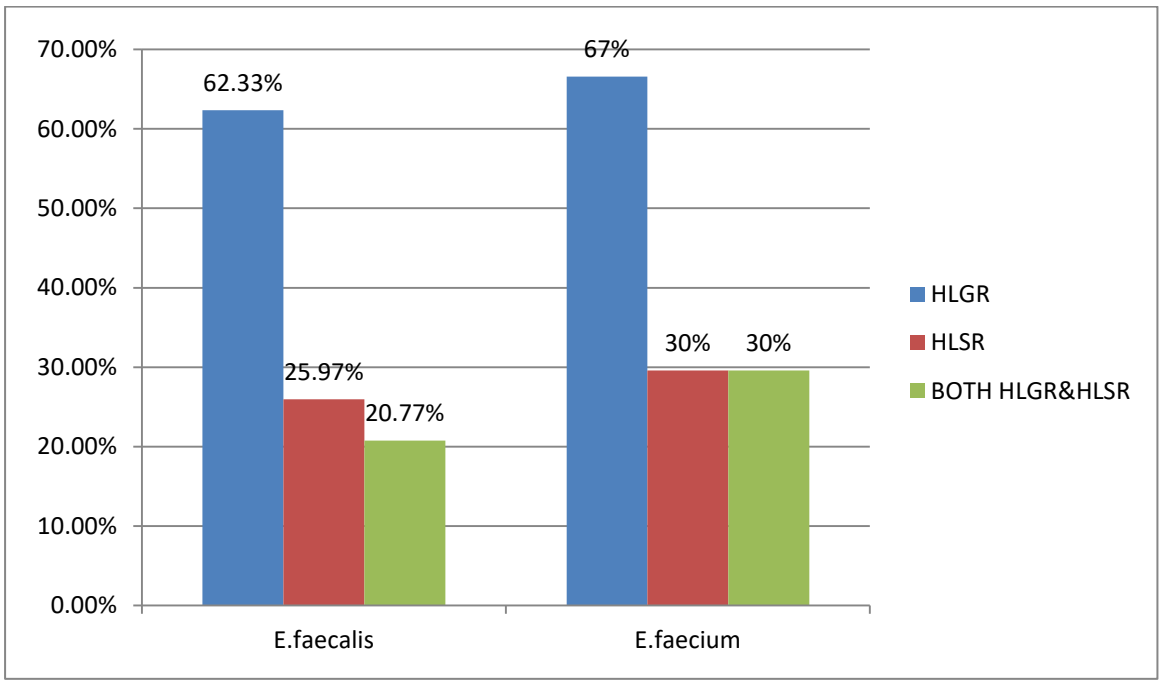
ENTEROCOCCAL SPECIES	TOTAL ISOLATES	RESISTANCE TO BOTH HLS & HLG	RESISTANCE TO HLS	RESISTANCE TO HLG
E.faecalis	77	16 (20.7%)	20(25.97%)	48(62.33%)
E.faecium	27	8 (29.6%)	8(29.6%)	18(66.66%)
TOTAL	104	24(23.07%)	28(26.9%)	66(63.46%)

Chart X (a) : High level aminoglycoside resistance (HLAR) among the isolated enterococcal species by disc diffusion method.



From the table X & Chart X (a) Out of total 104 enterococcal isolates, 66(63.46%) were HLGR, 28(26.9%) were HLSR and 24(23.07%) were resistant to both HLG and HLS.

Chart X (b) : High level aminoglycoside resistance (HLAR) among E. faecalis and E. faecium isolates by disc diffusion method.



From the Table X & Chart X (b) it was found that, Among the isolated 77 E.faecalis, 48(62.33%) were HLGR, 20(25.97%) were HLSR and both HLGR &HLSR were 16(20.77%).

Among the isolated 27 E.faecium, 18(66.6%) were HLGR, 8(29.6%) were HLSR and both HLGR &HLSR were 8(29.6%).

Comparision of E-Test and agar dilution method for detecting high level gentamicin resistance in enterococci:

66 High level gentamicin resistance (HLGR) Enterococcal isolates (E.faecalis -48, E.faecium -18), were subjected to E-test and agar dilution method for screening HLGR > 500µg/ml and the results were as follows

Table XI : Comparision of E-Test and agar dilution method for detecting high level gentamicin resistance in enterococci:

No. enterococcal isolate subjected for testing	MIC >500µg/ml of gentamicin	
	by E-test	by agar dilution method
66	66	66

From the Table XI it was found that, in the E-test all the High level gentamicin resistance isolates that were screened by the high content gentamicin disc (120µg) showed MIC >1024 µg/ml. In agar dilution screening method by using Brain Heart Infusion agar containing gentamicin 500 µg/ml, showed growth in all the 66 enterococcal isolate.

Prevalence of genes responsible for HLGR :

Among the total 104 enterococcal isolates, 66(63.46%) were High level gentamicin resistant(HLGR) which was subjected to the molecular characterization study showed the results as follows

Table XII :Prevalence of genes responsible for HLGR

Name of the HLGR gene	E.faecalis (n=48)	E.faecium (n=18)
aac(6)-Ie-aph(2)-Ia	48	18
(aph(2)-Ib	_	_
aph(2)-Ic	_	_
aph(2)-Id	_	_

From the table XII , it was found that, all the isolated enterococci (n=66) that are HLGR by screening test posses the gene that codes the bifuntional enzyme aac(6')-Ie-aph(2")-Ia.

Other genes that are responsible for high level gentamicin (aph(2")-Ib, aph(2")-Ic & aph(2")-Id) were not detected in this study.

DISCUSSION

Enterococci species have emerged as an important cause of health care acquired infections.Urinary tract infections,intra abdominal and pelvic infections,bloodstream infection are commonly encountered hospital acquired infections by these enterococcal species. Enterococci also cause surgical site infections, endocarditis, neonatal sepsis and meningitis. The major reason for existence of these organisms in hospital environment is due to the intrinsic resistance to commonly used antibiotics. Enterococci have the ability to acquire resistance mechanism to various antibiotics either by means of gene transfer by plasmids and transposons or by means of mutation. Serious enterococcal infections like endocarditis, meningitis and sepsis are usually treated with combination of two antibiotics that is one acts on cell wall synthesis like beta lactam or glycopeptides (pencillin,ampicillin or vancomycin and an aminoglycoside which inhibits bacterial protein synthesis(i.e gentamicin or streptomycin).The addition of cell wall acting agent,such as ampicillin or vancomycin, increases uptake of the aminoglycoside in to the cell,which results in synergistic bactericidal effect on enterococcus.If Enterococci have acquired gentamicin resistant genes (HLGR) that mediate production of aminoglycoside modifying enzymes, results in loss of synergism between cell wall acting agents and gentamicin.

Inherent resistance to the commonly used antibiotics makes these organism excellent survivors in health care places and cause health care

acquired infections. In this study, prevalence of high level gentamycin resistance among the isolated enterococcal species in the various clinical sample and molecular characterization of genes that are responsible for high level gentamycin (MIC>500µg/ml) resistance have been assessed.

In this present study, out of 396 samples processed, 368 showed growth and 28 were no growth. Out of this 368 isolates, 104 (28.26%) were Enterococcal species, 121 (32.88%) were other gram positive cocci and 143(38.85%) were gram negative bacilli.

In the present study, among 104 isolated Enterococcal species, 48 (46.15%) were from urine, 10 (9.6%%) from blood, 30 (28.8%) from pus and 16 (15.3%) from wound swab samples which was in concordant with the study by **Baragundi MC et al**[5] who observed that, out of 120 enterococcal isolates, 50(41.66%) were isolated from Urine sample, 35(29.1%) from blood sample, 20(16.66%) from pus sample and 15(12.5%) from wound swab samples. In both these studies showed that, Enterococci were commonly isolated more from urine samples,which was similar to the study by **Reza Faraji1**[85]**, Leblank et al**[52](36.6%)**.** The observation in the present study was more number of isolation of enterococcal species from the urine sample due to the normal residence of enterococci in the genitourinary tract.

In the present study, Enterococcal species were isolated from 13-45 years (36.53%) of age followed by 46-60 years (23%). Most of the enterococcal infections were associated with males 66(63.4%) than females 38(36.5%). According to **Kayoko Hayakasswa et al**[45] , mean age of the

enterococcal infection study population was in the range of 45-60 years,and also stated that most of the affected people in their study were male (53.6%). Analysis of adult-children distribution showed incidence of Enterococcal infections were more common in adults 84(80.76%) than children 20 (19.23%).

