DETECTION OF VANCOMYCIN RESISTANT ENTEROCOCCI WITH VAN A GENOTYPE IN CLINICAL ISOLATES FROM A TERTIARY CARE CENTRE

DISSERTATION SUBMITTED TO

In partial fulfillment of the requirement for the degree of

DOCTOR OF MEDICINE IN MICROBIOLOGY

(Branch IV) M. D. (MICROBIOLOGY)

of

THE TAMIL NADU DR. M. G. R MEDICAL UNIVERSITY

CHENNAI- 600032

DEPARTMENT OF MICROBIOLOGY

TIRUNELVELI MEDICAL COLLEGE

TIRUNELVELI- 11

MAY 2019

BONAFIDE CERTIFICATE

This is to certify that the dissertation entitled **"Detection of Vancomycin resistant Enterococci with Van A genotype in clinical isolates from a tertiary care centre"** submitted by **Dr. E. Manimala** to the Tamilnadu 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 her under direct supervision & guidance.

Head of the Department,
Department of Microbiology
Tirunelveli Medical College,
Tirunelveli.

CERTIFICATE

This is to certify that the Dissertation **"DETECTION OF VANCOMYCIN RESISTANT ENTEROCOCCI WITH VAN A GENOTYPE IN CLINICAL ISOLATES FROM A TERTIARY CARE CENTRE"** presented herein by **Dr. E.Manimala** is an original work done in the Department of Microbiology, Tirunelveli Medical College Hospital, Tirunelveli for the award of Degree of M.D. (Branch IV) Microbiology under my guidance and supervision during the academic period of 2016 -2019.

The DEAN

Tirunelveli Medical College,

Tirunelveli - 627011

DECLARATION

I solemnly declare that the dissertation titled **"DETECTION OF VANCOMYCIN RESISTANT ENTEROCOCCI WITH VAN A GENOTYPE IN CLINICAL ISOLATES FROM A TERTIARY CARE CENTRE"** is done by me at Department of Microbiology, Tirunelveli Medical College hospital, Tirunelveli. 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.

The dissertation is submitted to The Tamilnadu Dr. M.G.R. Medical University towards the partial fulfilment of requirements for the award of M.D. Degree (Branch IV) in Microbiology.

Place: Tirunelveli

Date:

Dr. E. ManiMala

Postgraduate Student,

M.D Microbiology,

Department of Microbiology,

Tirunelveli Medical College

Tirunelveli.

ACKNOWLEDGEMENT

My Research project is made possible with the support of many people. I take this opportunity to express my gratitude towards them.

I am grateful to The **Dean, Dr. Dr.S.M Kannan M.Ch,** Tirunelveli Medical College and, Tirunelveli Medical College hospital Tirunelveli for permitting me to carry out this study.

It is with great previlage and respect that I express my cordial and humble thanks to **Dr.C.Revathy,M.D.,** Professor and Head, Department of Microbiology, Tirunelveli Medical College, whose kindness, guidance and constant encouragement enabled me to complete this study.

I wish to thank **Dr. V.Ramesh Babu, M.D.**, Professor ,Department of Microbiology, Tirunelveli Medical College, for his valuable guidance for the study.

I am deeply indebted to **Dr.S.Poongodi@ Lakshmi,M.D.,** Professor, Department of Microbiology, Tirunelveli Medical College, who helped me offering most helpful suggestions and corrective comments.

I am very grateful to **Dr.P .Sorna jeyanthi,M.D.,** Professor, Department of Microbiology, Tirunelveli Medical College, for the constant support rendered throughout the period of study and encouragement in every stage of this work.

I am highly obliged to Senior Assistant Professors **Dr.B.Cinthujah,M.D., Dr. G.Velvizhi, M.D., Dr. G.Sucila Thangam, M.D, Dr. V.P.Amudha M.D**, **Dr. I.M Regitha M.D., Dr.S.Gowri, M.D ,Dr.Kanagapriya, M.D.**, **Dr. Nagalakshmi M.D., Dr.Subha M.D**., Department of Microbiology, Tirunelveli Medical College, for their evincing keen interest, encouragement, and corrective comments during the research period.

Special thanks are due to my co-postgraduate colleagues **Dr.Saishruti , Dr. L.Gracia Paul , Dr.Mayakumar and Dr.R.Uma Maheswari** for never hesitating to lend a helping hand throughout the study.

I would also wish to thank my seniors **Dr. D.Jeya Ganguli** ,**Dr. S. Punitha ranjitham, Dr.R.P.R.Suyambu Meenakshi, Dr.V.Uma Maheswari** and **Dr.Ambuja Sekhar** and my juniors **Dr.Roohee Zubaidha, Dr. S.K. Jayaswarya, Dr. V.Aswini , Dr. A. Sangeetha, Dr G. Malathi , Dr. V. Thanalingam, Dr. S. I. Saheed Askar, Dr. M. Srividya, Dr.R. Priyadharshini and Dr. Cini B Fernz** for their help, motivation and support.

Thanks are due to the, Messer **V.Parthasarathy, V.Chandran, S.Pannerselvam, Murugesan , S.Santhi, S.Venkateshwari, S.Arifal Beevi, S.Abul Kalam, A.Kavitha, ,T.Jeya, K.Sindhu, K.Mangai,Manivannan,K.Umayavel, Sreelakshmi** and other supporting staffs for their servicesrendered.

I thank my parents **Mr.G.Esakkiappa** and **Mrs.E.Rajalakshmi** for being my backbone and not only giving me moral support but tolerating my dereliction during the study.

I am indebted **to my husband Mr.G.Madasamy, my daughter M.M.Ishaa Gopika, my brother Mr.E.Muthu Nagararajan,Uncle, Aunty,Sister,Brother in law and all the family members** for not only their moral support but also for tolerating my dereliction of duty during the period of my study.

And of course, I thank the Almighty for His presence throughout my work. Without the Grace of God nothing would have been possible

CERTIFICATE – II

This is certify that this dissertation work title **"DETECTION OF VANCOMYCIN RESISTANT ENTEROCOCCI WITH VAN A GENOTYPE IN CLINICAL ISOLATES FROM A TERTIARY CARE CENTRE"** of the candidate **Dr.E. Manimala** with registration Number **201614302** for the award of **M.D.** Degree in the branch of **MICROBIOLOGY(IV).** 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 page and result shows **13 percentage** of plagiarism in the dissertation.

Guide & Supervisor sign with Seal.

TIRUNELVELI MEDICAL COLLEGE

INSTITUTIONAL RESEARCH ETHICS COMMITTEE
TIRUNELVELI, STATE OF TAMILNADU, SOUTH INDIA PIN 627011
91-462-2572733-EXT; 91-462-2572944; 91-462-2579785; 91-462-2572611-16
online@tvmc.ac.in, tirec@tvmc.ac.in; www.tvmc.ac.in

CERTIFICATE OF REGISTRATION & APPROVAL OF THE TIREC REF NO: 993/MICRO/2017

PROTOCOL TITLE: DETECTION OF VANCOMYCIN RESISTANT ENTEROCOCCI WITH VAN A GENOTYPE IN CLINICAL ISOLATES FROM A TERTIARY CARE CENTRE.
PRINCIPAL INVESTIGATOR: Dr. E.MANIMALA, MBBS.,
DESIGNATION OF PRINCIPAL INVESTIGATOR: PG STUDENT
DEPARTMENT & INSTITUTION: TIRUNELVELI MEDICAL COLLEGE, TIRUNELVELI

Dear, Dr.E.MANIMALA, MBBS., The Tirunelveli Medical College Institutional Ethics Committee (TIREC) reviewed and discussed your application during the IEC meeting held on 10.03.2017.

THE FOLLOWING DOCUMENTS WERE REVIEWED AND APPROVED

1.	TIREC Application Form	☐
2.	Study Protocol	☐
3.	Department Research Committee Approval	☐
4.	Patient Information Document and Consent Form in English and Vernacular Language	☐
5.	Investigator's Brochure	☐
6.	Proposed Methods for Patient Accrual Proposed	☐
7.	Curriculum Vitae of the Principal Investigator	☐
8.	Insurance /Compensation Policy	☐
9.	Investigator's Agreement with Sponsor	☐
10.	Investigator's Undertaking	☐
11.	DCGI/DGFT approval	☐
12.	Clinical Trial Agreement (CTA)	☐
13.	Memorandum of Understanding (MOU)/Material Transfer Agreement (MTA)	☐
14.	Clinical Trials Registry-India (CTRI) Registration	☐

THE PROTOCOL IS APPROVED IN ITS PRESENTED FORM ON THE FOLLOWING CONDITIONS

1. The approval is valid for a period of 2 year/s or duration of project whichever is later
2. The date of commencement of study should be informed
3. A written request should be submitted 3weeks before for renewal / extension of the validity
4. An annual status report should be submitted.
5. The TIREC will monitor the study
6. At the time of PI's retirement/leaving the institute, the study responsibility should be transferred to a person cleared by HOD
7. The PI should report to TIREC within 7 days of the occurrence of the SAE. If the SAE is Death, the Bioethics Cell should receive the SAE reporting form within 24 hours of the occurrence.
8. In the events of any protocol amendments, TIREC must be informed and the amendments should be highlighted in clear terms as follows:
   a) The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no, Clause no. etc.)
   b) The PI must comment how proposed amendment will affect the ongoing trial. Alteration in the budgetary status, staff requirement should be clearly indicated and the revised budget form should be submitted.
   c) If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented.
   d) If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IEC, only then can they be implemented.
   e) Approval for amendment changes must be obtained prior to implementation of changes.
   f) The amendment is unlikely to be approved by the IEC unless all the above information is provided.
   g) Any deviation/violation/waiver in the protocol must be informed.

STANDS APPROVED UNDER SEAL

Dr.K.Shantaraman MD
Registrar, TIREC
Tirunelveli Medical College, Tirunelveli – 627011
State of Tamilnadu, South India

Dr.J.Suresh Durai, MD
Member Secretary, TIREC
Tirunelveli Medical College, Tirunelveli – 627011
State of Tamilnadu, South India

URKUND

Urkund Analysis Result

Analysed Document:	DETECTION OF VANCOMYCIN RESISTANT ENTEROCOCCI WITH VAN A GENOTYPE IN CLINICAL ISOLATES FROM A TERTIARY CARE CENTRE.pdf (D42202360)
Submitted:	10/6/2018 10:05:00 AM
Submitted By:	manisingapore7676@gmail.com
Significance:	12 %

Sources included in the report:

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)
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 (D30585773)
DR.VENNILA PLAGIARISM.docx (D31213590)
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3734693/
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4892083/
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4800524/
http://www.entomoljournal.com/archives/2018/vol6issue1/PartD/5-6-313-289.pdf
http://iosrjournals.org/iosr-jdms/papers/Vol13-issue2/Version-3/E013231722.pdf
https://www.sciencedirect.com/science/article/pii/S0377123714000446
http://file.scirp.org/Html/2-2260098_43009.htm
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4518417/

Instances where selected sources appear:

24

CONTENTS

S.No	Title	Page No.
1	INTRODUCTION	**1-3**
2	REVIEW OF LITERATURE	**5-45**
3	AIMS & OBJECTIVES	**47**
4	MATERIALS AND METHODS	**49-62**
5	RESULTS	**64-86**
6	DISCUSSION	**88-94**
7	SUMMARY	**96-97**
8	CONCLUSION	**99-100**
9	BIBLIOGRAPHY	
10	ANNEXURE	

- Data Collection Proforma
- Preparation of Media
- Colour plates
- Master Chart
- Abbreviations

INTRODUCTION

The genus *Enterococcus* consists of Gram-positive, facultatively anaerobic organisms that are spectacle shaped and may appear on smear in short chains, in pairs or as single cells. Enterococci, though commensals in adult feces are essential nosocomial pathogens.

Enterococcal infections may of at least 12 species including *Enterococcus faecalis, E. faecium,E. durans, E. avium, E. casseliflavus, E. gallinarum, E. hirae, E.malodoratus, E. mundtii, E. pseudoavium, E. raffinosus,* and *E. solitarius*. Among enterococcal species, *E.faecalis* and *E. faecium* are the two major human pathogens accounting for 85-89% and 10-15% of all enterococcal infections, respectively.

Prior to the 1990s also, *enterococci* have been recognized as an important cause of bacterial endocarditis for almost a century. However, recently they are recognized as a cause of nosocomial infection and "superinfection" in patients receiving antimicrobial agents. The most common *Enterococci*-associated nosocomial infections are of the urinary tract, followed by surgical site infections and bacteremia.

The intrinsic antibiotic resistance pattern of *Enterococci*, along with their promiscuity in acquisition and dissemination of genetically versatile antibiotic resistance elements, presents serious challenges to the treatment of enterococcal infections. Infections by *Enterococci* have traditionally been treated with cell wall active agents (e.g., penicillin or ampicillin) in combination with an

aminoglycoside (streptomycin/gentamicin), More ever, emergence of high level aminoglycoside resistance (HLAR), β lactam antibiotics and to vancomycin by some strains has led to failure of synergistic effects of combination therapy.

Vancomycin is an effective antimicrobial for treating infection caused by gram positive organisms. Gram positive isolates are often routinely tested for vancomycin susceptibility. In the 1970s, hospital-associated enterococcal infections in the United States were mainly due to *E.faecalis*. More recently, *E.faecium* has emerged as therapeutically challenging organism because of its resistanceto vancomycin and pencillin. These VRE isolates also have a high level of resistance to aminoglycosides Resistance to glycopeptides is mediated by alteration of the drug target from D-alanine-D-alanine to D-alanine-D-lactate. So far, eight genotypes of glycopeptide resistance, which are different in the level and range of resistance and in transfrability of glycopeptides, have been described for enterococci. Five of the van genes are acquired (van A , B , D , E , G) and three (vanC1,C2,C3) are intrinsic. Multiple epidemics have been predominantly reported with *vanA* type . *vanA* gene cluster is located within transposon Tn*1546* and can be transferred through acquired resistance.

The CLSI recommends screening of enterococci for high level aminoglycoside resistance with both gentamicin and streptomycin isolated from blood cultures or specimens such as heart valve tissue. The emergence of multidrug resistant *Enterococci*, especially Vancomycin Resistant *Enterococci (*VRE), and its

spread has caused the occurrence of many hospital Out breaks worldwide. In the United States, vancomycin-resistance Enterococcus faecium accounted for 4 per cent of healthcare-asssociated infections. It is the second most common pathogen causing mortality and morbidity and the 3rd leading cause of hospital acquired blood stream infection.

The prevalence in Asian countries is decreased and probably due to recent emergence of this resistance in this continent and only a handful of studies to document. In India, the prevalence of VRE has been reported as 8%, 5.5% and 23% in New Delhi, Chandigarh, and Mumbai, respectively, all of vanB phenotype.

Enterococci have emerged as the leading causes of Multiple drug resistant hospital-acquired pathogens especially with the emergence of glycopeptide-resistant *enterococcus (*GRE) species.

REVIEW OF LITERATURE

DESCRIPTION OF GENUS:

Enterococcus was historically termed as a diverse genus identified as being 'faecal streptococci', associated with the gastrointestinal tract of human (Giraffa 2002). Thiercelin in 1899 first coined the term 'enterocoque' to describe a newly found Gram-positive diplococcus species.

Andrews and Horder in 1906, isolated the same organism from an endocarditis patient and named it '*Streptococcus faecalis'* (Murray 1990). Based on antigens identified as being group-specific, enterococci were placed in *Streptococcus* group D, while pyogenic streptococci belong to groups A, B, C, E, F or G using antisera. *Enterococci* were thus classified as group D streptococci because of their morphology and Lancefield antigenicity.

The antigenicity of the carbohydrate moiety of the cell wall is designated according to a system devised by Lancefield in the 1930s (Smith, Niven *et al.* 1938). The established lance field antigen of *Streptococcus* is a virulence determinant. For example, in group A *streptococci* it plays a significant role in resistance to platelet-derived antimicrobials in serum, neutrophil killing and the cathelicidin antimicrobial peptide LL-37 (van Sorge, Cole *et al.* 2014).

Many efforts have been made to classify these organisms into better taxonomic groups due to their great diversity. A new classification pattern was proposed by Sherman in 1937 that classified streptococci into four main groups which are

pyogenic, viridans, lactic *streptococci* and *enterococci*. In 1984 research carried out using nucleic acid hybridization revealed the latter

group showed only meagre association to streptococci (Sherman, Mauer *et al.*1937).

Subsequently based on molecular techniques, DNA hybridisation,DNA: rRNA hybridisation and 16S rRNA sequencing revealed that *S.faecalis* and *S. faecium* were only distantly related to other streptococci.

The new genus named *Enterococcus* was proposed and *S. faecalis* and *S.faecium* were removed from the genus *Streptococcus* and renamed as *Enterococcus faecalis* and *Enterococcus faecium*, respectively (Schleifer,Kilpper-Balz *et al.* 1984, Ludwig, Seewaldt *et al.* 1985). The classification of enterococci has been challenging because it is a heterogeneous group of Gram-positive cocci which is more closely related to the genera *Carnobacterium*, *Lactococcus* and *Vagococcus*, but still has many characteristics of the genus *Streptococcus* (Leclerc, Devriese *et al.* 1996).

Domain:	Bacteria
Division:	Firmicutes
Class:	Bacilli
Order:	Lactobacillales
Family:	Enterococcaceae
Genus:	***Enterococcus***

The genus of Enterococcus is composed of more than forty species (The National Center for Biotechnology Information, NCBI), classified on the basis of pigment production, motility and ability to generate acids from a range of carbohydrates (Fischetti, Novick et al. 2006).

Based on the chemotaxonomic and phylogenetic studies, the establishment of 16S rRNA sequences led to the description of seven clonal complexes within the genus namely (i*) E. faecalis, E. haemoperoxidus and E. moraviensis; (*ii) *E.faecium, E. durans, E. hirae, E. mundtii, E. pocinus, and E. villorum; (*iii) *E.avium, E. pseudoavium, E. malodoratus, and E. raffinosus;* (iv*) E.casseliflavus, E. gallinarum and E. flavescens;* (v) *E. cecorum and E.columbae;* (vi) *E. dispar and E. asini;* (vii) *E. saccharolyticus and*

E.sulfureus. Other species are E. gilvus, E. pallens and E. ratti (Klein 2003) While there are multiple species in the genus *Enterococcus,* two are associated with the majority of human infections, *E. faecalis* and *E. Faecium* (Magi, Capretti *et al.* 2003).

Characteristics of Enterococci:

The *enterococci* are gram-positive cocci typically arranged in pairs and short chains, non-motile and non-capsulate. The cocci are facultative anaerobes and grow optimally at 35°C, although most isolates can grow in the temperature range l0ºC to 45°C. They grow readily on blood agar , with large, white colonies appearing after 24 hours of incubation; the colonies are typically non-hemolytic but can be ∝-hemolytic or β -hemolytic. It grows readily on ordinary nutrient media and on MacConkey agar, on which it forms small (0.5-1 mm), usually magenta-colored colonies.

Distinctive Features of Enterococci:

The Enterococci possess several distinctive features separating them from streptocooci: The enterococci grow in the presence of 6.5 percent NaCl, 40 percent bile, at pH 9.6, at 45°C and in 0.1 percent methylene blue. It survives heating at 60°C for 30 min, a feature distinguishing it from streptococci, and also grows within a wider range of temperatures (10-45°C). On MacConkey

medium they produce deep pink colonies. Enterococci are PYRase test positive. They do not hydrolyze hippurate.

Enterococcal Species:

The genus was established in 1984 with the characterization of *Enterococcus faecalis* and *Enterococcus faecium*; however, a further 32 species have now been added to the genus on the basis of chemotaxonomic and phylogenetic studies. These additions were based on evidence provided by 16S rRNA sequencing studies.