In the present study, among the 104 isolated enterococcal species, 77 (74.03%) were E.faecalis, 27(25.96%) were E. faecium. .No other enterococcal species isolated during the study period. This correlates with study done by **Sreeja et al**[94] **(**E.faecalis 76%,E.faecium 24%**), Emaneini et al**[63] E.faecalis (70.3%), E.faecium (29.7%)**, Lathika et al**[51] **(**E.faecalis 75%, E.faecium23%), **Sivasankari et al**[92] **(**E.faecalis (78.8%), E.faecium (21.2%)**, Reza Faraji**[85]**,** E.faecalis (72.4%), E.faecium (13.79%) **Mohammad Mehdi Soltan et al**[69] E.faecalis (70.%), E.faecium (30%)**,** in contrast to the study by **Niharika et al**[71] **(**E.faecalis 51.4%,E.faecium 48.6%), **Chandrim et al**[17] **(**E.faecalis 46.9%, E.faecium 33.3%), **Haiying et al**[35] E.faecalis (28.2%), E.faecium (53.8%)**.**

Comparative findings from various studies :

Study by	E.faecalis	E.faecium
In the present study	74.03%	25.96%
Sreeja et al	76%	24%
Emaneini et al	70.3%	29.7%
Lathika et al	75%	23%
Sivasankari et al	78.8%	21.2%
Reza Faraji1,	72.4%	13.79%
Mohammad Mehdi Soltan Dallal et al	70%	30%
Niharika et al	51.4%	48.6%
Chandrim et al	46,9%	33.3%
Haiying et al	28.2%	53.8%

With respect to isolation of various species of enterococci ,E.faecalis &E.faecium were only isolated in the present study which was in accordant to the study conducted by **Sreeja et al[(94)], Emaneini et al [(65)]& Sivasankari et al[(92)]** and in contrast to the study by **Reza Faraji1**[(85)]**,** (*E.faecalis* , *E.feacium* , *E. hirea*, *E avium*, *E. gallinarium* , and *E. mundtii)* **,Schouten et al [(95)](***E.faecalis* , *E.feacium* , *E. hirea*, *E durans*, *E. gallinarium* , and *E.casseliflavus).* **MM Salem Bekhit et al[(64)] (***E.faecalis* , *E.feacium* , *E. hirea*, *E. gallinarium* , and *E.casseliflavus).*

Most of the studies mentioned above and also in the present study E.faecalis was the predominant species isolated among Enterococcus. It was noted in this study and others mentioned above,there was increasing trend in

the prevalence of E.faecium in the clinical settings. (Previously E.faecalis to E.faecium ratio was 10:1).

In this study the *E. faecalis* and *E. feacium* were the most prevalent species isolated . The reasons for this are, these species are normal flora of the alimentary tract, vagina,and mouth while other species are mostly found in the environment and also their ability to acquire resistance to various antimicrobial drugs and possesing more variable virulent factors when compared to the other enterococcal species.

Among the total 104 enterococcal isolates,,66(63.46%) were HLGR, 29(27.8%) were HLSR and 24(23.07%) were resistant to both HLG and HLS. According to the study by **Sivasankari et al[92], Martha L. Sanchez et al [58]& Elango padmasini et al[23],** HLGR & HLSR were 50%&38.4% ,31%&42% and 42.7%,&29.8% respectively.

HLGR & HLSR AMONG ENTEROCOCCAL SPECIES :

Study by	HLGR	HLSR
In the present study	63.46%	27.8%
Sivasankari et al	50%	38.4%
Martha L. Sanchez et al	31%	42%
Elango padmasini et al	42.7%	29.8%

In the present study, among the isolated 104 enterococcal species high level gentamicin resistance (HLGR) were 66(63.46%) ,29 (27.88%) were high

level streptomycin resistance (HLSR) and 24(23.07) were both HLGR &HLSR. The reason for higher high level gentamicin resistance (HLGR) than high level streptomycin resistance (HLGR) could be due to restricted indication for usage of streptomycin.(like in tuberculosis treatment etc,)

In the present study, among the isolated 77 E.faecalis, 48(62.33%) were high level gentamicin resistance(HLGR) which was concordant with **Mithra et al[61], Sarika et al[87] ,Randhawa et al[83]** & **Mohammad Mehdi Soltan Dallal et al[69]** where it was 67.2%, 60% , 68% and 61.5% respectively but lower in the study conducted by **Maoddab et al[62], Sivasankari et al[92], C. Fernandes et al 2011[26],**it was13%.,48.7%&53.5% respectively.The presence of high level gentamicin resistance (HLGR) is predictive of loss of synergy between cell wall acting agents such as vancomycin or ampicillin and gentamicin .**(Murray et al,1998)**

HLGR AMONG E. faecalis :

Study by	HLGR in E.Faecalis (%)
In the present study	62.33%
Mithra et al	67.2%
Sarika et al	60%
Randhawa et al	68%
Mohammad Mehdi Soltan Dallal et al	61.5%

Among the isolated 77 E.faecalis, 20(25.97%) were high level streptomycin resistance (HLSR) which was comparable to the study by **Haiying et al[35]** & **Mithra et al [61]**27.4% & 22.7% respectively, but it was

higher in the study conducted by **Niharika et al**[71] and **Martha L Sanchez**[58], it was 60.7% &42% respectively.

HLSR AMONG E.FAECALIS :

Study by	HLSR in E.Faecalis (%)
In the present	25.97%
Haiying et al	27.4%
Mithra et al	22.7%
Niharika et al	60.7%
Mohammad Mehdi Soltan Dallal et al	42%

Among the isolated 77E.faecalis. both high level gentamicin resistance (HLGR) & high level streptomycin resistance(HLSR) were 16(20.77%) which was concordant with the study done by **Verma et al**[100] ,it was 30.4%,but not with the study by **Sarika et al**[87], **Gulcin et al**[30], **Randhawa et al**[83] & **Niharika et al**[71] where it was 54%, 36% 43% & 49.2% respectively.

BOTH HLGR & HLSR AMONG E. FAECALIS :

Study by	Both HLGR & HLSR in E.Faecalis (%)
In the present	**20.77%**
Verma et al	**30.4%**
Sarika et al	**54%**
Niharika et al	**49.2%**
Gulcin et al	**36%**
Randhawa et al	**43%**

In the present study,among the isolated 27 E.faecium, 18(66.6%) were high level gentamicin resistance(HLGR) which was in concordant with

the study conducted by **Sivasankari S et al[92],Sanal C. Fernandes et al 2013[26] & Mohammad Mehdi Soltan Dallal et al [69]** 54.6% ,53% &79% respectively ,but it was higher than the study conducted by **Mithra et al[61], Lathika et al[51], Carol et al[15] & Gulcin et al[30]** it was 32.8%, 40% ,32.6% & 48% respectively.

HLGR AMONG E. FAECIUM :

Study by	HLGR in E.faecium (%)
In the present	**66.6%**
Sivasankari S et al	**54.6%**
Sanal C. Fernandes et al	**53%**
Mohammad Mehdi et al	**79%**
Mithra et al	**32.8%**
Lathika et al	**40%**
Carol et al	**32.6%**
Gulcin et al	**48%**

Among the isolated 27 E.faecium ,9 (33.3%) were high level streptomycin resistance (HLSR) but it was low in the study done by **Maoddab et al[62]& Niharika et al[71],** it was 15% & 8.9% respectively and high in the study by **Sivasankari S et al[92], Sanal C. Fernandes et al 2013[26]**where they reported 54.8% & 58.8% respectively.

HLSR AMONG E.FAECIUM :

Study by	HLSR in E.Faecium(%)
In the present	33.3%
Maoddab et al	15%
Niharika et al	8.9%
Sivasankari S et al	54.8%
Sanal C. Fernandes et al	58.8%

Out of 27 E.faecium, both high level gentamicin resistance (HLGR) & high level streptomycin resistance (HLSR) were 8(29.6%).

In the present study high level gentamicin resistance (HLGR) among isolated enterococci were higher than the most of studies done all over india.The reason could be that our hospital is tertiary care level and usage of antibiotics are more, presence of intensive care units (medical,surgical paediatric etc),prolonged hospital stay for chronic disease.

In the present study high level gentamicin resistance (HLGR) was found higher in E .faecium than E.faecalis which was concordant to the study done by **Gordon et al[33] and Mendiratta et al[21].**

In this present study, out of 104 enterococcal isolates, 8 (7.69%) were vancomycin resistant. The prevalence of VRE in this study was 7.69% which was in par with the results shown by **Baragundi et al[5],** where they reported 7.5% of vancomycin resistant enterococci (VRE). and was lower than the study of **Karmarker MG et al[44]**, it was 23% .

In the present study, antimicrobial susceptibility results of E. faecium showed 70.4%% and E. faecalis showed 16.9% ampicillin resistance which was in concordant with a study conducted by **Salem-Bekhit etal2012[64] and Sivasankari.S et al[92]**where they reported 70.4%,15.7% and 72.7%,24.3% respectively. **Agarwal J et al[1]** have reported significantly higher resistance to ampicillin among E. faecium isolates which was similar to our study and also they documented multiple drug resistant enterococci which was a similar finding in the present study.in the present study it was found all HLGR enterococcal isolates were sensitive to linezolid & teicoplanin.

In the present study out of 104 enterococcal isolates 63.4% were high level gentamicin resistance (HLGR), where in the study conducted by **Haiying Niu et al,[35]Dr.Narayan Shrihari et al[22]** they reported lower rate 42.7%.,44.2% respectively. In this present study out of 104 enterococcal isolates 26.9% were high level streptomycin resistance (HLGR), where in the study conducted by **Haiying Niu etal[35],Dr.Narayan Shrihari etal[22]** they reported lower rate 42.7%.,44.2%.

In the present study all high level gentamicin resistance (HLGR) enterococcal isolates screened by high content disc (gentamicin 120 µg) ,were also showed Minimum inhibitory concentration >500µg/ml by E-test method and agar dilution method. This finding was also observed in the study conducted by **Martha L.Sanchez et al[58] .**According to CLSI guidelines screening method for high level gentamicin resistance (HLGR) are disc diffusion method with high content gentamicin disc (120 µg), agar dilution

method and broth dilution method. But in this study and study by **Martha L.Sanchez et al**[58] showed that the usefulness of E-test to predict high level gentamicin resistance (HLGR) among enterococci.This has to be evaluated by further studies.

In the present study all high level gentamicin resistance (HLGR) enterococcal isolates(n=66) were submitted for polymerase chain reaction for molecular characterization of the genes responsible for high level gentamicin resistance (HLGR). Result showed out of four genes i.e (***aac(6')-Ie-aph(2")-Ia, aph(2")-Ib, aph(2")-Ic, aph(2")-Id)*** that determine high level gentamicin resistance (HLGR) in enterococci, the bifunctional gene **aac(6')-Ie-aph(2")-Ia,** was alone detected .Other genes were not detected which was concordant with the study done by **Elango Padmasini et al**[23] **& Mohammad Mehdi Soltan Dallal et al**[69] in which they detected aac(6')-Ie-aph(2")-Ia, only, other genes were not detected in their enterococcal isolates.

According to the study done by **Haiying Niu et al** [35], they reported the prevalence of high level gentamicin resistance (HLGR) genes among enterococci were as follows, aac(6')-Ie-aph(2")-Ia(89.3%), aph(2")-Ib(0%) ,aph(2")-Ic(7.1%) aph(2")-Id(10.7%) .

According to **Joseph W Chow et al** [39] (clinical infectious Diseases 2000; 31:586-9), the prevalence of high level gentamicin resistance (HLGR) genes among enterococci were as follows, aac(6')-Ie-aph(2")-Ia(79%), aph (2")-Ib(5%), aph(2")-Ic(1.6%), aph(2")-Id (14.%). According to **Mithra Khani**

et al[58]**,** the prevalence were aac(6')-Ie-aph(2")-Ia(61.6%), other genes not evaluated in this study.

According to the the study done by **M.Emaneini B.Khoramian**, **et al** [65] the prevalence of high level gentamicin resistance gene was aac(6')-Ie-aph(2")-Ia, that was found in 96.2% (26/27) of the isolates.

In the present study and other studies it was concluded that the most prevalent gene that is responsible for high level gentamicin resistance (HLGR) among enterococcal species is **aac(6')-Ie-aph(2")-Ia.**

SUMMARY

- A total of 396 samples were collected to study the High level gentamicin resistance and its molecular characterization in Enterococcal isolates.
- A total of about 104 Enterococcal strains were isolated from clinical specimens. Majority of the Enterococcal isolates were from urine 46.15% followed by pus 28.84%, wound swab 15.38% and blood 9.6%.
- Isolation of Enterococci were more in adults 84(80.76%) than in the children 20 (19.23%) and also more in male patients 66(63.46%) when compared to female patients 38 (36.53%).
- E. faecalis was the predominant species isolated in the present study, with an isolation rate of about 77/104(74.03%), followed by E. faecium 27/104(25.96%). Other species of enterococci were not isolated and with the highest isolation rate of E. faecalis & E. faecium was in the urine samples.
- High level gentamicin resistance were seen in 63.4% of Enterococcal isolates by disc diffusion method and similar results were also seen in E-test method & agar dilution method.
- In antibiotic susceptibility testing(AST), E.faecium showed the following resistant pattern, penicillin (37%), ampicillin (29.6%),

ciprofloxacin (37%) doxycycline (59%), High level gentamicin (66.6%), high level streptomycin (29.6%), teicoplanin (7.4%), vancomycin (14.8%).

- In antibiotic susceptibility testing (AST), E.faecalis showed the following resistant pattern, penicillin (27.2%), ampicillin (16.8%), ciprofloxacin (31%), doxycycline (61%), high level gentamicin (62.3%), high level streptomycin (25.9%), teicoplanin (2.6%), Vancomycin (5.19%%).

- Resistance to both high level streptomycin (HLSR) and high level gentamicin (HLGR) were observed in 23.07% of isolates. The high level gentamicin resistance was higher in E. faecium 18/27 (66.6%) than in E. faecalis 62.3% (48/77).

- All the HLGR enterococcal isolates were sensitive to teicoplanin and linezolid.

- All the 66 high level gentamicin resistance isolates (E. faecalis-48 and E. faecium-18) were subjected to Polymerase Chain Reaction for the detection of resistance determining genes ***aac(6')-Ie-aph(2")-Ia , aph(2")-Ib, aph(2")-Ic, aph(2")-Id.***

- In PCR assay, out of four genes, the gene ***aac(6')-Ie-aph (2")-Ia*** was alone detected in all the isolates. This gene codes the bifuntional aminoglycoside modifying enzyme which confers resistant to all the commonly used aminoglycosides except streptomycin.

- The present study showed the ***aac(6')-Ie-aph(2")-Ia gene*** *was the most prevalent* gene present in Enterococci which was similar in other studies.
- Routine testing for HLGR Enterococci is necessary to reduce the long stay of the patient and transmission of resistant Enterococci to other patients.

CONCLUSION

- Enterococci are one among the common bacterial organisms causing serious health care associated infections at Govt.Rajaji Hospital (GRH), Madurai Medical College Madurai.
- Among 104 Enterococcal isolates from various specimen, E.faecalis 77 (74.03%) and E.faecium 27(25.96%) were isolated.
- Most of isolated enterococci were sensitive to teicoplanin and linezolid.(S >90%)
- High level gentamicin resistance were seen in 63.4% of Enterococcal isolates by disc diffusion method and similar results were also seen in E-test method & agar dilution method.
- High level gentamicin resistance in E.faecalis and E.faecium were 62.33% & 66.66% respectively.
- Vancomycin resistance in E.faecalis and E.faecium were 5.19%% & 14.8% respectively.
- All the High level gentamicin resistance enterococcal isolates were sensitive to teicoplanin(100%) and linezolid.(100%)
- In PCR assay, out of four genes, the gene ***aac(6')-Ie-aph(2")-Ia*** was alone detected in all the isolates. This gene codes the bifuntional aminoglycoside modifying enzyme which confers resistant to all the commonly used aminoglycosides except streptomycin resulting in

BIBLIOGRAPHY

1. Agarwal. J., Kalyan. R, Singh M. High level aminoglycoside resistance and beta lactamase production in enterococci at tertiary care hospital in India. Jpn J. Infec. Dis. 2009, 62, 158-159.
2. Abdul Razak SH, Hasan, Abbas A. Al Duliami, Kariem S. Al. Ajeely, Zainab H. Antimicrobiol susceptibility patterns of enterococcal isolates and its relevance with biofilms formation and beta lactamase production. Diyala Journal of Medicine vol 1 ; No. 1, 2011.
3. A.C. Dada, A. Ahmad, G. Usup, L.Y. Heng, R. HamidHigh-level aminoglycoside resistance and virulence characteristics among Enterococci isolated from recreational beaches in Malaysia Environ Monit Assess, 185 (2013), pp. 7427-7443
4. Baldassarri L., Creti R., Recchia S., Pataracchia M., Alfarone, G., Orefili, G., Compocia, D., Montanaro, L., & Arciola, C.R (2006). Virulence factors in enterococcal infections of orthopedic devices. Int. J Artif organs 29, 402-406.
5. Baragundi MC, Sonth SB, Solabannavar SS, Patil CS, Yemul VL.Species prevalence and antimicrobiol resistance pattern of enterococcal isolates in a tertiary health care centre. Journal of clinical and diagnostic research 2010 ; 4 : 3405 – 3409.