E. faecalis	Schleifer and Kilpper-Balz 1984
E. faecium	Schleifer and Kilpper-Balz 1984
E. avium	Collins et al. 1984
E. casseliflavus	Collins et al. 1984
E. durans	Collins et al. 1984
E. gallinarum	Collins et al. 1984
E. malodoratus	Collins et al. 1984
E. hirae	Farrow and Collins 1985

E. mundtii	Collins et al. 1986
E. raffinosus	Collins et al. 1989
E. solitariusa	Collins et al. 1989
E. pseudoavium	Collins et al. 1989
E. cecorum	Devriese et al. 1983; Williams et al.1989
E. columbae	Devriese et al. 1990
E. saccharolyticus	Farrow et al. 1984; Rodrı´gues andCollins1990
E. dispar	Collins et al. 1991
E. sulfureus	Martinez-Murcia and Collins 1991
E. seriolicidaa	Kusuda et al. 1991
E. flavescensb	Pompei et al. 1992
E. asini	De Vaux et al. 1998
E. villorumc	Vancanneyt et al. 2001
E. haemoperoxidus	Svec et al. 2001
E. moraviensis	Svec et al. 2001

E. ratti	Teixeira et al. 2001
E. porcinusc	Teixeira et al. 2001
E. gilvus	Tyrrell et al. 2002
E. pallens	Tyrrell et al. 2002
E. phoeniculicola	Law-Brown and Meyers 2003
E. canis	De Graef et al. 2003

GENOME:

The genome size is about 2 to 3.5 Mb and the G+ C content is 37 to 45 mol %. The genome of *E.faecalis* V583, the first vancomycin resistant clinical isolate from United States is completely sequenced and is useful in various research purposes . The genome of > 80 *enterococcal* strains has been sequenced. The genetic diversity of *Enterococci* is due the acquisition of mobile DNA like plasmids, transposons and phages and also a result of recombination of " core" genomes. The medically important *E.faecium* harbors an accessory genome into which exogenous genetic elements like Phage DNA are incorporated .It also possesses pathogenicity island, which is a large genetic element carrying virulence associated genes and plasmids with antibiotic resistant determinants

HABITAT:

As outlined in Habitat, the enterococci are primarily members of the gastrointestinal microflora of humans, occurring in numbers as high as 108 colony forming units (CFU) per gram of feces of adult individuals (Noble 1978; Huycke et al. 1998). Enterococcal populations in the intestinal tract fluctuate in size according to the age and physiological condition of the human host, being more numerous during early life (Tannock and Cook 2002). Diet also seems to affect the numbers of enterococci in fecal samples. Although *E.faecalis* appears to be the enterococcal species most commonly detected in human feces, in all likelihood most *Enterococcus* species are normal inhabitants of the gastrointestinal tract of humans. Since the enterococci are opportunistic pathogens, the incidence of each species found in human infections probably reflects the distribution of the different *Enterococcus* species in the human gastrointestinal tract.

This site is believed to represent an important reservoir for strains associated with disease; from this location they may migrate to cause infections and can also disseminate to other hosts and environmental surfaces. *E. faecalis* is usually the most frequent enterococcal species isolated from human clinical specimens, representing 80–90 percent of the isolates, followed by *E. faecium* which is found in 5–10 percent of enterococcal infections. The other enterococcal species are identified less frequently. However, clusters of

infections with *E. casseliflavus* and *E. raffinosus* have been reported. Therefore, the distribution of species varies with each clinical setting. Although less frequently or even rarely, several of the other enterococcal species, including *E. avium, E. cecorum, E. dispar, E. durans, E. gallinarum, E. gilvus, E. hirae, E. mundtii, E. pallens, and E. faecalis variant strains, have also been isolated from human sources. E. columbae, E. haemoperoxidus, E. malodoratus, E. moraviensis, E. porcinus, E. , E. ratti, E. saccharolyticus, and E.sulfureus* have not been isolated from human sources.

The comparison of data from different publications and the evaluation of the real incidence of the different species of enterococci as members of the intestinal microflora or as members of the microflora in other body sites have been impaired due to differences in the methodology used and the changes in the taxonomy of the genus.

***Enterococcus* as a commensal:**

Commensalism is the relationship between two organisms in which one or both the organisms gets benefits and the other organism is not harmed. In the colon of nearly all humans and most animals enterococci are minor residents, present at ~108 colony forming units per g of faeces *Enterococci* have effectively evolved various genetic traits which helps maintain their stable colonisation. Commensal isolates of *E. faecium* and *E. faecalis* are genetically distinct compared to infection isolates. The differences may be unclear, however, since

immunocompromised patients are more susceptible to infection even with commensal strains

Enterococcal Infections :

The *enterococci* inhabit the gastrointestinal tract and the genitourinary tract in humans and other animals. Enterococci are frequent causes of nosocomial infections and may cause urinary tract infection, bacteremia, infective endocarditis, biliary tract infection, intra-abdominal abscess complicating diverticulitis, peritonitis and wound infection.

PATHOGENESIS OF ENTEROCOCCAL INFECTIONS:

To cause disease enterococci must colonise host tissues, defend against host immune mechanisms and express factors that enable persistence. Multiple factors are known that regulate the virulence of *Enterococcus* species, for example ability to colonise the gastrointestinal tract, ability to adhere to a variety of extracellular matrix components, including vitronectin, thrombospondin and lactoferrin, and ability to adhere to oral cavity epithelia, urinary tract epithelia and human embryo kidney cells (Fisher and Phillips 2009). Pathogenicity of enterococci has been related to several key virulence traits associated with adhesion, translocation and immune evasion.

Whereas enterococci are thought to account for no more than 1% of the adult intestinal microflora, the medical importance of enterococci outweighs their relative abundance . Of the 26 species that have been proposed to belong to the genus, only 11 (*E. avium, E. casseliflavus, E. durans, E. dispar, E. faecalis, E. faecium, E. gallinarum, E. hirae, E. mundtii, E. pseudoavium, and E. raffinosus)* have been described as associated with human disease . *E. faecalis* accounts for 80-90% of enterococcal isolates of clinical origin, with *E. faecium* the second most prevalent enterococcal species. Despite the lower frequency of isolation from clinical settings, *E. faecium* isolates are disparately resistant to treatment with antimicrobial chemotherapy.

Although normally commensal in nature, enterococci are responsible for approximately 10% of urinary tract infections and 16% of nosocomial urinary tract infections . They are also commonly isolated from wound infections of the abdominal area as well as those from crushing injuries . Enterococcal bacteremia is the third leading cause of nosocomial bacteremia with an estimated fatality rate of 28 to 58% . *Enterococci* are also responsible for between 5 and 20% of cases of bacterial endocarditis . *Enterococci* have been described as one of the most destructive agents that cause postoperative complications of cataract surgery .

Up to 13% of bacteriologically confirmed cases of neonatal sepsis have been attributed to enterococci . Those who are elderly or have an underlying compromising situation are predisposed to enterococcal infection, especially in

the hospital environment. This is a significant observation given the ability of enterococci to colonize surfaces of the hospital environment and persist on fingertips and dry surfaces. As a result, enterococci seeding the clinical environment may be more easily spread if infection control measures are poorly implemented.

CLINICAL SYNDROMES:

Urinary tract infections:

E*nterococci* have become the second most common agent recovered from nosocomial urinary tract infection (UTI). UTIs are the most common of the enterococcal infections: enterococci have been implicated in approximately 10% of all UTIs and in 16% of nosocomial UTIs. Enterococcal bacteriuria usually occurs in patients with underlying structural abnormalities and/or in those have undergone urologic manipulations.

Bacteremia and Endocarditis:

Enterococcal bacteremias can usually be traced to enterococcal infections at other sites besides theurinary tract (e.g., intravenous catheter infections, biliary tract infection, gastrointestinal/genitourinaryinfections) and are most often hospital-acquired. The organisms can also gain entry into the bloodstreamthrough intra-abdominal or pelvic abscesses, wounds, decubitus ulcers, or intravenous access devices.Risk factors for the development of enterococcal bacteremia include advanced age, immunosuppression,underlying

diseases and conditions (e.g., prematurity, diabetes, malignancy, congestive heart failure, renalinsufficiency, deep-seated infections, prior gastrointestinal, genitourinary, or respiratory tractinstrumentation, long-term hospitalization, indwelling devices, and the use of broad-spectrum antibioticshaving little or no anti-enterococcal activity (e.g., cephalosporins). Bacteremias caused by *E. Faecium* are associated with a poorer prognosis than those due to *E. faecalis* primarily because of increasedantimicrobial resistance among the former species and the inherent difficulties of adequately treating moreresistant isolates.

Enterococci, particularly *E. faecalis*, are also a common cause of prosthetic valve endocarditis. Endocarditis usually occurs in older male patients with underlying valvular disease orwith prosthetic valves and is generally subacute in clinical presentation, with patients having fevers, weight loss, malaise, and other vague constitutional symptoms. Endocarditis in these patients often follows procedures involving the gastrointestinal (e.g., transrectal prostatic biopsies, colonoscopy, fiberoptic sigmoidoscopy) or genitourinary (e.g., cystoscopy, prostatectomy) tracts. Complications of this infection include embolic phenomena frequently involving the CNS. In up to half of patients, enterococcal endocarditis results in acute heart failure that requires valve replacement

Enterococci are more causative agents of both community acquired and hospital associated endocarditis especially in elderly, debilitated patients with comorbid conditions. It can cause both native and prosthetic valves, mitral and aortic

valves affected commonly. *E.faecalis* is isolated more frequently than *E.faecium* and other species. Malignant and inflammatory conditions and procedures involving genitor urinary or gartro intestinal tracts is the source of origin.

Typical presentation involves a sub acute course with fever, malaise, weight loss, cardiac murmur and less than frequent peripheral signs. Heart failure is the common complication followed by embolic penomina, the most important end organ being brain. Mortality is mainly due to heart failure or embolization and the overall mortality rate ranges from 11% to 35%.

Meningitis:

Enterococcal meningitis is a rareenterococcal infection that may be seen in both adults and children.834 Enterococcal meningitis maydevelop spontaneously or as a postoperative infection. Individuals with spontaneous enterococcalmeningitis usually have enterococcal infection at other sites, have severe underlying disease, and haveconcomitant enterococcal bacteremia more frequently than those with postoperative infections. Underlying disease in patients with spontaneous meningitis include malignancy, diabetes, renal failure,and treatment with immunosuppressive agents. Patients with postsurgical enterococcal meningitis usually have an antecedent history of intracerebral hemorrhage, CNS neoplasms, head trauma, and hydrocephalus. Patients with this condition often have intraventricular catheters, surgical site CSF leakage, or ventriculoperitoneal shunts in place prior to diagnosis. In these cases, fever, obtundation, andmeningismus are usually present, and the CSF indices include

high white blood cell counts, elevatedprotein, and low or normal glucose. Postsurgical enterococcal meningitis may or may not be associated with enterococcal infections at other sites. In a review of enterococcal meningitis, *E. faecalis* accounted for 76% of isolates, and 15 of the 25 cases that were due to *E. faecium* were caused by vancomycin-resistant strains.

Intraabdominal,pelvic and soft tissue infections:

Intra-abdominal and pelvic infections are the next most commonly encountered infections. However,cultures from patients with peritonitis, intra-abdominal or pelvic abscesses, biliary tract infections, surgical-site infections, and endomyometritis are frequently polymicrobial, and the role of enterococci in this setting remains controversial. Enterococci are being recovered from wound infections at an increasing rate, which likely results from increased antibiotic usage and emerging resistance among these organisms.

Enterococci produce spontaneous peritonitis in cirrhotic patients and patients on chronic ambulatory peritoneal dialysis. It is usually isolated along with gram negatives and anaerobes and its presence indicates treatment failure and increases the postoperative complications, and death. The emergence and spread of VRE and multi-drug resistant *E.faecium* isolates worsens the situation further.

Enterococci ranks Third amongst the causative agents of hospital acquired surgical-site infections. *E.faecalis* is the common species isolated *Enterococci* often colonize the decubitus ulcer and diabetic foot and can be a source of bone infections.

Other infections:

Enterococcal infections of the respiratory tract or the central nervous system, as well as otitis,

sinusitis, septic arthritis, and endophthalmitis may occur but are rare. *Enterococci* are often found in wound andsoft tissue infections (e.g., burns, decubitus ulcers) with other facultative and anaerobic bacteria, andcomplications associated with such infections (e.g., enterococcal osteomyelitis) are rare

LABORATORY DIAGNOSIS:

Collection, transport and storage of specimens:

Standard procedures for collection and transport of blood, urine, or wound specimens should be followed. The specimens should be cultured as soon as possible with minimum delay. Trypticase soy or brain-heart infusion agar supplemented with 5% sheep blood is routinely used to culture enterococci. *Enterococci* grow well at 35ºC in the presence of CO2 but do not require a high level of CO2 for growth. If the clinical specimen is obtained from a contaminated site or is likely to containing bile esculin azide, colistin-nalidixic

acid, phenulethyl alcohol, chromogenic subrates, or cephalexin-aztreonam-arabinose agar should be used for isolation of enterococci.

Direct examination:

The direct microscopic examination of gram stained smears of normally sterile clinical specimens like blood is useful in diagnosing *Enterococcal* infections. However, only a presumptive report of "presence of Gram positive cocci" can be given in case of nonsterile specimens. Direct detection of *Enterococci* especially VRE from clinical specimens and surveillance specimens (feces, rectal swab) by using conventional and real-time PCR based methods have been developed and evaluated.

A multiplex real-time PCR assay (Light cycler septifasttest) for rapid detection and identification of major pathogens of nosocomial bacteremia in whole blood is available for use in the US.

Isolation procedures:

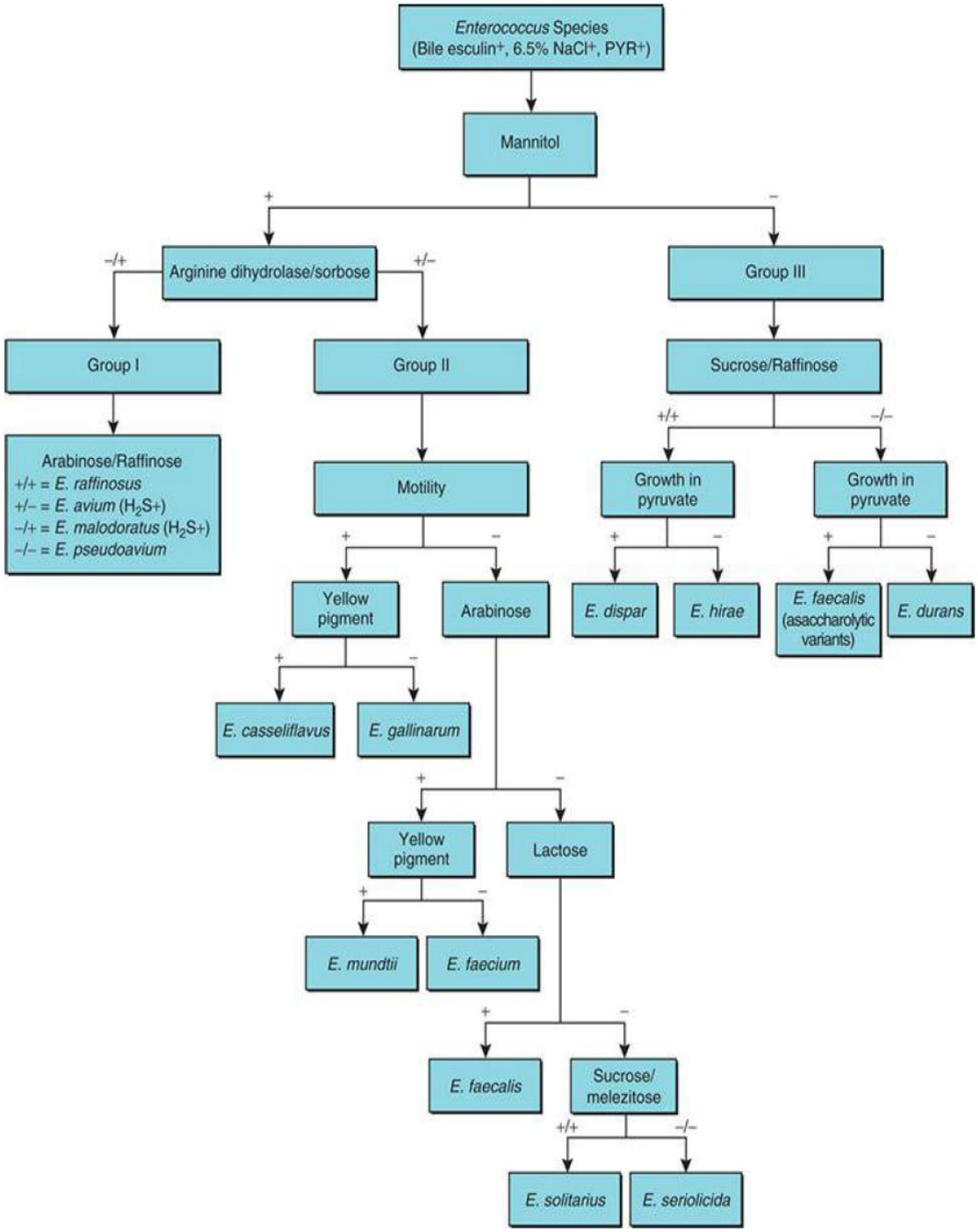
Clinical specimens from normally sterile body sites, can be plated onto tryptic soy agar, brain heart infusion agar or blood agar base containing either 5% sheep, horse or rabbit blood for primary isolation of *Enterococci*. Samples for blood culture are inoculated into conventional

blood culture systems. Most of the clinically relevant species grow well at 35 to 37ºC. For specimens obtained from non-sterile sites especially when contaminated with gram negative bacilli, selective media containing sodium

azide, bile salts, antibiotics and esculin , tetrazolium can be used for primary isolation. However not all *Enterococci* grow on selective media. Use of enrichment broth (Enterococcosel broth- BEA medium with 6µg vancomycin) increases the recovery rate of *Enterococci* especially VRE from feces and rectal samples especially surveillance specimens. Various chromogenic media from different manufacturers also have been evaluated for the primary isolation.

Identification of *Enterococcus* species:

The genus identification of a catalase negative, Gram positive coccus as " *Enterococcus"* is based on the above said tests in genus description. *Enterococal* species can be classified into five physiological groups of species as proposed by Facklam and Collins,based on acid production from mannitol and sorbose and hydrolysis of arginine . Further speciation is based on acid production from sugars like arabinose, sorbitol, raffinose, sucrose, pyruvate, trehalose and reduction of 0.04% tellurite, motility and pigment production.


Enterococcus Species
(Bile esculin+, 6.5% NaCl+, PYR+)
Mannitol
+
–
–/+
Arginine dihydrolase/sorbose
+/–
Group III
Group I
Group II
Sucrose/Raffinose
+/+
–/–
Arabinose/Raffinose
+/+ = *E. raffinosus*
+/– = *E. avium* (H_2S+)
–/+ = *E. malodoratus* (H_2S+)
–/– = *E. pseudoavium*
Motility
Growth in pyruvate
Growth in pyruvate
Yellow pigment
Arabinose
E. dispar
E. hirae
E. faecalis (asaccharolytic variants)
E. durans
E. casseliflavus
E. gallinarum
Yellow pigment
Lactose
E. mundtii
E. faecium
E. faecalis
Sucrose/ melezitose
E. solitarius
E. seriolicida

Various **commercial identification systems** – manual, semi automated and automated systems like API 20S, API Rapid ID32STREP, Crystal gram positive ID system, Gram positive identification card of Vitek system etc..are available. These are reliable for the detection of most common species *E.faecalis* and to a lesser extent *E.faecium.*

Molecular methods

The introduction of various molecular techniques has substantially improved the ability to discriminate enterococcal isolates and has provided critical insights into

epidemiological aspects of enterococcal infections. As a result of the use of more discriminatory typing methods, it has been possible to demonstrate that strains can be exogenously acquired by direct and indirect contact among patients. The first molecular techniques developed for typing of enterococci were the analysis of plasmid profiles and the restriction enzyme analysis (REA) of genomic DNA by conventional electrophoresis.