6. Bhat KG, Paul C, Bhat MG. High level aminoglycoside resistance in enterococci isolated from hospitalized patients. Indian J Med Res. 1997;105:198–9.
7. Bailey & Scott's Diagnostic Microbiology 13th edition, page No. 216.
8. Bailey & Scott's Diagnostic microbiology 13th endition, page No. 226.
9. Basics of clinical Microbiology by Patrick Murray,Tenth edition pg no. 1116-1119.
10. Bhat KG, Chitra P, Bhat M. High level aminoglycoside resistance in Enterococci isolated from hospitalized patients. *Indian J Med Res* 1997; 105:198-9
11. Brian L. Hollenbeck and Louis B. Rice, Intrinsic and acquired resistance mechanism in enterococcus Virulence 3:5, 421–433; August 15, 2012; G 2012 Landes Bioscience
12. Clinical and Laboratory standards Insitute (CLSI). Performance standards for antimicrobial disc susceptibility tests. Approved standard – MO2 – A11, M100-S25, MO2 ,MO7,- 2017, Wayne. PA, USA.
13. Clinical and Laboratory standards Institute (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, Approved standard -ninth edition, vol (34) 1 MO7 – A9, 2017 wayne, PA, USA.
14. Caron F, Pestel M, Kitzis MD, Lameland JF, Humbert G, Gutmaan L. Comparison of different beta lactams-glycopeptides-gentamicin

combinations for an experimental endocarditis caused by a highly glycopeptides resistant isolate of enterococcus faecium. J Infect Dis. 1995;171:106–12.

15. CAROL A.Laboratory Detection of High-Level Aminoglycoside-Aminocyclitol Resistance in Enterococcus spp. JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1988, p. 2270-2274
16. Catherine Barnhart, PharmD, Ronald Campbell Mechanism of amninoglycosides oct 2002
17. Chandrim Sengupta, High Level Gentamycin Resistance among *Enterococcus Species* Isolated From Clinical Samples in a Tertiary Care Hospital, Int J Med Health Sci. Oct 2015,Vol-4;Issue-4
18. Chow JW. Aminoglycoside resistance in enterococci. *Clinical Infectious Diseases*. 2000;31:586-9.
19. Cong-Ran Li,In vitro antibacterial activity of vertilmicin and its susceptibility to modification by the recombinant AAC(6)-APC(2) Enzyme, Antimicrobial agents and chemotherapy , nov 2018,p.3875-3882.
20. Davies J and Wright G. Bacterial Resistance to Aminoglycoside Antibiotics. *Trends in Microbiology* 1997;5:234-39.
21. DK Mendiratta, H Kaur V. Deotale, DC Thamke, Varang R, Narang P. Status of high level aminoglycoside resistant Enterococcus faecium and Enterococcus faecalis in a rural hospital of central India. Indian Journal of Med Microbiol (2008) 26(4) : 369-371.

22. Dr Narayan Shrihari, Dr. Kumudini T.S Speciation of enterococcal isolates and antibiotic susceptibility test including high level aminoglycoside resistance and minimum inhibitory concentration for vancomycin, Int J Biol Med Res. 2011; 2(4): 865 – 869

23. E. Padmasini, R. Padmaraj, S.S. RameshHigh level aminoglycoside resistance and distribution of aminoglycoside resistant genes among clinical isolates of *Enterococcus* species in Chennai, India Sci World J, 2014 (2014), p. 329157

24. Esther culebrasaminoglycoside resistance mediated by the bifunctional enzyme 6'-n-aminoglycoside acetyltransferase-2''-o-aminoglycoside phosphotransferase Frontiers in Bioscience 4, d1-8, January 1, 1999.

25. Facklam RR,Collins MD.Identification of Enterococcus species isolated from human infections by a Conventional test scheme.J Clin Microbiol 1989:27:731-4.

26. Fernandes SC, Dhanashree B. Drug resistance & virulence determinants in clinical isolates of Enterococcus species. Indian J Med Res. 2013 May;137(5):981-5

27. Goodman and Gilman's.Textbook of the Pharmacological Basis of Therapeutics. 12th edition pg no 1541

28. Goodman and Gillman's.Textbook of the Pharmacological Basis of Therapeutics. 12th edition pg no. 1539 .

29. Gupta V, Singla N. Antibiotic susceptibility pattern of Enterococci. Journal of clinical and diagnostic research 2007 ; 1 (5) : 385 – 388.

30. Gulcin balldir,High level resistance to aminoglycosides , vancomycin and linezolid in enterococcal strains, Journal of microbiology and infectious diseases,2013,3(3):100-103.

31. G.D. Wright**Aminoglycoside-modifying enzymes** Curr Opin Microbiol, 2 (1999), pp. 499-503

32. Gilbert D. Aminoglycosides. In: Mandell GL, Bennett JE, Dolin R, eds. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. 5th ed. Philadelphia: Churchill Livingstone, 2000:307-336.

33. Gordon S, Swenson JM, Hill BC, Piggot NE, Facklam RR, [3]Cooksey RC, et al. Antimicrobial susceptibility patterns of common and unusual species of enterococci causing infections in the United States. *J Clin Microbiol*. 1992;30:2373–78.

34. Harrison's principles of Internal medicine.18th edition.Vol 1.pg no 1180-1187

35. *Haiying Niu* The prevalence of aminoglycoside-modifying enzyme and virulence genes among enterococci with high-level aminoglycoside resistance in Inner Mongolia, China, Brazilian journal of micribiology 47 (2016) 691-696.

36. HD Isenberg , editor. Tests to detect high-level aminoglycoside resistance in enterococci. Vol. 1. Washington DC: American Society of Microbiol; 1992 Clinical microbiology procedure handbook; pp. 541–8.

37. H.P. Nepal, B. Khanal, A. Acharya, N. Gyawali, P.K. Jha, R. PaudelHigh-level gentamicin resistance and vancomycin resistance in clinical isolates of enterococci in a tertiary care hospital in eastern NepaL Nepal Med Coll J, 14 (2012), pp. 60-63.

38. J.W. Chow, M.J. Zervos, S.A. Lerner, *et al.*A novel gentamicin resistance gene in *Enterococcus* Antimicrob Agents Chemother, 41 (1997), pp. 511-514

39. Joseph W .Chow Aminoglycoside resistance in Enterococci,clinical infectious disease 2000;31:486-9s

40. Jett, BD, Huycke, MM & Gilmore, M.S. (1994) Virulence of Enterococci. Clin Microbiol Rev.7, 462 – 478.

41. Jyothi P, Metri B, Peerapur B. High level resistance toaminoglycosides in urinary isolates of enterococci. *Ann MedHealth Sci Res*. 2014;4(Special issue 1):S58–S59.9

42. Jones, M.E., Draghi, D.C, Thornsberry, C., Karlowsky, J.A., Sahm, D.F & Wenzel, R.P. (2004). Emerging Resistance among bacterial pathogens in the intensive care unit – a European study

43. J.J. Ferretti, K.S. Gilmore, P. CourvalinNucleotide sequence analysis of the gene specifying the bifunctional 6′-aminoglycoside acetyltransferase 2-aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and identification and cloning of gene regions specifying the two activities J Bacteriol, 167 (1986), pp. 631-638

44. Karmarkar MG, Gershom ES, Mehta PR, Enterococcal infections with special reference to phenotypic characterization and drug resistance. Indian J Med Res. 2004 ; 119 : 22-25.

45. Kayoko Hayakawa, Dror Marchaim, Emily T, Martin, Namita Tiwari et al (2012). Comparison of the clinical characteristics and outcomes associated with Vancomycin – Resistant Enterococcus faecalis and Vancomycin – Resistant E. Faecium bacteremia. Antimicrob. Agents chemother. 2012, 56(5) : 2452 – 2458.

46. K.J. Shaw, P.N. Rather, R.S. Hare, G.H. MillerMolecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes Microbiol Rev, 57 (1993), pp. 138-163

47. Koneman's Color Atlas and Text book of Diagnostic Microbiology, 6th edition, pg no. 1459-1460.

48. Koneman's Color Atlas and Textbook of Diagnostic Microbiology, 6th edition, page no. 1473.

49. Koneman's Color Atlas and Textbook of Diagnostic Microbiology – 6th edition, pg no. 700-704, 735,768-774

50. .K.J. Shaw, P.N. Rather, R.S. Hare, G.H. MillerMolecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifyingenzymes,Microbiol Rev, 57 (1993), pp. 138-163

51. Latika Shah prevalence of enterococci with higher resistance level in a tertiary care hospital: a matter of concern, National journal of medical research volume 2 issue 1 jan – march 2012

52. Leblank DJ, Lee LN, Inamin M, Cloning and nucleotide sequence analysis of aspectinomycin adenyltransferase AAD (9) determinant from Enterococcus faecalis. *Antimicrob agents chemother*, 1991; **35:** 1804-1810.

53. Mathur P, Kapil A, Chandra R, Sharma P, Das B. Antimicrobial resistance in *Enterococcus faecalis* at a tertiary care centre of northern India.

Indian J Med Res. 2003;118:25–8.

54. Mandell, Dougla's and Bennett's Principles and practice of infectious diseases. 8th edition, vol 2, pg no. 2328 – 2344

55. Mandell, Dougla's, Bennett's principle and practice of infectious disease – 7th edition, vol 1 : page no. 23.

56. Mandell's Dougla's, Bennett's principle and practice of infectious disease 7th edition, vol 2, page no. 2649-2651.

57. Monica Cheeshbrought-District Laboratory practice in tropical countries – Part II, pg no. 81, 125, 106.

58. Martha L. Sanchez, Useof the E-Test to predict high level resistance to aminoglycosides among enterococci, Journal of clinical microbiology .p 3030-3032.

59. Murray B.E (2000). Vancomycin – resistant enterococcal infections. N. Engl J Med. 342, 710 – 721.

60. Mingeot-Leclercq MP, Glupczynski Y, and Tulkens PM. Aminoglycosides: Activity and Resistance. *Antimicrobial Agents and Chemotherapy* 1999;43:727-37.

61. Mitra Khani Increasing Prevalence of Aminoglycoside-Resistant *Enterococcus faecalis* Isolates Due to the *aac(6')-aph(2")* Gene: A Therapeutic Problem in Kermanshah, Iran Jundishapur J Microbiol. 2016 March;9(3): e28923.

62. Moaddab SR Rafi A. prevalence of vancomycin and high level aminoglycoside resistant enterococci among high risk patients southeast asian J Trop Med Public Health 2003; 34;849-854.

63. M. Emaneini, R. Bigverdi, D. Kalantar, *et al.*Distribution of genes encoding tetracycline resistance and aminoglycoside modifying enzymes in *Staphylococcus aureus* strains isolated from a burn center Ann Burns Fire Disasters, 26 (2013), pp. 76-80

64. MM Salem – Bekhit, IMI Moussa, MM Muharram, FK Alanazy, HM Hefni. Prevalence and Antimicrobial resistance pattern of multidrug resistant enterococci isolated from clinical specimens. Indian Journal Med Microbiol 2012, 30(1) : 44-51.

65. M.Emaneini b.khoramian Prevalence of high-level gentamicin-resistant Enterococcus faecalis and Enterococcus faecium in an Iranian hospital J PREV MED HYG 2016; 57: E197-E200

66. .M.M. Feizabadi, P. Maleknejad, A. Asgharzadeh, S. Asadi, L. Shokrzadeh,

S. Sayadi Prevalence of aminoglycoside-modifying enzymes genes among isolates of *Enterococcus faecalis* and *Enterococcus faecium* in Iran Microb Drug Resist, 12 (2006), pp. 265-268

67. M. Sepúlveda, H. Bello, M. Domínguez, S. Mella, R. Zemelman, G. GonzálezMolecular identification of aminoglycoside-modifying enzymes among strains of *Enterococcus* spp. isolated in hospitals of the VIII Region of Chile Rev Med Chil, 135 (2007), pp. 566-572.

68. Mohanty S, Jose S, Singhal R, Sood S, Dhawan B, Das BK et al. Species prevalence and antimicrobial susceptibility of enterococci isolated in a tertiary care hospital of North India. South east Asian J. Troop Med Public Health 2005 ; 36 : 962 – 965.

69. Mohammad Mehdi Soltan Dallal High-Level Gentamicin-Resistant Enterococcal Isolates from Urinary Tract Infection in Iran. Infectious Diseases in Clinical Practice Volume 16, Number 1, January 2008

70. Moellering, R. C., O. M. Korzeniowski, M. A. Sande, and C. BWennersten. 1979. Species-specific resistance to antimicrobial synergism in Sireptococcus faecium and Streptococcus faecalis.J. Infect. Dis. 140:203-208.

71. Niharika Lall high level aminoglycoside resistant enterococcus species : a study Int J Cur Res Rev, Feb 2014/ Vol 06 (03) Page 17.

72. Opal S, Mayer K, and Medeiros A. Mechanisms of Bacterial Antibiotic Resistance. In: Mandell GL, Bennett JE, Dolin R, eds. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. 5th ed. Philadelphia: Churchill Livingstone, 2000:236-252.

73. Paterson D, Bodman J, Thong ML. High level aminoglycoside resistant Enterococcal blood culture isolates. Comm Dis Intell. 1996;20:532–5.

74. PM Giridhara Upadhyaya, KL Ravikumar, BL Umapathy. Indian Journal of Med Microbiol (2009) 27 (4) : 301 – 5.

75. Praharaj Ira, Sistla Sujatha, Parija Subash Chandra. Virulence factors in clinical and commensal isolates of enterococcus species. Indian Journal of pathology and microbiology 56 (1) Jan- Mar 2013.

76. Patterson JE, Zervos M. High-level gentamicin resistance in Enterococcus: Microbiology, genetic basis and epidemiology. *Rev Infect Dis* 1990; 12:644-51.

77. Pooja A Kamaria,Prevalence and antibiotic resistance pattern of clinical isolates of enterococci from blood samples in a tertiary care hospital, jamna gar, Gujarat, India.

78. Praharaj Ira, Sistla Sujatha, Parija Subash Chandra. Virulence factors in clinical and commensal isolates of enterococcus species. Indian Journal of pathology and microbiology 56 (1) Jan- Mar 2013.

79. Prakash V.P. (2005) Clinical prevalence, identification and molecular characterisation of Enterococci .

80. P. Jyothi, B. Metri, B. PeerapurHigh level resistance to aminoglycosides in urinary isolates of enterococci Ann Med Health Sci Res, 4 (Special issue 1) (2014), pp. S58-S59

81. Shaw KJ, Rather PN, Hare RS, et al. Molecular Genetics of Aminoglycoside Resistance Genes and Familial Relationships of the Aminoglycoside-Modifying Enzymes. *MicrobiologicalReviews* 1993; 57:138-63.

82. Rahangdale VA, Agarwal G, Jalgaonkar SV. Study of antimicrobial resistance in Enterococci. Indian Journal Med Microbiol (2007) vol 26, pg no. 285 – 287.

83. Randhawa VS, Kapoor L, Singh V, Mehta G. Aminoglycoside resistance in enterococci isolated from paediatric septicaemia in a tertiary care hospital in north India. Indian J Med Res. 2004;119:77–9

84. Renata O Soares, Ana claudia Fedi, Keli C Reiter, Juliana Caierao, Pedro A d'Azevedo Liandes. Bioscience virulence (2014) 5 : 5, 634-637.

85. Reza Faraji Prevalence and Antibiotic Resistance of *Enterococci* Species Isolated from Clinical Samples journal of pure and applied microbiology, Nov 2015. Vol. 9(Spl. Edn. 2), p. 153-159

86. Richards, M.J. Edwards, J.R. Culver, D.H & Gaynes, R.P. (2000). Nosocomial infections in combined medical – surgical intensive care units in the United States. Infect Control Hosp Epidemiol 21,pg no 510-515.

87. Sarika Jain, Ckinicoepidemiological profile and high level aminoglycoside resistance in enterococcal septicaemia from a tertiary care hospital east Delhi, International Journal of basic medical sciences,2011.vol 1(2): 354-360.

88. Srujana Mohanty, Swapna Jose, Ritu singhal ,Seema Sood, Benu Dhawan et al. Species prevalence and antimicrobiol susceptibility of enterococci isolated in a Tertiary care hospital of North India. South east Asian J Trop Med Public health 2005; 36(4) : 962-965.

89. S. Jain, A. Kumar, B. Kashyap, I.R. KaurClinico-epidemiological profile and high-level aminoglycoside resistance in enterococcal septicemia from a tertiary care hospital in east Delhi Int J Appl Basic Med Res, 1 (2011), pp. 80-83

90. S.F. Tsai, M.J. Zervos, D.B. Clewell, S.M. Donabedian, D.F. Sahm, J.W. Chow A new high-level gentamicin resistance gene: *aph(2)-Id*, in *Enterococcus* spp. Antimicrob Agents Chemother, 42 (1998), pp. 1229-1232

91. Seema sood, Meenakshi Malhotra, B.K. Das, Arti Kapil. Enterococcal infections and antimicrobial resistance. Indian J Med. Res. 128, Aug 2008, pg 111-121

92. Sivasankari S,Detection of high level aminoglycosides resistant enterococci in a tertiary care hospital,Journal 0f pharmacy and biological sciences ,volume 1, issue 5,dec 2013,p.53-57

93. Srujana Mohanty, Swapna Jose, Ritu singhal ,Seema Sood, Benu Dhawan et al. Species prevalence and antimicrobiol susceptibility of enterococci isolated in a Tertiary care hospital of North India. South east Asian J Trop Med Public health 2005; 36(4) : 962-965.