Ability to discriminating among enterococcal strains was noted with the use of techniques involving the analysis of chromosomal DNA restriction endonuclease profiles by pulsed-field gel electrophoresis (PFGE) by either field-inversion gel electrophoresis (FIGE) or ideally, counter-clamped homogeneous electric-field electrophoresis.

Multi locus enzyme electrophoresis (MLEE) , Ribotyping and the polymerase chain reaction (PCR)-based typing methods, such as the random amplified polymorphic DNA (RAPD)-PCR assay and the repetitive element sequence (REP)-PCR have also been used to investigate the genetic relationship among enterococcal strains. Sequencing of PCR products and restriction fragment length polymorphism (RFLP) analysis of PCR products have been used to trace and to determine differences among specific resistance genes in enterococci, therefore representing additional tools for typing resistant strains. Analysis of SmaI restriction digests of genomic DNA by PFGE is widely useful for studying enterococcal species.

Antimicrobial Resistance:

Resistance to several commonly used antimicrobial agents is a remarkable characteristic of most of the enterococcal species. Moreover, the majority of the information available is based on studies with *E. Faecalis* and *E. faecium*, the two species that are more frequently associated with human infections. Antimicrobial resistance can be classified as either intrinsic or acquired.

Intrinsic resistance is related to inherent or natural chromosomally encoded characteristics present in all or most of the enterococci. Furthermore, certain specific mechanisms of intrinsic resistance to some antimicrobial agents are typically associated with a particular enterococcal species or group of species. In contrast, the occurrence of acquired resistance is more variable, resulting

from either mutation in existing DNA or acquisition of new genetic determinants found in plasmids or transposons.

Intrinsic resistance of enterococci:

Enterococcal intrinsic resistance involves two major groups of antimicrobial therapeutic drugs: the aminoglycosides and the b-lactams. Because of the poor activity of several antimicrobial agents against enterococci due to intrinsic resistance, the recommended therapy for serious infections (i.e. endocarditis, meningitis, and other systemic infections, especially in immunocompromised patients) includes a combination of a cell-wall-active agent, such as a B-lactam (usually penicillin) or vancomycin, combined with an aminoglycoside (usually gentamicin or streptomycin).

These combinations overcome the intrinsic resistance exhibited by the enterococci and a synergistic bactericidal effect is generally achieved since the intracellular penetration of the aminoglycoside is facilitated by the cell-wall-active agent.

In addition to the intrinsic resistance traits, enterococci have acquired different genetic determinants that confer resistance to several classes of antimicrobials,including chloramphenicol, tetracyclines, macrolides, lincosamides and streptogramins, aminoglycosides, blactams, glycopeptides, and, more recently, quinolones.

Acquired resistance of enterococci to ß –Lactams and aminoglycosides antibiotics:

Aminoglycoside resistance:

Over the past decades, the occurrence of acquired antimicrobial resistance among enterococci, especially high-level resistance (HLR) to aminoglycosides, β -lactams, and resistance to glycopeptides (especially vancomycin), has been increasingly reported. These resistance traits are of particular clinical relevance as they confer resistance to agents used in the treatment of serious enterococcal infections and can abolish the activity of the therapeutic regimens with proven bactericidal activity against enterococci. Isolates that are resistant to the cell-wall-active agent or have HLR to aminoglycosides are resistant to the synergistic effects of combination therapy and constitute an even more serious problem concerning the effective management of enterococcal infections. Therefore, the detection of resistance to these groups of antimicrobial agents is important to predict the likelihood of synergy by using antimicrobial association as a therapeutic strategy.

Enterococcal isolates exhibiting HLR to one or more aminoglycosides have been described with increasing frequency (Murray 1990, 1998; Antalek et al. 1995;Leclercq 1997; Huycke et al. 1998; Strausbaugh and Gilmore 2000) and are now present in large proportions in several geographic areas. Strains expressing acquired HLR to aminoglycosides usually have minimal inhibitory

concentrations (MIC) >2000 lg/ml and cannot be detected by diffusion tests with conventional disks.

Special tests using high-content gentamicin and streptomycin disks (Sahm and Torres 1988), as well as a single dilution method, were developed to screen for this type of resistance (Swenson et al. 1995). Strains exhibiting HLR to penicillin and ampicillin due to altered penicillin-binding proteins have also disseminated widely in the past several years (Murray 1990, 1998; Boyce et al. 1992; Huycke et al. 1998; Strausbaugh and Gilmore 2000), and strains producing β-lactamase have been identified (Murray 1990; Gordonet al. 1992).

ß -Lactam antibiotic:

Many strains of enterococci, particulary *E. faecium*, are intrinsically resistant to β-lactam antibiotics because they possess proteins with low binding affinity for these drugs. In particular, the cephalosporins are uniformly ineffective against enterococci and shoud not be tested. In general, ampicillin is more effective against enterococci than penicillin in vitro (Leclercq *et al*, 1988).

Vancomycin:

Definition:

Vancomycin is a glycopeptide antimicrobial produced by the soil bacteria *Streptomyces orientalis*. It was developed and introduced in the 1950s. Another glycopeptide authorised for use in humans in Sweden is teicoplanin. Glycopeptides interfere with the cell wall production resulting in a destabilized cell wall and lysis of the bacteria. When the bacterial cell wall is synthesized, polysaccharide-pentapeptide complexes are linked together via a transpeptidation reaction in which the end amino acid of the pentapeptide is removed. Glycopeptides interferewith this process by binding tightly to the D-Alanyl-D-Alanin (D-Ala-DAla) end of the pentapeptide and hiding it from the transpeptidase that is to catalyse the cross-linking in the peptidoglycan synthesis.

Vancomycin is active against most Gram positive bacteria whereas the majority of Gram negatives are resistant. It is considered a drug of “last resort” and has been classified as critically important for human medicine for treatment of patients with severe infections with multi-drug resistant *Enterococcus* spp. and meticillin resistant *Staphylococcus aureus* (MRSA) as the main indications.

Mode of action:

Like many antibiotics (including the penicillins), vancomycin acts by interfering with the construction of cell walls in bacteria, blocking the enzymes necessary for bacterial cell wall assembly.

Resistance to vancomycin:

History:

Scientists introduced Vancomycin into hospitals more than forty years ago in response to new strains of Staphylococci developing resistance to penicillin. The introduction of methicillin decreased the use and importance of Vancomycin for a few years; however, when methicillin-resistant *S. aureus* strains appeared in the past two decades, the glycopeptide antibiotic was reinstated as a therapeutic agent.

Vancomycin is now seen as the last-resort drug because it is often the last opportunity that a physician may have to eliminate infections caused by multi-drug resistant bacteria.

Genes of vancomycin resistance:

Until today, nine different variants of vancomycin resistance in enterococci have been described (vanA, B, C, D, E, G, L, M and N; Table 1) (Hegstad*et al.*, 2010; Lebreton *et al.*, 2010; Xu *et al.*, 2010; Boyd *et al.*, 2008; Courvalin, 2006). Among those, the three most common variants are the vanA, B and C

types with *E. faecium* carrying the *vanA* genotype as the most common combination (Fisher & Phillips, 2009; Werner *et al.*, 2008). An additional variant (vanF) has also been described but thus far only in *Paenibacillus popilliae* (Patel *et al.*, 2000). Since the vanF variant has a high similarity in amino acid sequences to the vanA variant, *P. popilliae* has been suggested as a possible origin for vancomycin resistance in enterococci (Patel *et al.*, 2000). Other plausible sources are various glycopeptide producing organisms, even if genetic differences make an older common source more likely (Patel, 2003).

Common to all variants of Vancomycin resistance in enterococci is the ability to cause a change in the structure of the pentapeptide incorporated in the 3 dimensional web of peptidoglycans composing the bacterial cell wall: from the original D-Ala-D-Ala to either D-Ala-D-Lactate (D-Ala-DLac) or D-Ala-D-Serine (D-Ala-D-Ser) (Courvalin, 2006). This shift results in a reduced affinity for Vancomycin by 1000 and seven times respectively (Fisher & Phillips, 2009).

In all different variants of vancomycin resistance are several genes involved in the alteration of the cell wall structure which results in the resistance. The number and organisation of these genes are somewhat similar among the different variants. For the vanA variant, the genes are organized as in Figure4. *VanS* is a sensor gene which in the presence of a glycopeptide

phosphorylate, and thus activate the regulator gene *vanR* (Courvalin, 2006).

After activation of the gene complex, *VanH* mediates production of lactate from pyruvate which *vanA* uses to synthesize the alternative D-Ala-D-Lac end of the pentapetide (French, 1998). It is essential for resistance that production of the normal D-Ala-D-Ala end of the pentapetide does not continue. This is resolved by the *vanX* and *vanY* genes where *vanX* hydrolyzes and thereby interrupts the production of the pentapeptides, and *vanY* cleaves the pentapeptides that might still be produced (French, 1998; Arthur *et al.*, 1996). In the absence of a glycopeptide, *vanS* initiates dephosphorylation of *vanR* resulting in deactivation of the gene (Courvalin, 2006).The function of the *vanZ* gene is not understood (Courvalin, 2006).

The mechanism of resistance has been best characterized for the *vanA.* It consists of cluster of seven genes found on the transposable (mobile) genetic element Tn*1546.* In the presence of an inducer like vancomycin, transcription of the genes necessary for resistance to vancomycin is activated as a result of the interactions of a sensory kinase and a response regulator.

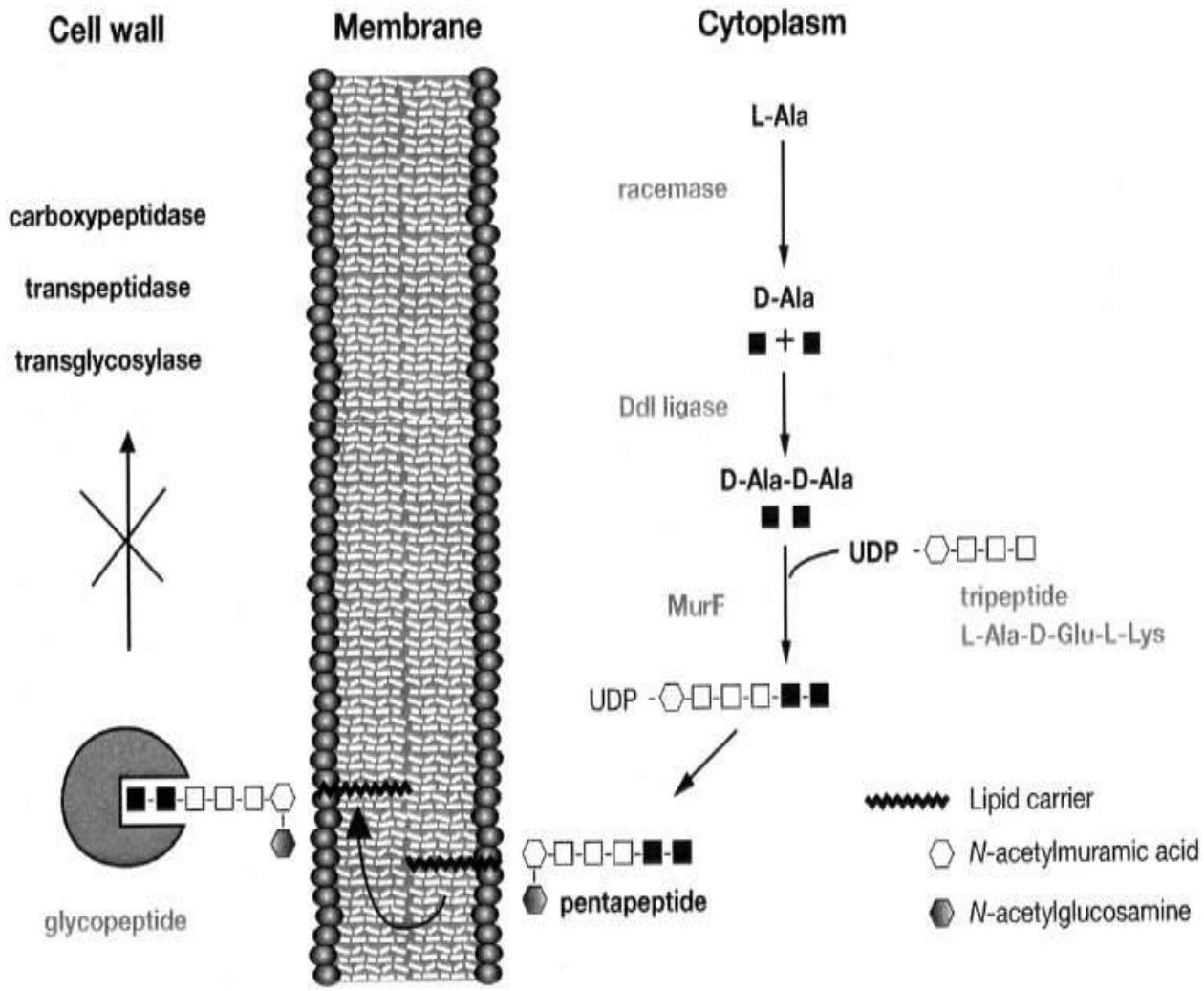


Figure : Peptidoglycan biosynthesis and the mechanism of vancomycin. Association of the antibiotic to the C-terminal d-Ala–d-Ala of late peptidoglycan precursors stops catalysed reactions by transpeptidases, transglycosylases, and carboxypeptidases reproduced from Courvalin 2006.

DIFFERENT TYPES OF VANCOMYCIN RESISTANCE FOUND IN *ENTEROCOCCUS SPP.*

TABLE 13-1 Genotypes and Phenotypes of Vancomycin-Resistant Enterococci

			Vancomycin Resistance		Teicoplanin Resistance					
Genotype (Gene Clusters)	Phenotype	Type of Resistance	Level	MIC's (μg/mL)	Level	MICs (μg/mL)	Expression of Resistance	Location	Conjugative Transfer	Species With Resistance Type
vanA	VanA	Acquired	High level	64 to ≥1,000	High	16–512	Inducible	Plasmid, transposon on chromosome	Yes	*E. faecium, E. faecalis, E. avium, E. casseliflavus, E. durans, E. gallinarum, E. hirae, E. mundtii, E. raffinosus*
vanB	VanB	Acquired	High–variable level	4–512	Susceptible	≤0.5	Inducible	Plasmid, chromosome, transposon, integrative conjugative element	Yes	*E. faecium, E. faecalis, E. casseliflavis, E. durans, E. gallinarum, E. hirae*
vanC1, vanC2, vanC3	VanC	Intrinsic	Low level	2–32	Susceptible	≤0.5	Constitutive	Chromosome	No	*E. casseliflavus (vanC2, vanC3), E. gallinarum (vanC1), E. flavescens (vanC3)*
vanD	VanD	Acquired	Moderate–high level	64–256	Low	4–32	Inducible/constitutive	Chromosome	No	*E. faecium, E. faecalis, E. avium, E. gallinarum, E. raffinosus*
vanE	VanE	Acquired	Low level	16	Susceptible	≤0.5	Inducible	Chromosome	No	*E. faecalis*
vanG	VanG	Acquired	Low–moderate level	≤16	Susceptible	≤0.5	Inducible	Chromosome or on integrative conjugative element	Yes	*E. faecalis*
vanL	VanL	Acquired	Low level	8	Susceptible	≤0.5	Inducible	Unknown, possibly chromosomal	No	*E. faecalis*
vanM	VanM	Acquired	High level	≥256	Susceptible to resistant	0.75 to ≥256	Inducible	Unknown	Yes	*E. faecalis*
vanN	VanN	Acquired	Low level	16	Susceptible	≤0.5	Constitutive	Plasmid	Yes	*E. faecium*

Phenotypic description:

VRE strains have been classified by phenotypes and genotypes. Six types of glycopeptide

resistance have already been described among enterococci. Three of them are the most common: the VanA phenotype, with inducible high-level resistance to vancomycin, as well as to teicoplanin, encoded by the vanA gene; the VanB phenotype, with variable (moderate to

high) levels of inducible resistance to vancomycin only, encoded by the vanB gene; and the VanC phenol type, with non inducible low-level resistance to vancomycin.

The VanA and VanB phenotypes are considered the most clinically relevant and are usually associated with *E. faecium* and *E. faecalis* strains while the VanC resistance is a intrinsic characteristic of *E. gallinarum* (vanC1 genotype) and E. casseliflavus (vanC2 and vanC3 genotypes) strains (Clark et al. 1998; Huycke et al. 1998; Murray 1998; Cetinkaya et al. 2000).

The remaining three types of enterococcal glycopeptides resistance seem to occur rarely and are encoded by genetic determinants that were recently recognized, named vanD (Perichon et al. et al. 2000).

The details of vancomycin resistance have been best documented with the *Van A* gene cluster found on the transposon, Tn1546 (Arther and Courvalin, 1993; Arther *et al*., 1993). *Van B* isolates were initially believed to be inducibly resistant to more modest levels of vancomycin but are susceptible to teicoplanin. *VanB* resistance determinants also reside on large mobile elements that can be transferred from 1 strain of enterococcus to another (Quintiliani *et al.*, 1993, 1994).

The *VanC* resistance phenotype was described in *E. gallinarum* which demonstrate low-level resistance to vancomycin and susceptible to teicoplanin. In the United States, *VanA* and *VanB* account respectively for approximately 60% and 40% of VRE isolate (Clark *et al*., 1993).

Certain limitations of this classification method have become evident over time. For example, the genetic determinants of the *VanA* Phenotype have now appeared in *E. gallinarum* and other enterococcal species (Dutka Malen *et al*., 1994).

Nevertheless, this phenotypic classification is still useful, because it usually corresponds well to the genetic classification and utilizes information that can be derived simply and inexpensively in laboratory (Elipoulos, 1997).

Genotypic classification of VRE:

VanA glycopeptide resistance:

The *vanA* gene and other genes involved in the regulation and expression of vancomycine resistance (*vanR, vanS, vanH, vanX*, and *vanZ*) are located on a 10,581.bp transposon (Tn1546) of *E. faecium*, which often resides on plasmid (Arther *et al*., 1993). The advantage of accumulating genes in plasmids is that, these regions of DNA can replicate independently of the bacterial genome, and can also be readily transferred from one cell to another. Resistance can therefore easily spread between species. In addition, these vancomycin-resistance genes are located on transposon elements, which can cut themselves out of one segment of DNA and move to another segment. These characteristics make it very easy for bacteria to transfer antibiotic resistance to other cells of the same (or different) species (Walsh and Christopher, 2001). VanA phenotype display

vancomycinandteicoplanin-inducible, transposon-mediated, high-level resistance to both vancomycin (MIC, 64 to1,000 µg/mL) and teicoplanin (MIC, 16 to 512 µg/mL).1147

***VanB* glycopeptide resistance:**

The *vanB* cluster genes is often located on the host chromosome and initially was thought not transferable to other bacteria. However, it can also occur on plasmids, and, even when it is chromosomal, this gene cluster has been transferable as part of large mobile elements, perhaps related to large conjugative transposons (Quintiliani *et al*., 1994). Strains with the *vanB* genotype (VanBphenotype) have acquired vancomycin-inducible resistance to various concentrations of vancomycin(MIC, 4 to 1,000 µg/mL) but remain susceptible to teicoplanin (MIC, 0.5 to 1 µg/mL), although rare *vanB* strains may also be resistant to the latter antibiotic.

***VanC* glycopeptide:**

The genes encoding the *VanC* type of vancomyin resistance are endogenous, species-specific components of *E. gallinarum (vanC-1)*and *E. casseliflavus/E. flavescens (vanC-2/vanC-3 (*Navarro and Courvalin , 1994*).*

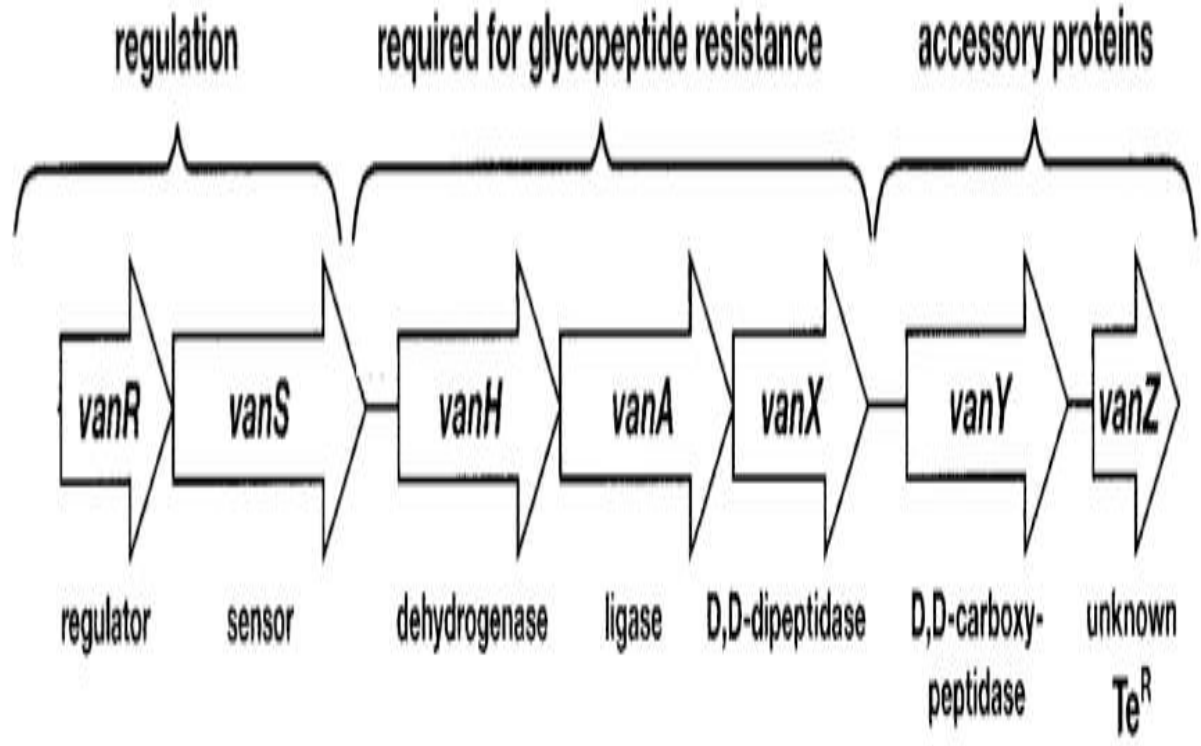
***VanE* glycopeptide resistance:**

The *vanE* gene has recently been described in *E. faecalis,* which is resistant to low levels of vancomycin, with Minimal Inhibitry Consentration (MICs)

16µg/ml (Butz *et al*. 1990). Isolates that have the *vanC* genotype display intrinsic,constitutive, low-level resistance to vancomycin (MIC, 2 to 32µg/mL) and are susceptible to teicoplamin(MIC, 0.5 to 1 µg/mL). The *vanC* genotype corresponds to the intrinsic glycopeptide resistance seen in *E.gallinarum*, *E. casseliflavus*, and *E. flavescens*. This *vanC* gene cluster is not transferred by conjugation to other organisms, is generally constitutively expressed, and is chromosomal in origin.

***VanD* glycopeptide resistance:**

The strain carring this resistance gene is *E. faecium* that inhibited by vancomycin at 64µg/ml . *VanD* appears to be located on the chromosome and is not transferable to other enterococci (Ostrowsky,1999). This *vanC* gene cluster is not transferred by conjugation to other organisms, is generally constitutively expressed, and is chromosomal in origin.



Organisation of VanA-type glyc opeptide resistance operon. The arrows show regulatory and resistance and the accessory coding sequences reproduced from Courvalin 2006.

Epidemiology and control of VRE:

Geographic distribution and spread within hospitals:

Since their initial recovery from patients in the UK and France, VRE have been found in many other countries, including Australia, Canada, Denmark, Italy, Malaysia, and the US (Woodford e*t al*., 1995).

Hospital outbreaks of infection or colonization have been reported with both *VanA* and *VanB* isolates (Boyce *et al*., 1995). Patients may be colonized simultaneously with more than one strain of VRE (Mato *et al*., 1996; Wade, 1995). Stool isolates of VRE have included a

number of different species such as *E. faecalis*, *E. faecium*, *E.gallinarum*, and *E. avium* (Bates *et al*., 1995). Fortunately, rates of stool colonization with VRE among hospitalized patients by far exceeds infection rates with these organisms (Lam *et al*., 1995;Montecalvo *et al*.,1995). Gastrointestinal tract colonizatin with VRE may persist for weeks or monthes, and single negative cultures may be intermixed with positive culters over time (Montecalvo *et al*., 1995). During outbreaks,enviromental cultures in hospital rooms have yielded VRE (Boyce *et al*., 1995; Mato *et al*., 1996 ; Slaughter *et al*., 1996).

VRE in the community:

In the USA, attention has focused on the epidemiology of VRE mainly in hospitals, and there is little evidence to suggest that transmission of VRE to healthy adults occurs to any significant extent in the community (Murray, 1995). In a study in Texas, investigators failed

to find any VRE in the feces of chickens (Murray, 1997). In addition,VRE could not be isolated from healthey volunteers in two studies (Murray, 1997 and Wade, 1995). Two cases of apparent community acquired VRE urinary tract infections in New York City have been reported (Friden *et al*., 1993).

The situation in Europe is quite different from that in the United States. In Europe, VRE have been isolated from sewage and various animal soureces (Bates, *et al*., 1995; Klare, *et al.,* 1995). It has been suggested that the use of glycopeptide-containing animal feeds in some reigons of Europe may have contributed to such differences . In one study, *VanA* resistant *E. faecium* was isolated from frozen poultry and pork and from the feces of 12 of 100 non hospitalized inhabitants in a rural area (Klare *et al*., 1995) *VanA* VRE have also be found in the feces or intestines of other farm animals (Devriese *et al*., 1996). These observations suggest a potential for VRE or the resistance genes of VRE to reach humans through the food chain or through contact with domesticated animals (Gordst *et al*., 1995) .

Reservoirs:

Although much has been learnt about the epidemiology of VRE in recent years, the most important reservoirs of VRE has not been reached. Subsequent investigations revealed that several of these patients resided in farms and that chickens and swine present on the farms were colonized with vancomycin-resistant *E. faecium.* VRE has subsequently been recoverd from various animal sources in different European countries . The occurrence of VRE in such animals could be related to the fact that avopracin (a glycopeptide) has been available as a feed additive for more than 15 years in the United Kingdom and other European countries (Aarestrup, 1995; Witte *et al*., 1995). It has been fed

to chickens, swine, and cattle. In the US, avopracin has not been used as a feed additive for animals, and culture surveys of a limited

number of chickens in several cities have failed to detect VRE (Harrison *et al*., 1995). Further studies of animal-based food products are needed to determine if food items represent a community reservoir for VRE in that country (Boyce, 1997).

Mode of transmission:

Transmission of VRE by hospital workers whose hands become contaminated with the organism while caring for patients is probably the most common mode of hospital acquired infection transmission (Tornieporth *et al*., 1996; Zervos *et al.,* 1987). Transmission of VRE may also occur by way of contaminated medical,surgical equipment, although this is probably much less important than transmission by the hands of personnel. Electronic thermometers contaminated with the outbreak strain were epidemiologically implicated in an outbreak described by Livornese *et al*., (1992).

Since *enterococci* may remain viable for several days to weeks on dry surfaces, it seems that contaminated surfaces may act as a source from which personnel may contaminate their hands or clothing (Boyce *et al*., 1994). However, further studies are necessary to determine the extent to which these items contribute to the transmission of VRE (Boyce *et al.*,1997).

Prevention and control:

The epidemiology of VRE has not been completely elucidated; certain patient populations are at high risk for VRE infection or colonization . These include critically ill patients or those with severe underlying disease or immunosuppression, such as intensive care unit (ICU) patients or patients in oncology or transplantation. Those who have had an intra-abdominal or cardiothoracic surgical procedure, and those who have had prolonged hospital stay or received multiple antimicrobial agents (Boyce *et al*., 1994; Boyle *et al*., 1993; Centers for Disease Control and prevention. 1993; Friden *et al*., 1993; Handwerger *et al*.,

1993; Karanfil *et al*., 1992 and Montecalvo *et al*., 1994). Because *enterococci* are part of the normal flora of the gastrointestinal tract and the female genital tract, most infections with those organisms have been attributed to the patient's endogenous flora (Murray, 1990). However, recent reports have demonstrated that *enterococci,* including VRE, can be spread by direct patient-to patient contact or indirectly via transient carriage on the hands of personnel (Boyce *et al*., 1994), Contaminated environmental surfaces (Boyce *et al*., 1994; Karanfil *et al*., 1992), or patient care equipment (Livornese *et al*., 1992).

In an effort to control the hospital acquired infection transmission of VRE, Hospital Infection Control Practices Advisory Committee (HICPAC) published recommendations in February 1995 (Centers for Disease Control and Prevention. 1995). These recommendations mainly focused on ; prudent use of

vancomycin, education of hospital staff, effective use of the microbiology laboratory, implementation of infection control measures (including the use of gloves and gowns) and isolation of patients, as appropriate to specific conditions (Boyce, 1997; Centers for Disease Control and prevention. 1995).

Education programs:

Continuing educational programs for hospital staff (including students, pharmacy personnel, nurses, laboratory personnel) should include information about the epidemiology of VRE (Centers for Disease Control and prevention, 1995). Because detection and containment of VRE require high performance standards for hospital personnel, special awareness and educational sessions may be indicated (Boyce, 1997).

Role of the microbiology laboratory in the detection of VRE:

Early detection of patients colonized or infected with VRE is an essential component of any hospital program designed to prevent nosocomial transmission of VRE (Boyce, 1997). Once the prevalence of VRE reaches high levels within an institution, prevention of transmission is more difficult. The microbiology laboratory i**s** the 1st line of defense against the spread of VRE in the hospital . The ability of the laboratory to identify *enterococci* and to detect Vancomycin resistance accurately is essential in recognizing VRE colonization and infection and avoiding complex, costly containment efforts that are required

when recognition of the problem is delayed (Centers for Disease Control and Prevention,1995).

In addition, cooperation and communication between the laboratory and the infection control program would facilitate control efforts (Friden, *et al*., 1993; Karanfil, *et al*., 1992).

Enterococci may also be tested for vancomycin resistance by using Polymerase Chain Reaction (PCR) assays designed to detect the genes responsible for glycopeptide resistance in these organisms (Clark,*et al*., 1993; Swenson, *et al*., 1992). Such testes may be particulary helpful in detecting *VanB* or *VanC* containing strains with low-level resistance to vancomycine (Swenson, *et al*., 1992). Testing VRE isolates for susceptibility to teicoplanin by using simple disk diffusing tests will differentiate between *VanA* (teicoplanin-resistant) and *VanB* (teicoplanin-susceptible) strains in most instances (Satake, *et al*., 1999)

AIMS AND OBJECTIVES

1. To isolate and speciate enterococci from cases of nosocomial infection.

2. To determine the antibiotic susceptibility pattern of the isolates and the pattern of vancomycin resistance.

3. Screening for vanA gene using molecular methods.

MATERIALS AND METHODS

This study was conducted during the period from April 2017 to May 2018 at the Department of Microbiology, Tirunelveli Medical College , Tirunelveli.

INCLUSION CRITERIA:

During the study a total of about 100 non duplicate clinical isolates of *Enterococci* were collected from different clinical samples like clinical specimens such as urine, blood,

pus, tissue fluids obtained from both in-patient and out-patient departments of Tirunelveli Medical College. The *Enterococcal* species were identified by standard biochemical techniques.

EXCLUSION CRITERIA:

- Patients who were already on treatment with vancomycin .

A detailed history regarding previous hospital admission within two years, antibiotic intake in previous six months was elicited from every patient.

Ethical clearance:

As this study involved the clinical samples from the patients, institutional ethical clearance was obtained before the commencement of the study.

Informed consent:

Informed consent was obtained from all persons involved in this study.

Proforma:

A filled in proforma was obtained from the patients with details like name, age, sex, ward, clinical diagnosis, risk factors, surgical intervention, hospital stay, previous use of Vancomycin or any other antibiotics and other parameters relevant to the study.

Sample storage:

The *Enterococcal* isolates were sub-cultured on to nutrient agar slope and stored at 2 to 8^0C. The *Enterococcal* isolates were sub-cultured every fortnight.

Primary isolation and identification of *Enterococci* :

Samples:

During the study period sample collected from were urine, blood, pus, tissue fluids were collected from those patients attending outpatient& inpatient Department in Tirunelveli Medical College . Samples which were received was processed within two hours of receipt as per standard procedures.

Microscopy:

A primary smear is made from the sample and stained with Gram stain, gram positive cocci which appear mainly in pairs slightly ovoid in shape and may appear in short chains, or as single cells were suspected of being enterococcus.

Biochemical reactions:

Catalase test:

It was done by slide test or tube test.

Slide test-

A single colony taken from nutrient agar plate was placed over the clean glass slide, to this one drop of 3% H2O2(hydrogen peroxide) was added, effervescence was not observed. When effervescence appeared it was a negative test.

Tube test-

1ml of 3% H2O2 was taken in a small test tube, small amount of bacterial growth was introduced with the help of glass rod or plastic applicator stick , and presence of effervescence was not observed.

Bile esculin agar:

The suspected *Enterococcal* isolates were then inoculated onto Bile esculin agar (containing 40% bile) , incubated aerobically at 37^0C overnight. The next day the isolates showing black discoloration of the medium due to hydrolyse of esculin to esculetin and dextrose which reacts with ferric citrate were identified as BEA positive.

Aesculin agar plates were inoculated with the suspected isolates and incubated at 37^0C for 48 hr. aerobically. Isolates that produced blackening of the medium were tentatively identified as

enterococci.

Growth in 6.5% NaCl:

Two or three colonies of the suspected enterococci were inoculated into nutrient broth containing 6.5% NaCl. The inoculated broth was incubated at 37^0C for 3 days aerobically. Growth was indicated by development of turbidity.

Heat tolerance test:

The suspected *Enterococcal* isolates along with the control strains were tested for heat tolerance by inoculating them into BHI broth and incubating them along at 60^0C for 30 minutes in a water bath. Subcultures from the broth were done on blood agar and Mac Conkey agar before incubation and at intervals of 10 min, 20min and 30 minutes after incubation . ATCC *E.faecalis 29212* was used as a positive control.

The growth of the positive control was checked before reading other isolates. The ATCC control strain growth both before and after heating the broth at 60^0C for 30 minutes was noted. The isolates showing growth before and after 30min of incubation at 60^0C were taken as heat tolerant *Enterococcal* isolates .

Salt tolerance:

Salt tolerant property of the suspected *Enterococcal* isolates were tested by inoculating 2 to 3 identical colonies of suspected isolates along with control strains into a tube containing nutrient broth with 6.5% sodium chloride and incubated at 37^0C for 24-72 hours. 1% bromo cresol purple was added as an indicator to detect yellow discoloration on growth. The broth showing turbidity with or without yellow discoloration was taken as positive reaction and was confirmed by subculturing the broth on blood agar / Mac Conkey agar. The salt tolerant isolates grew well even in the presence of 6.5% NaCl. Salt tolerant , BEA positive isolates, which were able to grow on MacConkey agar and at temperatures of > 45ºC were identified as *Enterococci* and selected for further speciation.

Speciation of the *Enterococcal* isolates was done based on the Faklam and Collins conventional identification scheme. *Enterococci* were classified into the physiological groups I-V based primarily on arginine dihydrolysis, fermentation of mannitol and sorbose .

Further speciation was based on acid production from specific carbohydrates and motility and pigment production.

Arginine dihydrolysis:

Arginine dihydrolysis was tested by inoculating the isolate into a tube of Moeller' s decarboxylase broth containing arginine and a control tube (without arginine), overlaid with sterile liquid paraffin and incubated for seven days at 37^0C . Control strains were also included in the test. Development of deep purple colour due to alkalinisation after an initial change to yellow colour was read as positive reaction.

Persistant yellow color indicates negative reaction.

Identification of *Enterococcus* species

Identification	***E.faecalis***	***E.faecium***
Gram Stain	Cocci in pairs & short chain	Cocci in pairs & short chain
Catalase	Negative	Negative
Motility	Nonmotile	Nonmotile
Blood Agar	α – or non hemolytic, small, cream	α – or non hemolytic, small, cream

	colored,smooth colonies with entire edge	colored,smooth colonies with entire edge
MacConkey agar	Lactose fermenting, majenta coloured colonies	lactose fermenting, majenta coloured colonies
Bile esculin agar	Positive	Positive
Heat tolerance	survives a temperature of 60ºc for 30 minutes.	survives a temperature of 60ºc for 30 minutes.
Arginine dihydrolysis	Positive	Negative

ANTIBIOTIC SUSCEPTIBILITY TESTING :

Antibiotic susceptibility testing of all the *Enterococcal* isolates and the screening and confirmatory tests for the detection of specific resistance mechanism of Glycopeptide resistance were performed as per CLSI Standards.

ANTIBIOGRAM BY KIRBY-BAUER DISC DIFFUSION

METHOD :

The antibiotic susceptibility pattern of the isolates was determined by on Mueller –Hinton agar (MHA) .The bacterial inoculums was prepared by inoculating few identical colonies in a Nutrient broth and incubated for 3-6hrs. It was standardized with 0.5 Mc Farland turbidity

standard (1.5×108 CFU/ml) before inoculation, if the bacterial suspension is too thick it should be diluted to match the standard and if it is less turbid, it has to incubated further.

After standardization, a sterile swab is dipped in that broth and the excess fluid is squeezed out by pressing on the side of the test tube, and it was streaked on the surface of the agar three times, turning the plate at 60º each time to produce a lawn culture of the organism. Then it is allowed to dry and the antibiotic discs were placed over the lawn culture within 15 minutes of inoculation.

The antibiotics tested were as follows Antibiotic susceptibility testing by kirby-Bauer disk diffusion method as per the CLSI (clinical Laboratory Standards Institue) guideline again Vancomycin (30 microgram) , Teicoplanin (30 microgram) , Linezolid (30 microgram) , Penicillin G (10 microgram), Tetracycline (30 microgram) , Ciprofloxacin (5 microgram) , Chloramphenicol (30 microgram), Doxycycline (30 microgram) and HLG (120 microgram).