94. Sreeja S, Babu PRS, Prathab AG, The prevalenceand the characterization of the enterococcusspecies from various clinical samples in a tertiary *J Clin Diagn Res*, 2012; **6:** 1486

95. Schouten MA, Hoogkamp-Korstanje JAA,Meis JFG and Voss A, Prevalence of vancomycin resistant Enterococciin Europe. *Eur J ClinMicrobiol Infect Dis*, 2000; **19:** 816-822

96. Topley & Wilson's microbiology & microbial infections. Vol I pg no. 3,6,10, 517.

97. Topley & Wilson's Microbiology & Microbiol infections Vol II – pg no. 882 – 897.

98. Udo EE, AL-Sweih N, Philips OA, Chugh TD. Species prevalence and antibacterial resistance of Enterococci isolated in Kuwait hospitals. J Med Microbiol. 2003;52:163–8.

99. Uttley,A.H.C.,Collins,C.H.,et al 1988.Vancomycin resistant enterococci.The lancet,I,57-58.

100. Verma D, Sinha S.Prevalence and comparison of high-level aminoglycoside resistance invancomycin-sensitive and vancomycin resistant *Enterococcus* at atertiary care hospital in Rohillkhand region *Int J Med Res Rev*2015;3(10):1162-1166.

101. Zarrilli Molecular epidemiology of high-level aminoglycoside-resistant enterococci isolated from patients in a university hospital in southern Italy journal of Antimicrobial Chemotherapy (2005) 56, 827–835

102. YA Marothi, H Agnihotri, Dubey D. Enterococcal resistance – an overview. Indian Journal of Med Microbial (2005), 23 (4) :214 – 219.

.

loss of synergism between cell wall acting drugs and aminoglycosides except streptomycin.

- The higher prevalence of High level gentamicin resistant enterococci have posed serious problem in the management serious enterococcal infection where aminoglycosides (gentamicin or streptomycin) is indicated along with the cell wall acting agents, thus limiting the therapeutic options.
- Judicious use of aminoglycosides, and regular surveillance of all Enterococcal isolates for High level gentamicin resistance is necessary for the prevention of nosocomial transmission of resistant strain.
- Appropriate surveillance, stringent infection control practice and hospital infection control committee guidance is very important to control the spread of High level gentamicin resistant Enterococci.

ANNEXURE-1

PREPARATION OF GRAM STAIN:

GRAM STAIN REAGENTS

1. Methyl violet – Primary stain

    Methyl violet 10g

    95% ethyl alcohol 100ml.

    Distilled water 1L

2. Gram's lodine – Mordant

    lodine 10g

    Potassium lodide 20g

    Distilled water 1 L

3. Acetone – Decolouriser

4. Dilute CarbolFushsin – Counter stain

    Basic fushsin 0.3 g

    95% Ethyl alcohol 10 ml

    Phenol crystals, melted 5 ml

    Distilled water 95 ml

Basic fuchsin was dissolved in alcohol 5% phenol solution was added and was allowed to stand overnight. Then the solution filtered through coarse filter paper.

ANNEXURE-2

PREPARATION OF MEDIA

PREPARATION OF NUTRIENT AGAR

Contents:

- Peptone – 5 g
- Beef extract –1.5 g
- Yeast extract –1.5 g
- Sodium choloride – 5 g
- Agar – 15g

28 g of the contents were suspended in 1000 ml of distilled water. It was heated to boiling to dispense the medium completely. Medium was sterilized by autoclaving at 121 degree C at 15 lbs pressure for 15 minutes.

PREPARATION OF BLOOD AGAR

Nutrient agar 100 ml

Sheep blood (defibrinated) 10 ml

- The sterile nutrient agar was melted by steaming and cooled to 45 deg C
- 5%-10% sheep blood was added aseptically with constant shaking.
- The blood was mixed with molten nutrient agar thoroughly but gently, to avoid froth formation. To remove the bubbles, media was flamed.
- Immediately poured into petri dishes and allowed to set.

PREPARATION OF MUELLER – HINTON AGAR Contents:

Beef extract 2.0 gm

Acidicase peptone 17.5 gm

Starch 1.5 gm

Agar 17.0 gm

Distilled water 1000 ml

Final pH 7.4+0.2

Dissolved the ingredients in one liter of distilled water.Mixed thoroughly. Heated with frequent agitation and boiled for one minute. Dispensed and sterilized by autoclaving at 121 deg. C for 15 minutes.Should not be overheated.When remelting the sterile medium, heated as briefly as possible.

CONSENT FORM

நோயாளியின் பெயர்: _____ வயது: _____ இனம்: _____

விலாசம்: _____

தகவல் அளிக்கப்பட்டஒப்புதல் படிவம்

மேற்குறிப்பிட்டமருத்துவஆய்வில் ஓர் பங்கேற்பாளராகசேர்க்கப்பட்ட இதன் மூலம் நான் சுதந்திரமாகஎன் ஒப்புதலைஅளிக்கிறேன்.

இந்தமருத்துவஆய்வின் நோக்கம் மற்றும் முக்கியத்துவம் பற்றிமற்றும் அதனால் ஏற்படும் எனது பொறுப்புகள் பற்றி எனக்கு தகவல் தெரிவிக்கின்றார். இதோடு கூடுதலாக,நான்

தேதியிட்டஎனக்கு அளிக்கப்பட்ட நோயாளிக்கான தகவல் தாள் மற்றும் தகவல் அளிக்கப்பட்ட ஒப்புதல் படிவத்தில் அடங்கியவிபரங்கள் பற்றிபடித்து புரிந்து கொண்டுள்ளேன். மருத்துவர் போதிய மற்றும் விரிவானவ ிதத்தில் என் பங்கேற்பு பற்றித் தீர்மானிக்க எனக்குப் போதிய நேரம் இருந்தது.

இந்தமருத்துவ ஆய்வுநடத்தப்பட்ட மிக முக்கிமானதாக என் மருத்துவரின் குறிப்புகளை நான் பின்பற்றுவேன். எந்த காரணமும் அளிக்காமல், எனக்கு எந்த நஷ்டமும் ஏற்படாமல் எந்த நேரத்திலும் ஆய்வை விட்டுவிலக எனக்கு உரிமை உண்டு.

இந்தமருத்துவ ஆய்வில் சேகரிக்கப்படும் எனது சொந்த தகவல், குறிப்பாக எனது மருத்துவ ரெகார்டுகளில் எனதுபெயர் மற்றும் பாலினம் மற்றும் இனம் குறிக்கப்படும் என்பதற்கு நான் சம்மதிக்கிறேன் இந்ததகவல் ஆனது

1. எலக்ட்ரானிகல் முறையில் அல்லதுஒருபகுதிகாகிதவடிவில் பதிவுசெய்யப்படும் பத்திரமாகவைக்கப்படும் மற்றும் மதிப்பீடுசெய்யப்படும்.
2. விஞ்ஞான மதிப்பீடு மற்றும் கூடுதல் விஞ்ஞான உபயோகத்திற்காக மற்றும் அளிக்கப்படும்.
3. உகந்த தேசிய மற்றும் சர்வதேச ரெகுலேட்டரி அதாரிட்டிகளுக்கு அனுப்பப்படும்.

இதோடு மட்டுமின்றி அங்கீகரிக்கப்பட்ட பிரதிநிதிகள் எனது சொந்த விபரங்கள் உடனான மருத்துவ ரெகார்டுகளை பரிசோதிக்கலாம். விஞ்ஞான மதிப்பீடு மற்றும் மருத்துவ ஆய்வின் செயல் திறனுக்காக தகவலை முழுமையாக சரியாகப் பரிமாற்றம் செய்ய இது உதவுகிறது.

நான் இந்தஆய்வில் இதுவரை பங்கேற்று இருக்கவில்லை மற்றும் இந்த ஆய்வு ஆரம்பிக்கும் முன்பு 30 நாட்களில் நான் மற்றொரு ஆய்வில் பங்கேற்றிருக்கவில்லை என்பதை உறுதி செய்கிறேன்.

நோயாளிக்கான தகவல் தாளின் ஒரு அசல் உடன் கையெழுத்ததிட தகவல் அளிக்கப்பட்ட ஒப்புதல் படிவத்தை நான் பெற்றுள்ளேன்.