All the materials and antibiotic disc were procured from Himedia laboratories Pvt.Ltd. Mumbai. The inoculated plates were incubated aerobically at 37ºC overnight. Next day the zone of inhibition of the bacterial growth around each disc was measured using ruler under reflected light except for Vancomycin and read through transmitted light. The interpretation as susceptible, intermediate and resistant were done according to the CLSI guidelines.(Appendix)

DETECTION OF VANCOMYCIN RESISTANCE BY VANCOMYCIN SCREEN AGAR :

Presumptive identification of Vancomycin resistance was done by Vancomycin screen agar (i.e) brain heart infusion (BHI) agar containing 6 µg /ml Vancomycin. 10µl of 0.5 McFarland suspension of the isolate ,along with positive and negative control strains, was spot inoculated onto the agar surface and incubated aerobically **for 24hrs** at 35±2ºC.Growth of > 1 colony indicated presumptive Vancomycin resistance which was confirmed by determining the Minimum inhibitory concentration (MIC) for vancomycin.

MIC for vancomycin- resistant Enterococcus by E strip method :

Minimum inhibitory concentration (MIC) value for Vancomycin were determined using Hi Comb MIC Strip (Hi-media, Mumbai). Any *Enterococcus* was considered VRE if the MIc was ≥16microgram/ml.

E-test was done to determined the minimum inhibitory concentration of Vancomycin for all the clinical isolates of enterococci. The E-test is comprised of two strips: Strip A: 240-0.01 µg and Strip B: 4-0.001 µg (Hi Comb, MIC test, HIMEDIA laboratories).The results were interpreted as per CLSI guidelines.

MIC MINIMUM INHIBITORY CONCENTRATION FOR TEICOPLANIN:

E-test was done to determined the minimum inhibitory concentration of Teicoplanin for the VRE isolates .The glycopeptide teicoplanin MIC was also tested in the same method as described above for Vancomycin using Hi Comb, MIC test, The E-test is comprised of two strips: Strip A: 240-0.01 µg and Strip B: 4-0.001 µg HIMEDIA laboratories. Any *Enterococcus* was considered Teicoplanin resistant if the MIC was ≥32 microgram/ml.

MOLECULAR METHOD FOR THE DETECTION OF VRE:

Polymerase chain reaction (PCR) assay was performed for the detection of Vancomycin resistance genes in *Enterococci* especially in *E.faecium, and*

E.faecalis by the PCR Kit procured from Helini Biomolecules,Chennai. The DNA was extracted from the *Enterococcal*

isolates by using Helini Pure Fast Bacterial Genomic DNA Mini Spin Purification Kit and subjected to PCR and the gene product viewed by gel electrophoresis.

Extraction of DNA from the *Enterococcal* isolates :

1. 1ml of overnight bacterial culture centrifuged at 6000rpm for 5 min.
2. Supernatant discarded
3. Pellet is suspended in 0.2ml PBS.
4. 180µl of Lysozyme digestion buffer and 20µl of Protinase K added,
5. Incubated at 37ºC for 15 min.
6. 400µl of Binding buffer, 5µl of control template and 20µl Protinase K added, Mixed well by inverting several time.
7. Incubate at 56ºC for 15 min.
8. Added 300µl of Ethanol and mixed well.
9. Transferred entire sample into the PureFast spin column. Centrifuged for 1 min. Discard the flow-through and place the column back into the same collection tube.
10. Added 500µl wash buffer-1 to the PureFast spin column. Centrifuge for 30-60 seconds and discarded the flow-through. Place the column back in to the same collection tube.

11. Added 500µl wash buffer-2 to the PureFast spin column. Centrifuge for 30-60 seconds and discarded the flow-through. Place the column back in to the same collection tube.

12. Discard the flow-through and centrifuge for an additional 1 min. This step is essential to avoid residual ethanol.

13.Transferred the PureFast spin into a fresh 1.5ml micro-centrifuge tube.

14.Added 100µl of Elution Buffer to the centre of PureFast spin column membrane.

15.Incubate for 1 min at room temperature and centrifuge for 2 min.

16.Discard the column and store the purified DNA at -20ºc. Quality and Quantity of extracted DNA is checked by loading in 1% agrose gel and 5µl of extracted DNA is used for PCR amplification.

PCR Procedure:

1. Reactions set up as follows:

Components	Quantity
HELINI RedDye PCR Master mix	10µl
HELINI RedDye PCR -Primer mix	5µl
Purified bacterial DNA	5µl
Total volume	20µl

2.Mixed gently and spin down briefly

3. place into PCR machine and program it as follows;

Initial Denaturation: 95ºC for 5 min

Denaturation : 94ºC for 30 sec

Annealing : 58ºC for 30 sec } **35 cycles**

Extension : 72ºC for 30 sec

Final extension : 72ºC for 5 min

Loading:

1. Prepared 2% agarose gel. [2gm of agarose in 100ml of 1X TAE buffer]

2. Run electrophoresis at 50V till the dye reaches three fourth distance and observe the bands in UV Transilluminator.

Agarose gel electrophoresis:

1. Prepared 2% agarose. (2gm agarose in 100ml of 1X TAE buffer and melted using micro oven)

2. When the agarose gel temperature was around 60ºC, added 5µl of Ethidium bromide.

3. Poured warm agarose solution slowly into the gel platform.

4. Kept the gel set undisturbed till the agarose solidifies.

5. Poured 1XTAE buffer into submarine gel tank.

6. Carefully placed the gel platform into tank. Maintained the tank buffer level 0.5cm above then gel.

7. PCR samples are loaded after mixed with gel loading dye along with 10µl HELINI 100 bp DNA Ladder.

[100bp, 200bp, 300bp, 400bp, 500bp, 600bp, 700bp, 800bp, 900bp, 1000bp and 1500bp]

8. Run electrophoresis at 50V till the dye reaches three fourth distance of the gel.

9. Gel viewed in UV Transilluminator and observed the bands pattern.

RESULTS

5.1. STUDY DESCRIPTION

This study was conducted at the Department of Microbiology, Tirunelveli Medical College, over a period of one year from April 2017 to March 2018. Out of 996 culture positive samples received in the Microbiology laboratory , a total of 100 enterococcal species were isolated.

These enterococcal isolates were subjected to antimicrobial susceptibility testing by disc diffusion method for the following antibiotics: Penicillin, Tetracycline, Linezolid, Ciprofloxacin, Chloramphenicol, Teicoplanin, Doxycycline, High level Gentamycin and Vancomycin. Susceptibility of the isolates to vancomycin was tested by vancomycin screen agar and E strip method. Those isolates found resistant to vancomycin was screened for MIC of Teicoplanin by E strip and Van A gene using PCR.

5.2. STATISTICAL ANALYSIS

Data collected were entered in Microsoft Excel and analysed using statistical analysis software Statistical Package for Social Services (SPSS) v.16. Appropriate descriptive and inferential statistics were calculated. p values <0.05 were statistically significant.

5.3. ISOLATION OF ENTEROCOCCI

Among the 100 enterococcal isolates, 56 isolates were from male patients and 44 from female patients. Most of the isolates (27%) were from patients aged between 16 and 30 years. The mean age of the patients was found to be 35.6

years with a SD of 21.5 years. The age of the patients ranged from a minimum of 2 years to a maximum age of 84 years. The table below shows the age and sex distribution of the patients.

Table 1 Age and sex distribution of the patients

Age of the patients	**Sex of the patients**		**Number of Enterococcal isolates (N=100)**
	Male	**Female**	
Less than 15 years	12 (60%)	8 (40%)	20 (20%)
16 years to 30 years	13 (48%)	14 (52%)	27 (27%)
31 years to 45 years	9 (45%)	11 (55%)	20 (20%)
46 years to 60 years	12 (67%)	6 (33%)	18 (18%)
More than 60 years	10 (67%)	5 (33%)	15 (15%)
Total	56 (56%)	44 (44%)	100

Table 2 Distribution of isolates among Inpatients and Outpatients

Type of patients	Percentage
Out patients	31
Inpatients	69

Out of the 100 samples, 69 samples were collected from inpatients and remaining 31 from outpatients.

Figure 1 Distribution of isolates among inpatients and outpatients

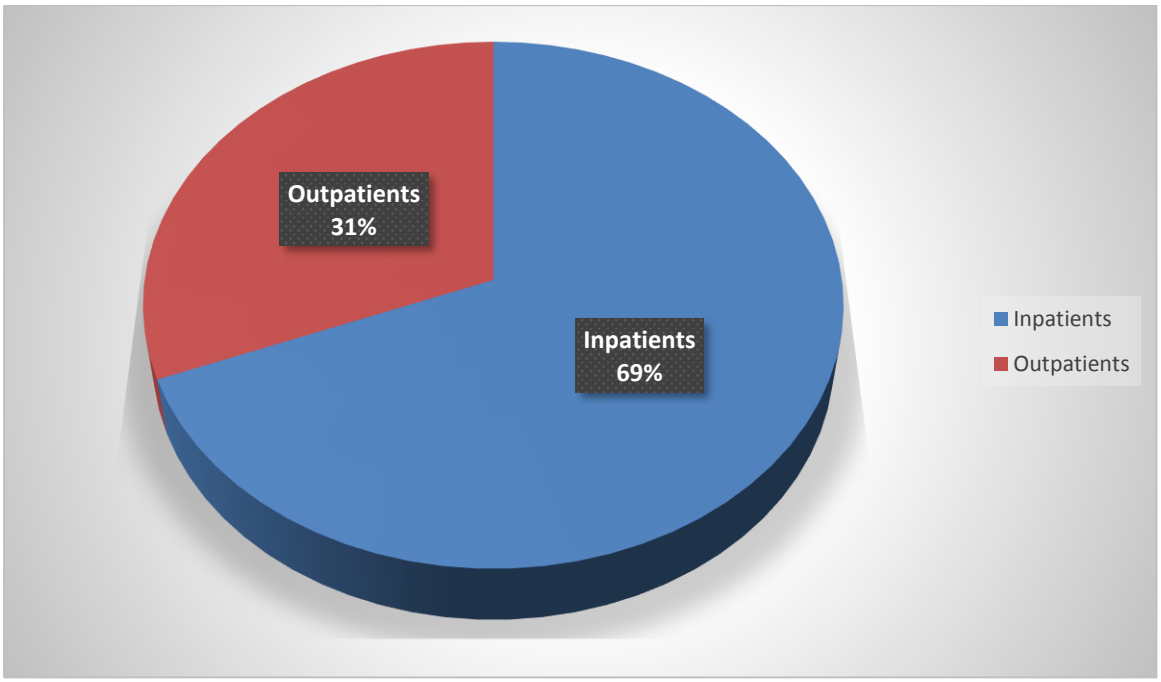


Table 3 Distribution of the Enterococcal isolates in different clinical samples

Sample	Percentage
Urine	93
Sputum	4
Pus	1
Vaginal swab	1
Ascitic fluid	1

Enterococci were commonly isolated from urine (93%), followed by sputum (4%), ascitic fluid (1%), pus from leg ulcer (1%) and vaginal swab (1%).

Figure 2 Distribution of the Enterococcal isolates in different clinical samples

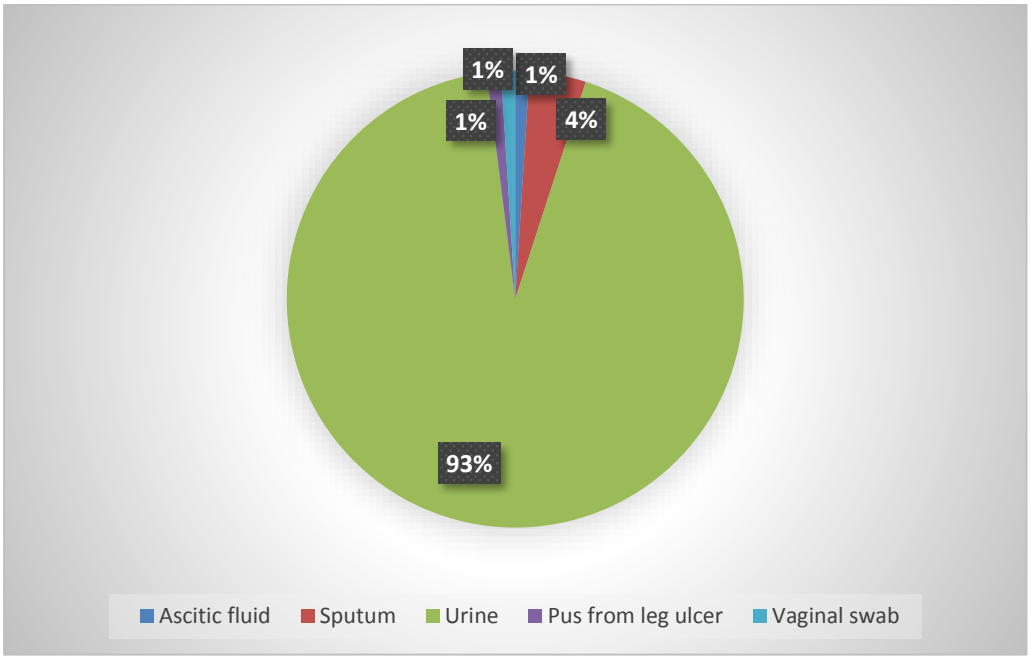


Table 4 Distribution of Enterococcal isolates in different wards

Ward	**Number of isolates (N=100) (%)**
Medicine	22 (22%)
Paediatrics	21 (21%)
Urology	17 (17%)
Surgery	13 (13%)
Maternity	9 (9%)
IMCU	8 (8%)
Nephrology	3 (3%)
CMCHIS	2 (2%)
Gynaecology	2 (2%)
Thoracic	2 (2%)
Trauma	1 (1%)

Most of the urinary isolates (43%) were from Medicine and Pediatric ward followed by Urology ward (17%), surgery ward (13%), maternity ward (9%) and IMCU (8%).

Figure 3 Distribution of Enterococcal isolates in different wards

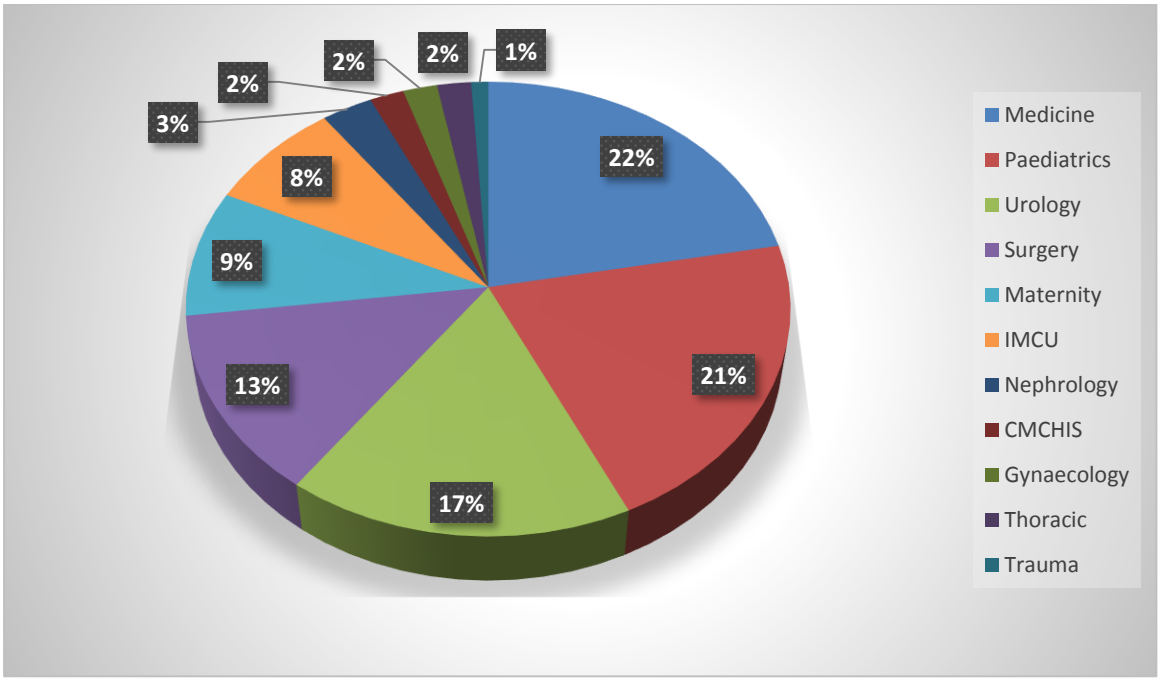


Table 5 Species distribution among isolates

Species isolated	Percentage
E.faecalis	90
E.faecium	10

The isolates were identified to be *Enterococcus faecalis* (90%) and *Enterococcus faecium* (10%).

Figure 4 Species distribution among isolates

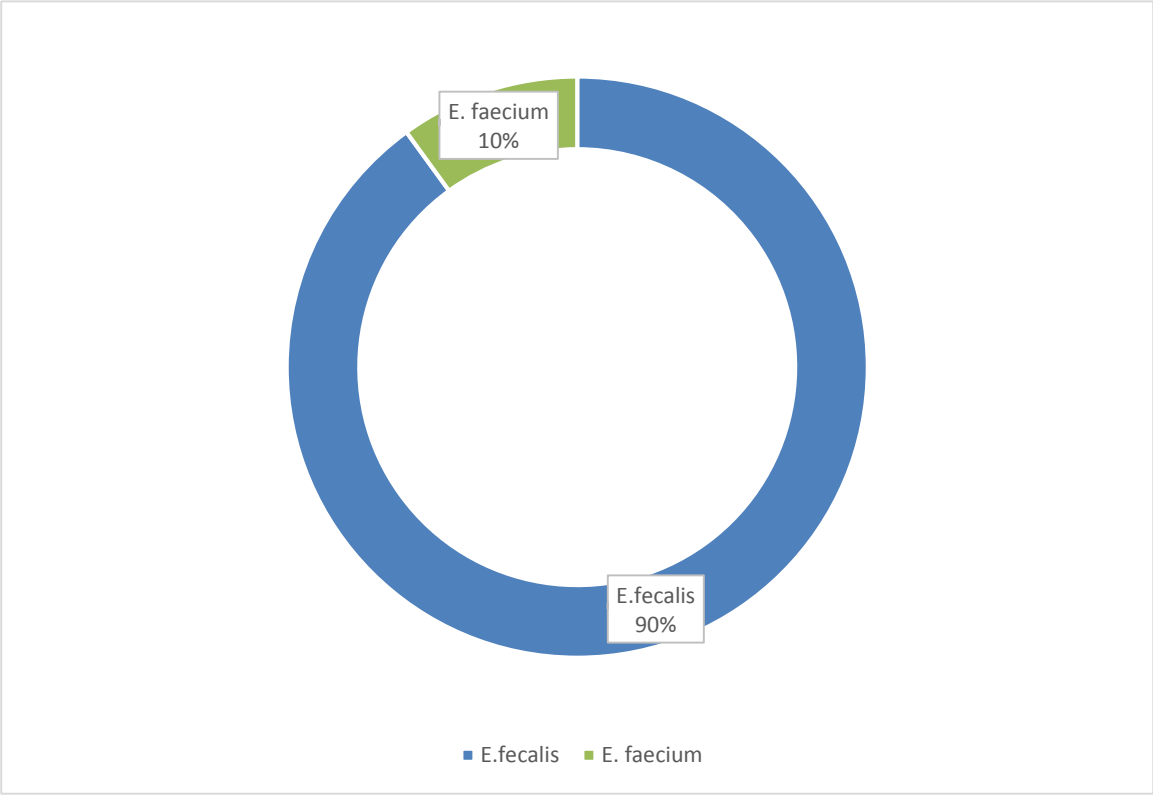
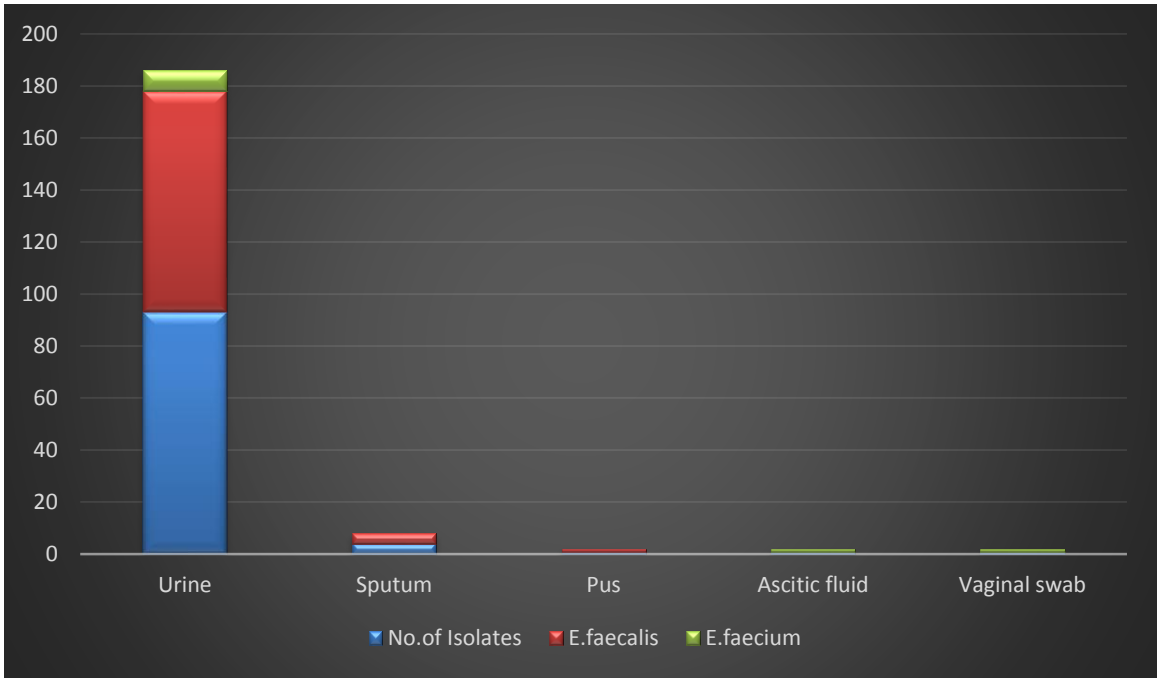


Table 6 Species distribution in different clinical samples

Sample	**No.of Isolates**	***E.faecalis***	***E.faecium***
Urine	93	85	8
Sputum	4	4	-
Pus	1	1	-
Ascitic fluid	1	-	1
Vaginal swab	1	-	1
Total	100	90	10

The above table shows the species of the Enterococcus in different clinical samples. All the four sputum isolates and pus isolates were found to be E.faecalis whereas the ascitic fluid isolate and vaginal swab isolate was found to be E.faecium.

Figure 5 Species distribution in different clinical samples



5.4. ANTIBIOTIC SUSCEPTIBILITY PATTERN OF THE ISOLATES

5.4.1. Disc diffusion method

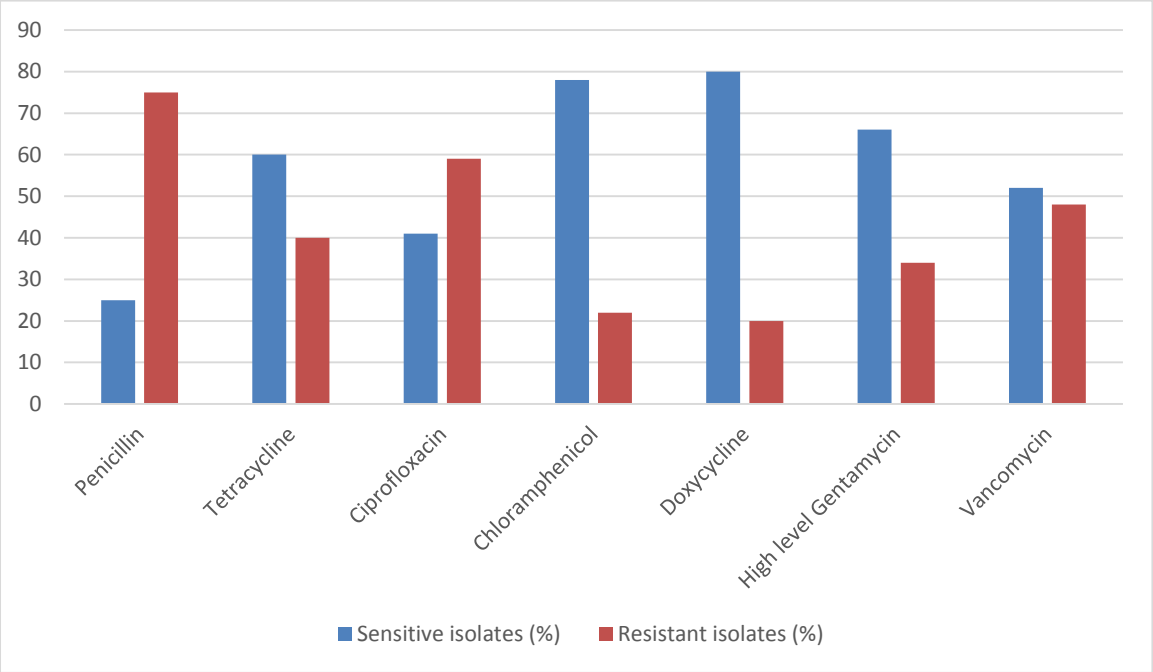
The table below shows the antibiotic susceptibility pattern of the enterococcal isolates by Kirby bauer disc diffusion method on Mueller Hinton agar according to CLSI guideline Highest prevalence of resistance was observed against Penicillin (75%), followed by Ciprofloxacin (59%) and Vancomycin (48%). On the other hand, Doxycycline was found to be sensitive for 80% of isolates, followed by Chloramphenicol (78%) and High level Gentamicin (66%).

Table 7 Antimicrobial sensitivity pattern of the Enterococcal isolates by disc diffusion method (N=100)

Antibiotic	**Sensitive isolates (%)**	**Resistant isolates (%)**
Penicillin	25 (25%)	75 (75%)
Tetracycline	60 (60%)	40 (40%)
Ciprofloxacin	41 (41%)	59 (59%)
Chloramphenicol	78 (78%)	22 (22%)
Doxycycline	80 (80%)	20 (20%)
High level Gentamycin	66 (66%)	34 (34%)
Vancomycin	52 (52%)	48 (48%)

It could be seen from the below figure that only 52% of the isolates were sensitive Vancomycin by disc diffusion method.

Figure 6 Antimicrobial susceptibility pattern of the isolates



5.4.2. Multi drug resistant isolates

Table 8: Multi drug resistant isolates

Total no of isolates	MDR isolates
100	66

Out of the 100 enterococcal isolates, 66 isolates were found to be multi drug resistant (resistant to three or more antibiotics).

Figure 7 Proportion of Multi drug resistant isolates

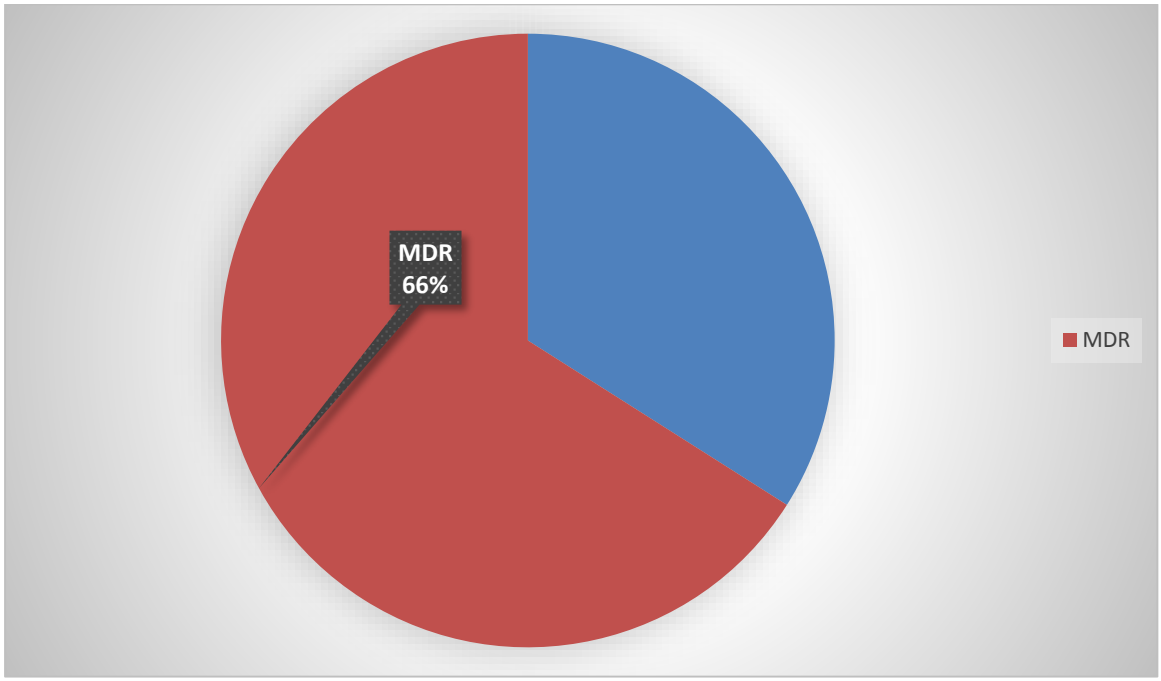
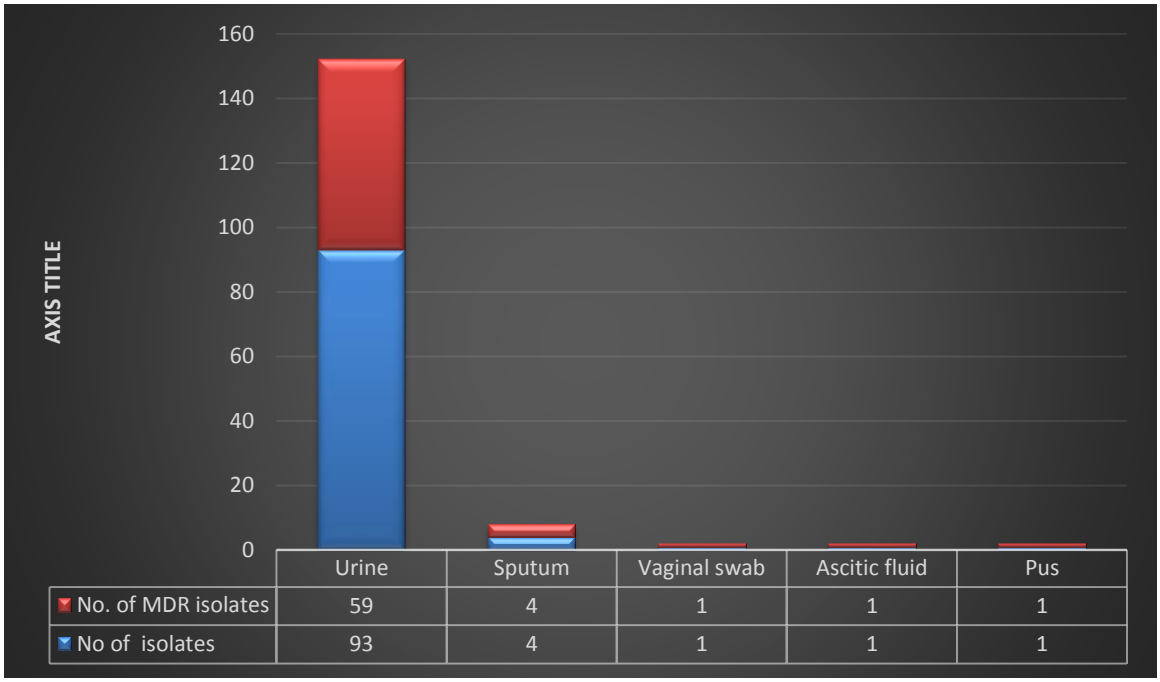


Table 9 Distribution of MDR isolates from various clinical samples

Sample	No of isolates	No. of MDR isolates
Urine	93	59 (63.4%)
Sputum	4	4(100 %)
Vaginal swab	1	1(100 %)
Ascitic fluid	1	1(100 %)
Pus	1	1(100 %)
Total	100	66

All the four sputum isolates, isolates from vaginal swab, pus and ascitic fluid were found to be multidrug resistant. Almost 63% of the urine isolates were found to be MDR. Nearly 60% of the MDR isolates were found to be resistant to vancomycin by diffusion method.

Figure 8 Distribution of MDR isolates from various clinical samples



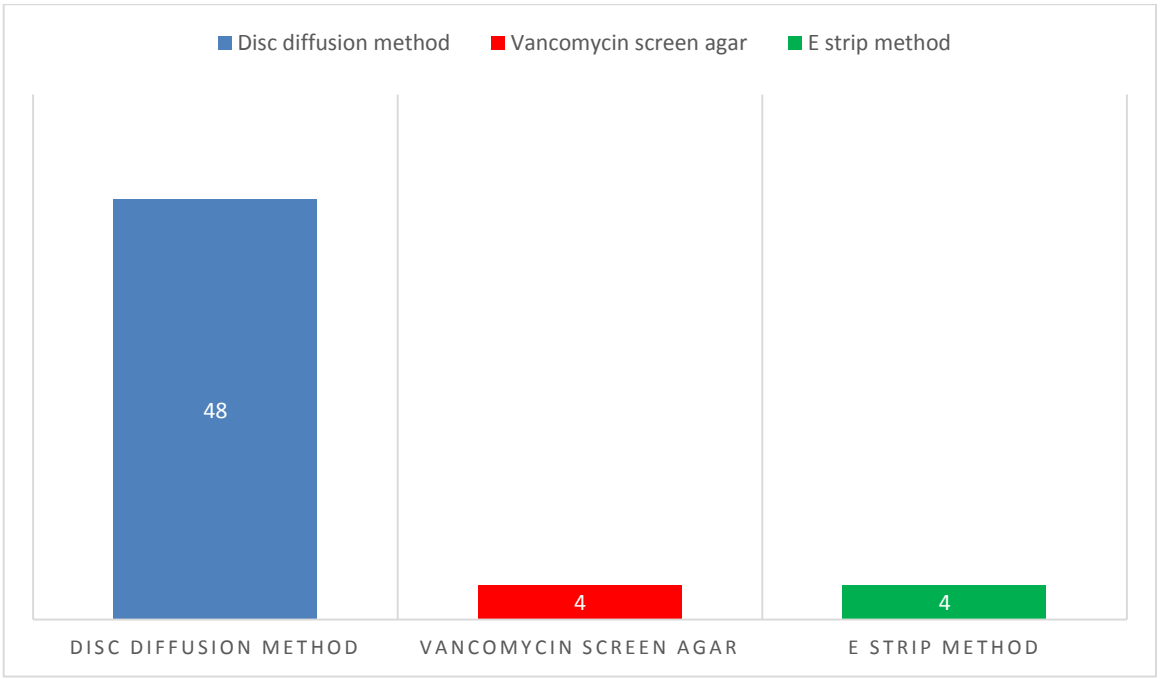
5.4.3. Vancomycin susceptibility pattern by different methods

Vancomycin screen agar and E strip method showed only four isolates to be resistant against Vancomycin. whereas, disc diffusion showed 48 isolates to be resistant against Vancomycin. This is depicted in the below figure.

Table 10 Vancomycin susceptibility pattern by different methods

Testing method	% of VRE
Disc diffusion method	48
Vancomycin screen agar	4
E strip method	4

Figure 9 Vancomycin susceptibility pattern by different methods



5.4.4. Vancomycin screen agar Vs disc diffusion method

There was a difference in the antibiotic susceptibility pattern found by disc diffusion method and Vancomycin screen agar

Table 11 Antibiotic susceptibility pattern. Disc diffusion method Vs Vancomycin screen agar

Disc diffusion method	**Vancomycin screen agar**		**Total (N=100)**
	Resistant isolates	**Sensitive isolates**	
Resistant isolates	4 (8.3%)	44 (91.7%)	48
Sensitive isolates	0	52 (100%)	52
Total	4 (4%)	96 (96%)	100

The above table shows that all the isolates found to be sensitive to vancomycin in disc diffusion method was also found to be sensitive in vancomycin screen agar method. However, only 8.3% of isolates found to be resistant to vancomycin in disc diffusion method was found to be resistant in vancomycin screen agar method.

5.4.5. E strip method Vs disc diffusion method

There also exists a difference in the antibiotic susceptibility pattern found by disc diffusion method and E strip method.

Table 12 Antibiotic susceptibility pattern. Disc diffusion method Vs E strip method

Disc diffusion method	**E strip method**		**Total (N=100)**
	Resistant isolates	**Sensitive isolates**	
Resistant isolates	4 (100%)	44 (46%)	48
Sensitive isolates	0	52 (54%)	52
Total	4	96	100

The above table shows that all the isolates found to be sensitive to vancomycin in disc diffusion method was also found to be sensitive in E strip method. However, only 8.3% of isolates found to be resistant to vancomycin in disc diffusion method was found to be resistant in E strip method. All the 4 VRE showed MIC above 16ug/ml.

This shows that the disc diffusion method is highly sensitive in detecting resistant isolates of Enterococcus (100%). But its specificity in detecting the resistance against Vancomycin remains low (54%).

5.4.6. E strip method Vs Vancomycin screen agar method

There seemed to be no difference in the antibiotic susceptibility pattern of the isolates found by Vancomycin screen agar and E strip method.

Table 13 Antibiotic susceptibility pattern. Vancomycin screen agar Vs E strip method

Vancomycin screen agar	**E strip method**		**Total (N=100)**
	Resistant isolates	**Sensitive isolates**	
Resistant isolates	4 (100%)	0	4
Sensitive isolates	0	96 (100%)	96
Total	4 (4%)	96 (96%)	100

The above table shows that all the isolates found to be sensitive to vancomycin in vancomycin screen agar method was also found to be sensitive in E strip method. Likewise, all the isolates found to be resistant to vancomycin screen agar method was also found to be resistant in E strip method. This shows that the Vancomycin screen agar method is as sensitive and as specific as E strip method for detecting antibiotic susceptibility of the enterococcal isolates.

5.5. PATTERN OF VANCOMYCIN RESISTANCE

The mean age of the patients from whom the vancomycin resistant enterococci were isolated was 38 years with a SD of 24.4 years. The mean age of the patients from whom the vancomycin sensitive strains were isolated was 35.5 years with a SD of 21.6 years. This difference was not found to be statistically significant (p value – 0.82, Independent sample t test). Similarly, vancomycin resistance of the isolates did not find any statistically significant difference between males and females (p value – 0.43, x^2test). The table below shows the pattern of vancomycin resistance among various age group and gender.