நோயாளி:

_____ _____ _____

பெயர் பெரிய எழுத்துகளில் கையெழுத்து தேதி

சாட்சி:

_____ _____ _____

பெயர் பெரியஎழுத்துகளில் கையெழுத்து தேதி

நோயாளிக்கு உறவுமுறை: _____

நான் டாக்டர் _____ மேற்கண்ட பெயருடைய நோயாளிக்கு ஆய்வின் நோக்கம் மற்றும் தன்மைபற்றி விளக்கியுள்ளேன் என்பதை உறுதி செய்கிறேன். மேலும் நான் அனைத்து ஆய்வுசம்பந்தப்பட்ட கேள்விகளுக்கும் பதில்கள் அளித்துள்ளேன். மற்றும் ஆய்வின் நிபந்தனைகளை அவர்களுக்கு விளக்கியுள்ளேன் என்பதை உறுதி செய்கிறேன்.

மருத்துவர்: _____ _____ _____

பெயர் பெரிய எழுத்துகளில் கையெழுத்து தேதி

PROFORMA

Name:	Serial No:
Age:	Lab No:
Sex:	OP/IP No:
Education:	D.O.A:
Occupation:	D.O.D:
Income:	Provisional Diagnosis:
Address:	

Chief complaints:

Fever

Dysurea

Frequency

Urgency

Lower abdominal/ flank pain

H/O Present illness:

Associated conditions- instrumentation/ surgery in urinary tract

Calculi

Diabetes mellitus

Chronic kidney and liver diseases

Benign Prostatic Hypertrophy

Pregnancy

Immuno compromised state

Treatment History: H/O anti biotic intake, duration

Past History: H/O Similar episode in the past

Instrumentation/ surgery in urinary tract

Family History:

Personal History:

General Examination: Stature, nourishment, anaemia, jaundice, cyanosis, clubbing, lymphadenopathy, pedal edema.

Vital signs: Temperature, pulse rate, respiratory rate, blood pressure.

Systemic examination: Abdomen

Inspection: shape of the abdomen

Position of the umbilicus

Movements of the abdominal wall

Skin and surface of the abdomen

Palpation : Mass

Tenderness (Suprapubic)

Rigidity

Organomegaly

Percussion : Any free fluid

Auscultation : Bowel sounds

Bruit

Examination of groin and genital region

P/V:

P/R:

Examination of other systems

CVS: RS; CNS:

Definitive Diagnosis

WORKSHEET

Specimen: Urine

Method of collection : MSU/Indwelling catheter/Cystoscope/Suprapubic aspiration

I. Macroscopic Examination: Color

Turbidity

II. Microscopic Examination: Wet mount

Gram staining

III. Culture : Nutrient agar

MacConkey agar

Blood agar

CLED agar

IV. Biochemical Reactions:

Gram staining :

Motility :

Catalase :

Sugar fermentation tests :

Arginine hydrolysis test :

Special Tests:

Micro organism isolated :

V. Anti Microbial Susceptibility test:

VI. Screening for HLGR 1. Antibiogram(resistant with 120µg disc o gentamicin, E-test , agar dilution test

VII Confirmation of HLGR and molecular characterization by genotypic method (PCR)

						ANTIBIOGRAM								
S.NO:	LAB ID NO:	AGE	SEX	SPECIMEN/SPECIES	CLINICAL DIAGNOSIS	AMPI	CIP	DOXY	HLS	HLG	TEICO	VAN	PEN	HLGR (PCR)
1	3970	30	M	E.faecalis-U	UTI	S	R	R	R	R	S	S	s	aac(6)-Ie-aph(2)-Ia.
2	1592	65	F	E.faecium-P	LEG ULCER	R	S	S	R	R	S	S	s	aac(6)-Ie-aph(2)-Ia.
3	3339	8 MON	Fch	E.faecalis-B	PUO	R	R	S	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
4	4012	18	M	E.faecalis-U	CKD	S	S	R	S	S	R	S	S	
5	1603	64	Mch	E.faecalis-P	INJURY	S	R	R	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
6	1634	54	F	E.faecalis-P	DIABETIC ULCER	S	R	R	R	S	S	S	S	
7	4108	69	Mch	E.faecium-U	UTI	R	R	S	R	R	S	R	S	aac(6)-Ie-aph(2)-Ia.
8	1643	29	F	E.faecalis-U	UTI	S	S	R	S	S	S	S	S	
9	3372	67	Fch	E.faecalis-B	PUO	S	R	R	S	S	S	S	S	
10	4302	51	M	E.faecalis-U	UTI	R	R	S	S	R	S	R	S	aac(6)-Ie-aph(2)-Ia.
11	3398	7	Mch	E.faecium-B	PUO	R	R	R	R	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
12	4318	71	F	E.faecalis-U	UTI	S	R	R	S	S	S	S	S	

13	3409	60	M	E.faecalis-B	PUO	S	S	R	R	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
14	3421	19	F	E.faecalis-B	ENTERIC FEVER	S	R	R	S	R	R	R	R	aac(6)-Ie-aph(2)-Ia.
15	1704	57	F	E.faecalis-P	WOUND INFECTION	S	R	R	S	S	S	S	R	
16	1726	44	F	E.faecium-WS	POST OP WI	R	R	S	R	S	S	S	S	
17	4354	74	F	E.faecalis-U	UTI	R	S	S	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
18	1765	38	M	E.faecalis-P	ABSCESS-THIGH	S	R	R	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
19	4390	46	M	E.faecalis –U	UTI	R	S	S	R	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
20	1801	65	F	E.faecalis-WS	WOUND INFECTION	S	R	R	R	S	S	S	S	
21	4441	60	F	E.faecalis-U	UTI	S	R	R	S	S	S	S	S	
22	4469	55	M	E.faecium-P	ABSCESS	R	R	R	R	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
23	4482	16	M	E.faecalis-U	UTI	S	R	R	R	S	S	S	S	
24	4507	62	M	E.faecalis –U	PUO	R	R	S	R	S	S	S	S	
25	1857	19	F	E.faecalis-P	DM-ULCER	S	S	R	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
26	4568	45	F	E.faecalis-U	CKD	R	R	R	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
27	3488	19	M	E.faecalis-B	PUO	S	R	R	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
28	1902	53	M	E.faecalis-P	ABSCESS-LT LEG	S	R	S	S	S	S	R	S	

29	3503	69	F	E.faecium-B	PUO	R	R	R	R	R	S	R	S	aac(6)-Ie-aph(2)-Ia.
30	4605	67	M	E.faecalis-U	CYSTITIS	S	R	R	R	S	S	S	S	
31	1989	31	F	E.faecium-P	INJURY-RA	R	R	S	R	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
32	3575	50	F	E.faecalis –B	FFE	R	S	S	R	S	S	S	S	
33	4684	7	Fch	E.faecalis-U	PUO	S	R	R	S	R	R	S	S	aac(6)-Ie-aph(2)-Ia.
34	3611	72	M	E.faecalis –B	FFE	R	R	S	S	S	S	S	S	
35	3675	12	Mch	E.faecium-P	ABSCESS	R	S	S	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
36	4722	19	M	E.faecalis –U	UTI	R	S	R	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
37	2008	46	M	E.faecalis-WS	POST OP WI	S	R	R	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
38	3690	80	Mch	E.faecalis-U	ENTERIC FEVER	S	R	R	S	S	S	S	R	
39	4782	24	F	E.faecalis-U	PID	S	R	R	R	S	S	S	S	
40	2024	71	F	E.faecalis-WS	WOUND INFECTION	S	R	R	S	R	S	R	S	aac(6)-Ie-aph(2)-Ia.
41	2046	60	F	E.faecalis-P	ABSCESS	S	S	R	S	S	S	S	S	
42	2111	20	M	E.faecalis –P	WOUND INFECTION	S	S	S	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
43	2243	62	F	E.faecalis-WS	POST OP WI	S	R	S	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
44	2279	7	Mch	E.faecalis-P	ULCER	S	R	R	R	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
45	4814	31	F	E.faecalis-U	UTI	R	R	R	R	S	S	S	S	
46	3732	52	F	E.faecalis-U	SEPTICEMIA	R	S	S	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.