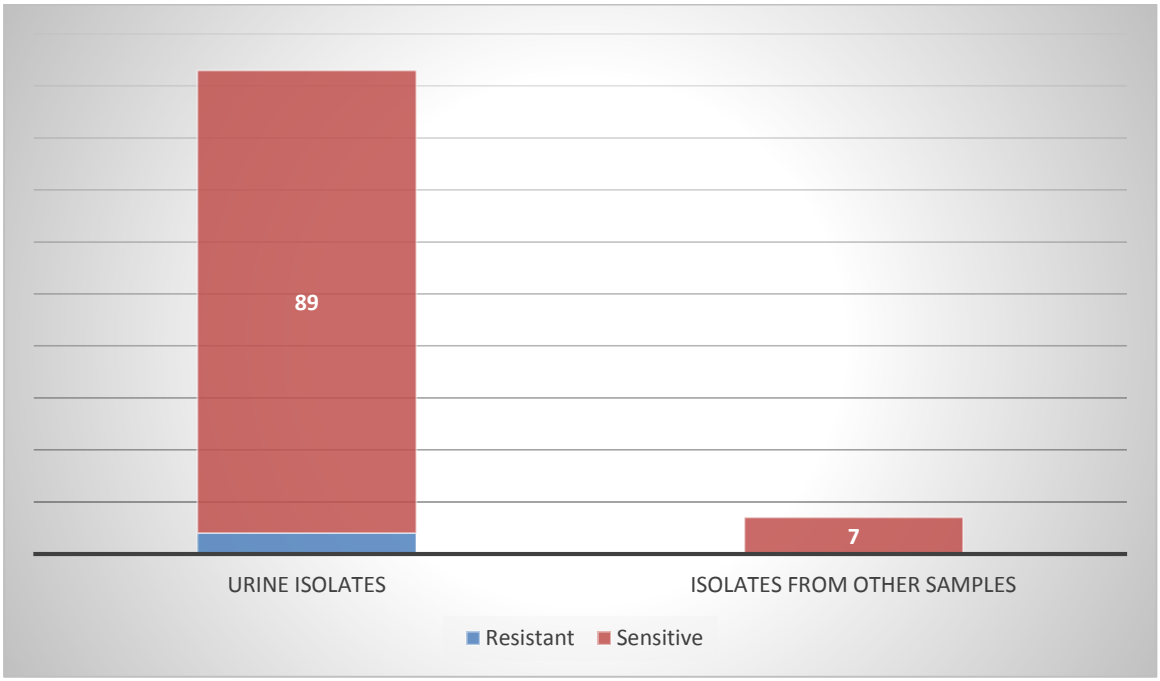
Table 14 Pattern of Vancomycin resistance among different age groups and gender

Patient details		Vancomycin susceptibility		Total (N=100)
		Resistant isolates	Sensitive isolates	
Age	**Less than 15 years**	1 (5%)	19 (95%)	20
	16 years to 30 years	1 (3.7%)	26 (96.3%)	27
	31 years to 45 years	0	20 (100%)	20
	46 years to 60 years	2 (11.1%)	16 (88.9%)	18
	More than 60 years	0	15 (100%)	15
Sex	**Male**	3 (5.4%)	53 (94.6%)	56
	Female	1 (2.3%)	43 (97.7%)	44
Outpatient		2 (6.5%)	29 (93.5%)	31
Inpatient		2 (2.9%)	67 (97l1)	69
Total		4 (4%)	96 (96%)	100

5.5.1. Vancomycin resistance pattern in different samples

All the four vancomycin resistant isolates were found to be from urine samples. Other samples which includes pus from leg ulcer, sputum, ascitic fluid and vaginal swab were found to be sensitive to vancomycin. However, statistical significance could not be drawn as there were only few sputum, pus and vaginal isolates as compared to urine isolates. It should also be noted that majority of the urine isolates (43%) were from medicine and pediatric ward.

Figure 10 Pattern of vancomycin resistance in different clinical samples



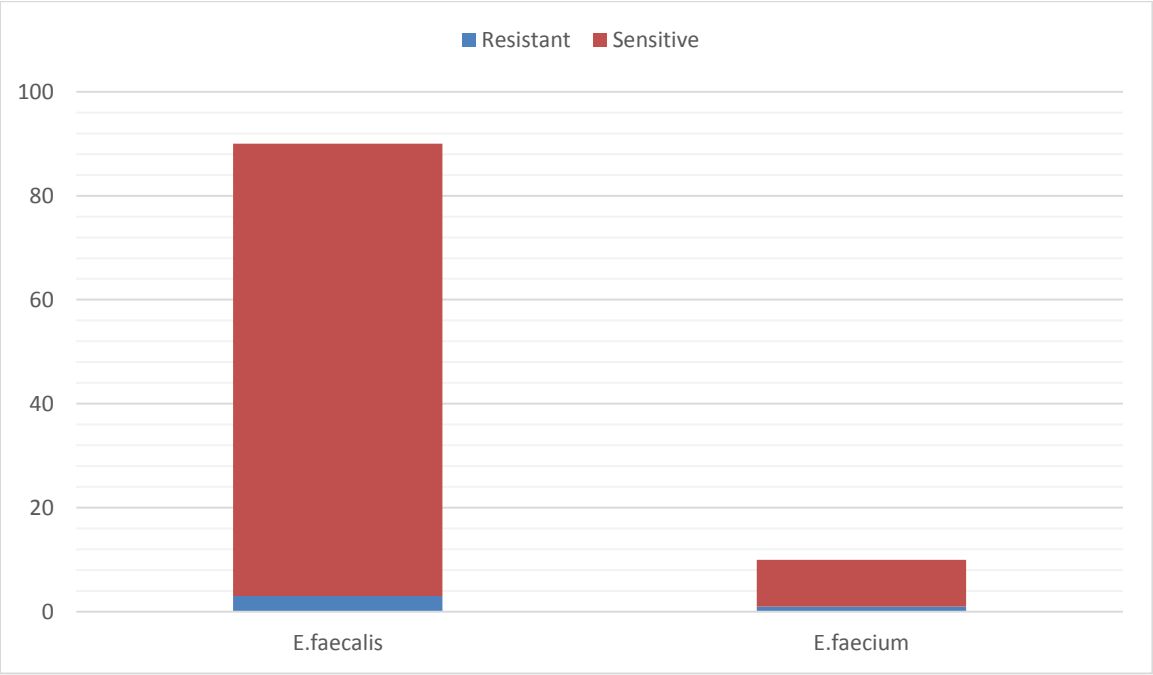
5.5.2. Vancomycin resistance pattern among different Enterococcal species

Nearly 10% of E.faecium was found to be resistant to vancomycin, whereas only 3% of E.faecalis was found to be resistant to vancomycin. There is no statistically significant difference in vancomycin susceptibility between the two species.

Table No* 15 *pattern of Vacomycin resistance among different species

Species	Total number	Vancomycin resistant isolates
E.faecalis	90	3
E.faecium	10	1
Total	100	4

Figure 11 Pattern of vancomycin resistance among different species



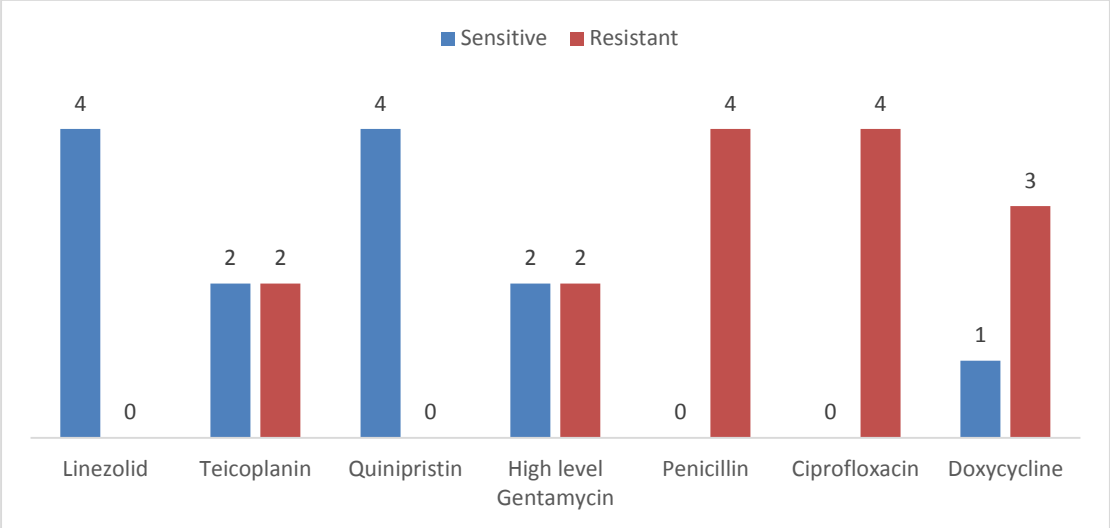
5.5.3. Antimicrobial susceptibility of Vancomycin resistant isolates

Out of 100 enterococcal isolates, only four isolates were found to be resistant to Vancomycin by E strip method. The below chart shows the antimicrobial susceptibility of those vancomycin resistant enterococcal isolates.

Table 16 Antimicrobial susceptibility of the Vancomycin resistant isolates

Antibiotic	Sensitive	Resistant
Linezolid	4	-
Teicoplanin	2	2
Quinipristin	4	-
High level Gentamycin	2	2
Penicillin	-	4
Ciprofloxacin	-	4
Doxycycline	1	3

Figure 12 Antimicrobial susceptibility of the Vancomycin resistant isolates



All the four vancomycin resistant enterococcal isolates were also found to be resistant for penicillin and ciprofloxacin. Fifty percent of the isolates were sensitive for High level Gentamycin and Teicoplanin .All the 4 isolates were sensitive to Linezolid and Quinipristin .

Table 17 Phenotypic charecterisation of VRE

VRE isolates n=4	MIC of Vancomycin by E strip ug/ml	MIC of Teicoplanin by E strip ug/ml	Phenotype
VRE 1	>60	>16	Van A
VRE 2	>120	>16	Van A
VRE 3	>60	<8	Van B
VRE 4	>60	<8	Van B

.>=more than. < less than .

5.6. SCREENING FOR VanA GENE

All the four Vancomycin resistant isolates were subjected to screening for VanA gene using PCR. It was found that all the four isolates were negative for VanA gene.

Table 18 Screening for Van A gene by PCR

Total VRE isolates	Van A gene
4	Negative

DISCUSSION

Enterococcus species have been recognised as a pathogen causing dieases like bacteremia, endocarditis, complicated urinary tract infections, intra abdominal infections, pelvic infections, wound and soft tissue infections etc. VRE has become an important nosocomial pathogen because of its rapid spread, high mortality rates associated with infections, limited option for treatment, and the possibility of transferring vancomycin resistance genes to other more virulent and more prevalent pathogens such as *Staphylococcus aureus.* This study was conducted to detect prevalence of vancomycin resistant enterococci in clinical isolates by phenotypic and genotypic methods.

A total of 100 enterococcal isolates were collected from 996 culture positive samples received over one year period. The majority of the isolates were from urine (93%) followed by sputum(4%),pus (1%),ascitic fluid (1%) and vaginal swab (1%) In the study by V.gupta et al majority of the isolates were from urine (49%) and blood (5%).

In the study group 56 % were males and 44 % were females, Majority of the isolates were from the age group of 16-30 years. 69% of the study group consisted of inpatients and 31% were outpatients.

90% of the isolates were identified to be *E.faecalis* and 10 % was *E.faecium* . Both these species are significantly associated with clinical disease, Parameswarappa et at in their study have found *E.faecalis* to be the predominant isolate followed by *E.faecium.* Chakraborthy et al also reported *E.faecalis* as the

predominant Enterococcal species (80%-90%) in their study followed by E.faecium(5%-15%). Several species of enterococci are currently recognized, but generally 90% of enterococcal infections are caused by E.faecalis and 10% are caused by E.faecium. Although a few studies have documented an increase in the prevalence of *E.faecium*, in this study the prevalence of this species was considerable low. *E.faecium* infections has been found to be more resistant to penicillin and aminoglycosides which is attributed to the production of enzyme 6-acetyl transferase and more penicillin binding proteins.

*Enterococcai a*re not generally regarded as high virulent bacterial pathogens, however, resistance to many antimicrobial drugs complicates the treatment of enterococcal infections. Acquired resistance to high concentrations of ampicillin ,aminoglycoside, and glycopeptides antibiotics, specifically vancomycin, has exacerbated this problem.

Antibiotic susceptibility pattern of the isolates was tested by Kirby Bauer disc diffusion method on Mueller Hinton agar. Highest prevalence of resistance was observed against pencillin (75%) followed by ciprofloxacin (59%) and vancomycin (48%). Of the 100 isolates, 66 % were multiple drug resistant..

Both E.faecalis and E.faecium exhibited >59% resistance for ciprofloxacin in this study. High level of ciprofloxacin resistance has been reported by Anbumani et al and Shah et al where ciprofloxacin resistance accounted for

58% and 62% respectvely. The isolates in this study showed 80% sensitivity to doxycycline.

High level gentamicin resistance(HLGR) was observed in 34% of the enterococcal isolates in our study. This finding correlates with the Anbumani et al, Shah et al and Fernandez at al studies where HLGR was 56%,53% and 53% respectively. Studies conducted in New Delhi and Mumbai have reported HLGR prevalence to be as high as 70 and 100 percent ,respectively.

In this study, the occurance of HLGR among the enterococcal isolates had no significant difference seen between *E.faecalis* and *E.faecium* isolates. However Mendiratta et al have reported greater resistance to HLG among *E.faecium* as compared to E.faecalis isolates.

Initial screening for VRE by disc diffusion methods detected 48 isolates as vancomycin resistant. However MIC detection by Hicomb E strip method and Vancomycin screen agar method detected only 4 isolates as vancomycin resistant. Presumptive identification of Vancomycin resistance was done by Vancomycin screen agar (i.e) brain heart infusion (BHI) agar containing 6 µg /ml Vancomycin. 10µl of 0.5 McFarland suspension of the isolate ,along with positive and negative control strains, was spot inoculated onto the agar surface and incubated aerobically for 24hrs at 35±2ºC.Growth of > 1 colony indicated presumptive Vancomycin resistance.In this study 4 isolates were detected as VRE in Vancomycin screen agar .

As per CLSI guidelines the MIC of VRE is above 16µg/ml . Minimum inhibitory concentration (MIC) value for Vancomycin were determined using Hi Comb MIC Strip (Hi-media, Mumbai). Any *Enterococcus* was considered VRE if the MIc was ≥16microgram/ml. In this study,MIC obtained for vancomycin by Hicomb E-test for three resistant *E.faecalis* isolates were more than 60µg, 60µg, 120µg and the MIC obtained for the resistant *E.faecium* isolate was above 60µg respectively .

These findings suggest that through the disc diffusion method is highly sensitive in detecting resistant isolates of Enterococcus (100%) its specificity is low (54%)

In this study the VRE isolation was 4% . Studies from Indore and Nagpur reported 14.29 & and 11.38% VRE ,respectively (Chitin S et al, Rahangdale VA et al). A study from Lucknow reported VRE in 55, 17% of isolates (Tripathi A et al). The prevalence of VRE varies based on geographic location, antibiotic use and the subject population.

In India, the prevalence of VRE has been reported to be between 0-30 %. In our study,4 isolates were found to be resistant to vancomycin. Among the VRE 3 were *E.faecalis* 1 was *E.faecium* . This is similar to the finding by Agarwal et al who found vancomycin resistance to be greater among *E.faecalis* isolates.

The mean age of patients from whom VRE was isolated was 38 years. All the 4 VRE isolates were found to be from urine samples. Vancomycin resistance enterococci was found in 3 *E.faecalis* and 1 *E.faecium* VRE isolates.

All the 4 VRE isolates were found to be resistant to pencillin and ciprofloxacin . Of the 4 VRE isolates 2 VRE were found to be susceptible to high level gentamicin. Hence, that infections could be treated with a combination of a high level aminoglycoside and a β lactam antibiotic The presence of high level gentamycin resistance and concurrent resistance to Pencillin or Ampicillin and vancomycin has been reported in some studies. An USA based study demonstrated that gentamycin resistance plasmid might cotransfer vancomycin resistance plasmids. Hence the detection of high level gentamycin resistance along with vancomycin resistant enterococci represents a significant problem in this region.

On studying the susceptibility pattern of VRE isolates to supplemental drugs like linezolid, Quinupristin and chloramphenicol , all of the he VRE isolates were susceptible to Linezolid and Quinipristin (100% sensitivity). 100% of the isolates showed resistance to chloramphenicol. The study carried out by V,Gupta,et al, from Chandigarh , India and MM Salem Behkit et al. from Iran have reported, 100% sensitivity of VRE isolates to linezolid which is similar to our study. Agarwal et al has also reported 100% sensitivity to linezolid in their study .Perlada.D.et al, from Australia also have reported 100% sensitivity to

linezolid and 100 % sensitivity to Chloramphenicol. But in our study 100% of VRE isolates were resistant to Chloramphenicol.

This study showed 50% of VRE isolates were sensitive to Teicoplanin. VRE strains have been classified by phenotypes and genotypes. Six types of glycopeptides resistance have been described among enterococci. Three of them are the most common: the VanA phenotype, with inducible high-level resistance to vancomycin, as well as to teicoplanin, encoded by the vanA gene; the VanB phenotype, with variable (moderate to high) levels of inducible resistance to vancomycin only, encoded by the vanB gene; and the VanC phenol type,with non inducible low-level resistance to vancomycin.

The VanA and VanB phenotypes are considered the most clinically relevant and are usually associated with E. *faecium* and E. *faecalis* strains while the VanC resistance is a intrinsic characteristic of E. *gallinarum* (vanC1 genotype) and E. casseliflavus (vanC2 and vanC3 genotypes) strains (Clark et al. 1998; Huycke et al. 1998; Murray 1998; Cetinkaya et al. 2000).

In this study by comparing the MIC of Vancomycin and Teicoplanin among the 4 VRE isolates,2 were of the Van A phenotype and 2 were of the Van B phenotype .All the 4 VRE isolates were subjected to Van A gene screening by PCR. However it was found that all the four isolates negative for Van A gene. It could be due to mutations in the Van A gene .Ramya Rengaraj et al have also noted an phenotype-genotype incongruence of Van A gene in their study and

suggested mutations in Van A gene as its cause. The VRE isolates in this study could be harbouring other Van genes. PCR remains the gold standard for diagnosis of Vancomycin resistance. Emerging Vancomycin resistance among Enterococcus is a cause for concern as this leads to a great difficulty in treating serious infections caused by them.

SUMMARY

- Out of 996 clinical specimens like urine, sputum,pus and Ascitic fluids 100 Enterococcal isolates were recovered . Majority of the specimens were from inpatients (69%) then from outpatients (31%).
- Majority of the isolates were from urine (93%) followed by sputum (4%) and pus (1%) ascitic fluid (1%)vaginal swab (1%).
- A total of about 8 (8%) *Enterococcal* isolates were from Intensive Medical care unit and the isolation rate from other specialities were Nephrology 3(3%), Urology 17(17%), Surgery 13 (13%), Medicine 22 (22%) and Paediatrics 21 (21%).
- Higher isolation rate about 56% (56/100) was observed in Male patients when compared to Female patients 44% (44/100).
- The age of the patients ranged from a minimum 2 years to a maximum age of 84 years. Most of the isolates (27%) were from patients aged between 16 and 30 years.The mean age of the patients was found to be 35.6 years.
- *E. Faecalis* was the predominant *Enterococcal* species with an isolation rate of about 90% in our study, followed by *E.faecium* 10%.
- The antibiotic susceptibility pattern showed highest prevalence of resistance against Pencillin (75%),followed by ciprofloxacin (59%) .On the other hand, Doxycycline was found to be sensitive for 80% of isolates followed by Chloramphenicol 78% and High level Gentamycin 66%.
- The High level gentamicin resistance was observed in 34% of isolates.

- Out of 100 Enterococcal isolates,66 isolates were found to be multi drug resistant (resistant to three or more antibiotics). Almost 63.4% of the urine isolates were found to be MDR. All the 4 sputum isolates, isolates from vaginal swab,pus and ascitic fluid were found to be multidrug resistant.
- 4 isolates (4%) were presumptively identified as vancomycin resistant by vancomycin screen agar containing 6 µg/ml vancomycin.
- The 4 isolates found to be resistant in vancomycin screening agar were also found to be resistant to Vancomycin by Hicomb E strip method. Of the 4 VRE isolates, 2 were Teicoplanin resistant by Hicomb E strip method
- All the 4 VRE isolates were resistant to Pencillin and Ciprofloxacin. 50% percent of the isolates were sensitive for High level Gentamycin and Teicoplanin. 100% percent of the isolates were sensitive to Linezolid and Quinipristin.
- 2 VRE were of van A phenotype and 2 were of van B phenotype
- All the 4 VRE as resistant isolates were subjected to detection of Vancomycin Resistance gene - Van A by Polymerase Chain Reaction.
- As per the PCR results, a total of 4 isolates including 1. *E.faecium* and three *E.faecalis* were not found to be of VanA Genotype.