47	4856	15	M	E.faecium –P	ABSCESS	R	R	S	R	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
48	4912	13	F	E.faecalis-U	PUO	S	R	R	R	S	S	S	S	
49	5045	47	M	E.faecalis –U	UTI	R	R	R	R	R	S	R	S	aac(6)-Ie-aph(2)-Ia.
50	2318	24	F	E.faecium-P	ULCER	R	S	R	R	S	S	S	R	
51	2434	5	Mch	E.faecalis-P	WOUND INFECTION	S	R	R	S	R	R	S	R	aac(6)-Ie-aph(2)-Ia.
52	5132	52	F	E.faecalis –U	PUO	S	R	R	S	S	S	S	R	
53	5189	33	M	E.faecalis-U	CKD	R	R	S	R	S	S	S	R	
54	2566	63	M	E.faecium-WS	SURGICAL SITE INFECTION	R	R	R	R	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
55	3817	52	M	E.faecium-B	SEPSIS	R	R	R	R	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
56	2610	28	M	E.faecalis-WS	ULCER	S	S	R	S	S	S	S	S	
57	3972	20	F	E.faecium-U	PUO	R	S	R	R	R	R	R	S	aac(6)-Ie-aph(2)-Ia.
58	4015	5	Mch	E.faecalis-U	PUO	S	R	R	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
59	2721	59	F	E.faecalis –P	ABSCESS	R	R	R	S	R	S	S	R	aac(6)-Ie-aph(2)-Ia.
60	5416	49	M	E.faecalis-U	UTI	R	S	R	S	S	S	S	R	
61	2914	17	M	E.faecalis-P	ULCER	S	R	R	R	R	S	S	R	aac(6)-Ie-aph(2)-Ia.
62	5603	67	F	E.faecalis-U	UTI	S	R	S	S	R	S	S	R	aac(6)-Ie-aph(2)-Ia.
63	3001	74	M	E.faecalis-WS	WOUND INFECTION	S	R	R	S	S	S	S	S	

64	5712	41	F	E.faecium-U	UTI	R	R	S	R	S	S	S	S	
65	5841	18	F	E.faecium-U	UTI	R	R	S	R	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
66	5985	8	Fch	E.faecalis-U	PUO	R	R	R	R	R	S	S	R	aac(6)-Ie-aph(2)-Ia.
67	3074	51	F	E.faecalis-P	WOUND INFECTION	S	S	S	S	R	S	S	R	aac(6)-Ie-aph(2)-Ia.
68	6092	26	M	E.faecalis-U	UTI	S	R	R	S	S	S	S	R	
69	6149	57	M	E.faecium-U	CKD	R	S	R	R	R	S	R	R	aac(6)-Ie-aph(2)-Ia.
70	4235	7	Mch	E.faecalis-U	SEPSIS	R	R	R	R	S	S	S	R	
71	6212	25	M	E.faecium-U	PID	R	R	R	R	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
72	6336	45	M	E.faecalis –U	PUO	S	S	S	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
73	3118	7	Mch	E.faecalis-WS	POST OP WI	S	R	R	S	R	S	S	R	aac(6)-Ie-aph(2)-Ia.
74	3190	18	M	E.faecalis -WS	WOUND-LL	S	S	R	R	R	S	S	R	aac(6)-Ie-aph(2)-Ia.
75	4444	49	M	E.faecium-U	FFE	R	R	S	R	S	S	S	S	
76	3263	28	F	E.faecalis-WS	ULCER	R	S	S	R	R	R	S	R	aac(6)-Ie-aph(2)-Ia.
77	6474	72	M	E.faeium-U	UTI	R	R	R	R	R	S	R	S	aac(6)-Ie-aph(2)-Ia.
78	6510	39	M	E.faecalis-U	UTI	S	R	R	S	R	S	S	R	aac(6)-Ie-aph(2)-Ia.
79	6596	3 months	Mch	E.faecalis-U	SEPTICEMIA	S	R	R	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
80	4613	50	M	E.faecalis-U	ENTERIC FEVER	S	R	R	S	R	S	S	R	aac(6)-Ie-aph(2)-Ia.
81	6640	39	F	E.faecium-U	PID	R	R	S	R	R	S	R	R	aac(6)-Ie-aph(2)-Ia.

82	6712	3	M	E.faecalis-U	FFE	S	S	R	R	S	S	S	R	
83	3352	26	M	E.faecalis-WS	WOUND INFECTION	S	R	R	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
84	3411	69	M	E.faecalis-P	CVA WITH ULCER	S	R	R	S	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
85	3619	43	M	E.faecium-P	CELLULITIS	R	R	R	R	S	S	S	S	
86	5114	27	M	E.faecium-U	UTI	R	R	S	R	S	S	S	S	
87	6875	66	M	E.faecalis-U	UTI	S	R	R	R	S	S	S	S	
88	6891	8	Mch	E.faecalis-P	PUO	S	S	S	S	R	S	S	R	aac(6)-Ie-aph(2)-Ia.
89	3706	44	M	E.faecalis-P	ULCER	S	R	R	S	R	S	S	R	aac(6)-Ie-aph(2)-Ia.
90	5484	65	M	E.faecium-P	PUS	S	R	R	R	R	R	R	S	aac(6)-Ie-aph(2)-Ia.
91	3784	3	Mch	E.faecalis-WS	WOUND INFECTION	S	R	R	S	R	S	S	R	aac(6)-Ie-aph(2)-Ia.
92	6959	30	M	E.faecium-U	UTI	S	R	R	R	S	S	S	R	
93	5808	14	M	E.faecalis-P	PUS	S	S	S	S	R	R	S	S	aac(6)-Ie-aph(2)-Ia.
94	6992	43	M	E.faecalis-U	PUO	S	R	R	R	R	S	R	R	aac(6)-Ie-aph(2)-Ia.
95	3819	71	M	E.faecalis-WS	POST OP WI	R	R	R	R	R	S	S	S	aac(6)-Ie-aph(2)-Ia.
96	7013	25	M	E.faecalis-P	ABSCESS	S	R	R	S	R	S	S	R	aac(6)-Ie-aph(2)-Ia.
97	3856	18	F	E.faecium-P	ABSCESS	S	S	S	R	S	S	R	S	
98	3872	50	F	E.faecium-WS	WOUND INFECTION	S	R	R	R	S	S	S	R	

99	7046	6	Fch	E.faecalis-U	UTI	S	R	S	R	R	R	S	S	aac(6)-Ie-aph(2)-Ia.
100	6111	11	Mch	E.faecalis-P	PUS	S	S	S	R	S	S	S	R	
101	3892	24	M	E.faecium-WS	WOUND INFECTION	R	R	R	R	R	S	R	R	aac(6)-Ie-aph(2)-Ia.
102	6179	19	M	E.faecalis –U	UTI	S	R	R	S	R	R	S	S	aac(6)-Ie-aph(2)-Ia.
103	3900	57	M	E.faecalis-P	CHRONIC OM	S	R	R	S	R	S	S	R	aac(6)-Ie-aph(2)-Ia.
104	6202	50	M	E.faecalis-P	UTI	S	R	R	R	R	S	S	S	aac(6)-Ie-aph(2)-Ia.

MADURAI MEDICAL COLLEGE
MADURAI, TAMILNADU, INDIA -625 020
(Affiliated to The Tamilnadu Dr.MGR Medical University, Chennai, Tamil Nadu)

ETHICS COMMITTEE CERTIFICATE

Name of the Candidate	:	Dr.J.Vijay anand
Course	:	PG in MD., Microbiology
Period of Study	:	2015-2018
College	:	MADURAI MEDICAL COLLEGE
Research Topic	:	A study of detection of high level gentamycin resistant among enterococcal species and its molecular characterization in a Tertiary care centre
Ethical Committee as on	:	17.03.2017

The Ethics Committee, Madurai Medical College has decided to inform that your Research proposal is accepted.

Member Secretary

Chairman

Prof.Dr.V.Nagaraajan
M.D., MNAMS, D.M., Dsc.,(Neuro), Dsc.(Hon)
CHAIRMAN
IEC - Madurai Medical College
Madurai

Dean / Convenor

DEAN
Madurai Medical College
Madurai-20

URKUND

Urkund Analysis Result

Analysed Document: INTRO to CONCLU.docx (D31324476)
Submitted: 10/15/2017 10:29:00 AM
Submitted By: shrianand2010@yahoo.com
Significance: 10 %

Sources included in the report:

review of literature.docx (D31286984)
methods &material (2).docx (D31286982)
dddiscussion.docx (D31286871)
A STUDY OF ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF ENTEROCOCCUS SPECIES WITH SPECIAL REFERENCE TO VANCOMYCIN RESISTANCE BY PHENOTYPIC AND GENOTYPIC METHODS FROM VARIOUS CLINICAL SAMPLES IN A TERTIARY CARE HOSPITAL.docx (D30298042)

Instances where selected sources appear:

20

CERTIFICATE –II

This is certify that this dissertation work titled **A STUDY ON DETECTION OF HIGH LEVEL GENTAMICIN RESISTANT AMONG ENTEROCOCCAL SPECIES AND ITS MOLECULAR CHARACTERIZATION IN A TERTIARY CARE CENTRE** 0f the candidate Dr. J. Vijay anand with registration Number 2015141102 for the award of M.D; in the branch of MICROBIOLOGY. I personally verified the urkund .com website for the purpose of plagiarism check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows 10% percentage of plagiarism in the dissertation.

Guide & supervisor sign with seal