CONCLUSION

Enterococci are emerging as an important pathogen causing variety of nosocomial infections and also cause community acquired infections contributing significantly to patients morbidity and mortality.

The emergence of Vancomycin resistant *Enterococci* worsens the problem further because of the Multidrug resistance exhibited by these agents leaving fewer therapeutic options for the clinicians in treating the serious life threatening VRE infections.

In our study we isolated a total of 100 *Enterococcal* isolates from various clinical samples with *Enterococcus faecalis and Enterococcus faecium* as the predominant species.

Predominant species. They should Resistant to multiple antibiotics like penicillin, ciprofloxacin and exhibited higher rate of high level aminoglycoside resistance. 4 isolates were identified as vancomycin resistant enterococci by Vancomycin Hicomb E stip. This method can be adopted in resource limited settings for the detection of vancomycin resistant phenotype of Enterococci. All the and isolates were negative for Van A gene by PCR.

The prevalence of VRE varies based on geographic location, antibiotic use of the subject population. This study emphasises the need for conducting frequent surveillance. Programmes for prompt identification of VRE in hospitals and community.

This also highlights the need for implementation of stringent infection control measures like rational use of antibiotics especially restricting the use of

Vancomycin to minimum, proper containment and effective treatment of VRE infections, strict hand washing practices, education of the healthcare workers and other personnel involved in the patient management. These measures are to be strictly followed to bring down the mortality and morbidity associated with these hospital acquired VRE infections.

BIBLIOGRAPHY

1. Antalek, M.D., Mylotte, J.M., et al. 1995. Clinical and molecular epidemiology of Enterococcus faecalis bacteremia, with specialreference to strains with high-level resistance to gentamicin. ClinInfect Dis, 20, 103–9.
2. Arias.A .C, Murray B.E . Enterococcal Infections. In :Principles of Internal Medicine, Harrisons editor.18th ed. Mc Graw Hill Medical
3. Agarwal J, Kalyan R, Singh M. High-level aminoglycoside resistance and β-lactamase production in Enterococci at a tertiary care hospital in India. *Jpn J Infect Dis* 2009; *62* :158-9.
4. Arthur, M., Depardieu, F., Cabanie, L., Reynolds, P., Courvalin, P., 1998.Requirement of the VanY and VanX D,D-peptidases for glycopeptide resistance in enterococci. Mol. Microbiol. 30:819-830
5. Boyce, J.M., Opal, S.M., et al. 1992. Emergence and nosocomial transmission of ampicillin-resistant enterococci. Antimicrob AgentsChemother, 36, 1032–9.
6. Buschelman, B.J., Bale, B.J. and Jones, R.N. 1993. Species identification and determination of high-level aminoglycoside resistance among enterococci. Comparison study of sterile body fluid isolates, 1985–1991. Diagn Microbiol Infect Dis, 16, 119–22.
7. Boyce, J. M., 1997. Vancomycine-resistant *enterococcus*: detection. Epidemiology and control measures. Infect. Dis. Clin. North Am.11:367-384
8. Boyce, J. M., Mermel, L. A., Zervos, M. J., Rice, L. B., Potter-Bynoe, G.,Giorgio, C., and Medeiros, A. A., . 1995. Controlling vancomycineresistant enterococci. Infect. Control Hosp. Epidemiol. 16:634-637.
9. Boyce, J. M., Opal, S. M., Chow, J. W., Zervos, M. J., G. Potter-Bynoe, G., Sherman, C. B., Romulo, R. L., Fortna, S., and A. A. Medeiros,A. A., 1994. Outbreak of multi-drug resistant *Enterococcus faecium* with transferable VanB class vancomycine resistance. J. Clin. Microbiol. 32:1148-1153
10. Boyd DA, Willey BM, Fawcett D, et al. Molecular characterization of *Enterococcus faecalis* NO6-0364 with low-level vancomycin resistance harboring a novel D-ala-D-ser gene cluster,*vanL*. Antimicrob Agents Chemother 2008;52:2667–2672.
11. Chitin S, Katara G, Hemvani N, Pareek S, Chitnis DS. *In vitro* activity of daptomycin & linezolid against methicillin resistant *Staphylococcus aureus* & vancomycin-

resistant enterococci isolated from hospitalized cases in Central India.*Indian J Med Res* 2013; *137* : 191-6.

12. Clark, N. C., Cooksey, R. C., Hill, B. C., Swenson, J. M., and Tenover, F.C., 1993. Characterization of glycopeptide-resistant enterococci from US hospitals. Antimicrob. Agent Chemother. 37:2311-2317.
13. Clinical Laboratory Standards Institute (CLSI). *Performance standards for antimicrobial susceptibility testing*, 21stinformational supplement (M100-S21). Wayne, PA: CLSI;2011.
14. Devriese, L. A., Leven, M., Goosens, H., Vandamme, P., Pot, B., Hommez, J., and Haesebrouck, F., 1996. Presence of vancomycinresistant enterococci in farm and pet animals. Antimicrob. Agents Chemother. 40:2285-2287.
15. Dutka Malen, S., Blaimont, B., Wauters, G., and Courvalin, P., 1994. Emergence of high-level resistance to glycopeptides in *Enterococcus gallinarum* and *casseliflavus*. Antimicrob. Agents Chemother. 38:1675-1677.
16. Eliopoulos, G. M., 1993. Increasing problems in the therapy of enterococcal infections. Eur. J. Clin. Microbiol. Infect. Dis. 12:409-412.
17. Elipoulos, G. M., 1997. Vancomycin-resistant enterococci:mechanism and clinical relevance. Infect. Dis. Clin. North Am. 11:851-865.
18. Facklam, R.R. and Collins, M.D. 1989. Identification of Enterococcus species isolated from human infections by a conventional test scheme. J Clin Microbiol, 27, 731–4.
19. Facklam, R.R. and Elliott, J.A. 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. Clin Microbiol Rev, 8, 479–95.
20. Facklam, R.R., Sahm, D.F. and Teixeira, L.M. 1999. Enterococcus. In: Murray, P.R., Baron, E.J., et al. (eds), Manual of clinical microbiology. Washington, DC: ASM Press, 297–305.
21. Facklam, R.R., Carvalho, M.G.S. and Teixeira, L.M. 2002. History, taxonomy, biochemical characteristics, and antibiotic susceptibility testing of enterococci. In: Gilmore, M.S., Clewell, D.B., et al. (eds), The enterococci: pathogenesis, molecular biology, and antibiotic resistance Washington, DC: ASM Press, 1–54.
22. Fernandez-Guerrero ML, Goyenechea A, Verdejo C, et al. Enterococcal endocarditis on native and prosthetic valves: a review of clinical and prognostic factors with

emphasis on hospital-acquired infections as a major determinant of outcome. Medicine (Baltimore) 2007;86:363–377.

23. Friden, T. R., Munsiff, S. S., Low, D. E., Willey, B. M., William, G.,Faur, Y., Eisner, W., Warren, S., and Kreiswirth, B., 1993.Emergence of vancomycine-resistant enterococci in New York City.Lcent. 342:76-79.
24. Ghanem G, Hachem R, Jiang Y, et al. Outcomes for and risk factors associated with vancomycinresistant *Enterococcus faecalis* and vancomycin-resistant *Enterococcus faecium* bacteremia in cancer patients. Infect Control Hosp Epidemiol 2007;28:1054–1059.
25. Gilmore, M.S., Coburn, P.S., et al. 2002. Enterococcal virulence. In: Gilmore, M.S., Clewell, D.B., et al. (eds), The enterococci: pathogenesis, molecular biology, and antibiotic resistance. Washington, DC: ASM Press, 301–54.
26. Gordon, S., Swenson, J. M., Hill, B. C., Piggot, N. E., Facklau, R. R., Cooksey, R. C., Thornsberry, C., Jarvis, W. R., and Tenvor, F. C. 1992. Antimicrobial susceptibility patterns of common and unusual species of enterococci causing infections in the United States. J. Clin. Microbiol. 30:2373-2378.
27. Gordts, B., Van Landuyt, H., Ieven, M., Vandamme, P., and Goossens, H., 1995. Vancomycin-resistant enterococci colonizing the intestinal tracts of hospitalized patients. J. Clin. Microbiol. 33:2842-2846.
28. Guardado R, Asensi V, Torres JM, et al. Post-surgical enterococcal meningitis: clinical and epidemiological study of 20 cases. Scand J Infect Dis 2006;38:584–588.
29. Harrison, T. S., Qaiyumi, S., Morris, J. G., J. r., and Schwalble, R. S., program Abstr. 35th Intersci. Conf. Antimicrob. Agents Chemother., abstr. J. 78, 1995.
30. Hayakawa K, Marchaim D, Palla M, et al. Epidemiology of vancomycin-resistant *Enterococcus faecalis*: a case-control study. Antimicrob Agents Chemother 2013;57:49–55.
31. Hancock, L.E. and Gilmore, M.S. 2000. Pathogenicity of enterococci. In: Fischetti, V.A. and Novick, R.P. (eds), Gram-positive pathogens. Washington, DC: ASM Press, 251–8.
32. Handwerger, S., Raucher, B., Altarac,D., Manka, J., Mrchione, S., Sing,K. V., Murray, B. E., wolff, J., and Walters,B., 1993. Nosocomialoutbreak due to *Enterococcus faecium* highly resistant tovancomycine, penicillin, and gentamicin. Clin. Infect. Dis. 16:750-755.

33. Huycke, M.M., Sahm, D.F. and Gilmore, M.S. 1998. Multiple-resistant enterococci: the nature of the problem and an agenda for the future. Emerg Infect Dis, 4, 239–49.
34. Lam, S., Singer, C., Tucci, V., Morthland, V. H., Pfaller, M. A., and Isenberg,H. D., 1995. The challenge of vancomycine-resistant enterococci: a clinical and epidemiologic study. Am. J. Infect. Control. 23:170-180.
35. Leclercq, R., Derlot, E., Duval. J., et al., 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in *E. Faceium.* N Engl. J.Med. 319:157-161.
36. Leclercq ,R., Dutka-Malen, S., Brisson-Noel, A., Molinas, C., Derlot, E., Arthur, M., Duval, J., Courvalin ,P., 1992. Resistance of Enterococci to aminoglycosides and glycopeptides. Clin. Infect. Dis. 15:495-501.Leclercq R, Courvalin, P., 1997. Resistance to glycopeptides in enterococci. Clin. Infect Dis. 24:545-554
37. Livornese, L. L., Dias, S., Samel, C., Romanowski, B., Taylor, S., May,P., Pitsakis, P., Woods, G., Kaye, D., and Levison, M. E., 1992.Hospital-acquired infection with vancomycin-resistant *Enterococcusfaecium* transmitted by electronic thermometers. Ann. Intern. Med.117:112-116.
38. armarkar MG, Gershom ES, Mehta PR. Enterococcal infections with special reference to phenotypic characterization & drug resistance. *Indian J Med Res* 2004;*119* (Suppl): 22-5.
39. Karanfil, L. V., Murphy, M., Josephson, A., Gaynes, R., Mandel, Hill, B.C., and Swenson, J. M., 1992. Acluster of vancomycine-resistant *Enterococcus faecium* in an intensive care unit. Infect. Control Hosp. Epidemiol. 13:195-200
40. Klare, I., Heier, H., Claus, H., Bohme, G., Marin, S., Seltmann, G., Hakenbeck, R., Antanassova, V., and Witte, W., 1995. *E. faecium* strains with vanA-mediated high-level glycopeptide resistance isolated from animals foodstuffs and fecal samples of humans in the community. Microb. Drug Resist. 1:265-272.
41. Klare, I., Heier, H., Claus, H., and Witte, W., 1993. Environmental strains of *Enterococcus faecium* strains with inducible high-level resistance to glycopeptides. FEMS Microbiol. Lett. 106:23-29.
42. Klare, I., Heier, H., Claus, H., Bohme, G., Marin, S., Seltmann. G., Hakenbeck, R.,Santanassova, V., and Witte,W., 1995. Enterococcus faecium strains with VanA-mediated high-level glycopeptide resistance isolated from food stuffs and fecal samples of humans in the community. Microb. Drug Resist. 1:265-272.

43. Klare, I., Heier, H., Claus, H., Reissbroadt, R., and Witte,W., 1995. VanA-mediated high-level glycopeptide resistance in *E. faecium* strains from animal husbandry. FEMS Microbiol. Lett. 125:165-172.
44. Knoll BM, Hellman M, Kotton CN. Vanomycin-resistant *Enterococcus faecium* meningitis in adults: case series and review of the literature. Scand J Infect Dis 2013;45:131–139.
45. Karmarker MG Gershom ES et al. enterococcal infection with special reference to phenotypic characterization and drug resistance.Indian J Med Res 2009;119:22-5
46. Mayhall, C. Glen., "Prevention and Control of Vancomycin McDonald, L. C., Kuehnert, M. J., Tenover, F. C., and Jarvis, W. J., 1997. Vancomycin-resistant enterococci outside the health-care setting: prevalence, sources, and public health implications. Emerg. Infect. Dis. 3:311-317.
47. Mato, R., delancestre, H., Carraher, M., Roberts, R. B., and Tomasz, A.,1996. Multiplicity of genetic backgrounds among vancomycineresistant*Entrococcus faecium* isolates recoverd from an outbreak in a New York City Hospital. Microb. Drug Resist. 2:309-317.
48. Mendiratta DK, Kaur H, Deotale V, Thamke DC, NarangR, Narang P. Status of high level aminoglycoside resistan in *Enterococcus faecium* and *Enterococcus faecalis* in hospital of central India. *Indian J Med Microbiol* 2008; *26* :369-71.
49. Murray BE. The life and times of the Enterococcus. Clin Microbial Rev 1990; 3:46-65. Back to cited text no.1
50. Murray, B. E., 1995. Editorial response, Clin. Infect. Dis. 20:1134-1136.
51. Murray, B. E., 1997. Vancomycin-resistant enterococci. Am. J. Med. 101:284-293.
52. Moellering, R.C. Jr 1992. Emergence of Enterococcus as a significant pathogen. Clin Infect Dis, 14, 1173–8.
53. Mondino, S.S.B., Castro, A.C.D., et al. 2003. Phenotypic and genotypic characterization of clinical and intestinal enterococci isolated from inpatients and outpatients in two Brazilian hospitals. Microb Drug Res, 9, 2, 167–74.
54. Navarro, F., Courvalin, P., 1994. Analysis of genes encoding D- alanine-D-alanine ligase-related enzymes in *Enterococcus casseliflavus* and*Enterococcus flavescens*. Antimicrob Agents Chemother. 38:1788-1793.
55. Noble, C.J. 1978. Carriage of group D streptococci in the human bowel. J Clin Pathol, 31, 1182–6.

56. Ostrowsky, B. E., Clark, N. C., Thuvin-Eliopoulos, C., Venkataraman, L., Samore, M. H., Tenover, F. C., Eliopoulos, G. M., Moellering, R.C., j. r., and Gold, H. S., 1999. A cluster of VanD vancomycinresistant*Enterococcus faecium*: molecular characterization and clinical epidemiology. J. Infect. Dis. 180:1177-1185.
57. Parvathi S, Appalaraju B. Isolation, characterisation and antibiogram of Enterococci from clinical samples. Indian J pathol Microbial 2003;46:501-3 Back to cited text no.2
58. Perichon, B., Reynolds, P., Courvalin, P., 1997. VanD-typeglycopeptide-resistant *Enterococcus faecalis* BM4339. Antimicrob Agents Chemother. 41:2016-2018
59. Pintado V, Cabellos C, Moreno S, et al. Enterococcal meningitis: a clinical study of 39 cases and review of the literature. Medicine (Baltimore) 2003;82:346–364.
60. Quintiliani, R., J. r., and Courvalin, P., 1994. Conjugal transfer of the vancomycin resistance determinant vanB between enterococci involves the movement of large genetic elements from chromosome to chromosome. FEMS Microbiol. Lett. 119:359-364.
61. Quintiliani, R., J. r., Evers, S., and Courvalin, P., 1993. The vanB gene cofers various levels of self-transferable resistance to vancomycin enterococci. J. Infect. Dis. 167:1220-1223.resistance in Gram-Positive Coccal Microorganisms: Fire Prevention and Fire Fighting."
62. Rahangdale VA, Agrawal G, Jalgaonkar SV. Study of antimicrobial resistance in enterococci. *Indian J MedMicrobiol* 2008; *26* : 285-7.
63. Ross PW. Streptococcus and enterococcus. In colle JG, Fraser BP, Marimion BP, et al., editors. Mackie and McCartney practical Medical Microbiology. 14TH ed. London: churchill Livingstone; 1996.p. 263-74. Back to cited text no 4.
64. Sahm, D.F. and Torres, C. 1988. High-content aminoglycoside disks for determining aminoglyside-penicillin synergy against Enterococcusfaecalis. J Clin Microbiol, 26, 257–60.
65. Schaberg, D.R., Culver, D.H. and Gaynes, R.P. 1991. Major trends in the microbial etiology of nosocomial infection. Am J Med, 91,79S–82S.
66. Shah et al. 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 ;25-27

67. Schleifer, K.H. and Kilpper-Balz, R. 1984. Transfer of Streptococcus faecalis and Streptococcus faecium to the genus Enterococcus nom. rev. as Enterococcus faecalis comb. nov. and Enterococcus faecium comb. nov. Int J Syst Bacteriol, 34, 31–4.
68. Schleifer, K.H. and Kilpper-Balz, R. 1987. Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci and lactococci: a review. Syst Appl Microbiol, 10, 1–19.
69. Smith, T. H. A. W. S., Mair, M. E., Sharp., and Halt, j. g., 1986. Bergys Manual of syst. Bacteriol.8 edition. Willian and Walkins, London, U. K. Spiegel, C. A., 1988. Laboratory detection of high-level aminoglycosideaminocyclitol resistance in *Enterococcus* spp. J. Clin. Microbiol.26:2270-2274.
70. Satake, S., Clark, N., Rimland, D., Nolte, F. S., And Tenover, F. C.,1997. Detection of vancomycin-resistant enterococci in fecal samples by PCR. J. Clin. Microbiol. 35:2325-2330.
71. Swenson, J. M., Ferraro, M. J., Sahm, D. F., P, Charache, and Tenover, F. C., 1992. New vancomycine disk diffusion breakpoints forenterococci. The National Committee for Clinical Laboratory Standards Working Group on Enterococci. J. Clin. Microbiol. 30:2525-2528.
72. Swenson, J.M., Ferraro, M.J., National Committee for ClinicalLaboratory Standard Working Group on Enterococci, et al. 1995.Multilaboratory evaluation of screening methods for detection of highlevelaminoglycoside resistance in enterococci. J Clin Microbiol, 33,3008–18.
73. Sood s, Malhotra M, Das BK, Kapil A. Enterococcal infections & antimicrobial resistance. Indian Med Res 2008;128:111-21. Back to cited text no.3
74. Strausbaugh, L.J. and Gilmore, M.S. 2000. Enterococcal infections. In:Stevens, D.L. and Kaplan, E.L. (eds), Streptococcal infections: clinicalaspects, microbiology, and molecular pathogenesis. New York: Oxford University Press, 280–301.
75. Stern, C.S., Carvalho, M.G.S. and Teixeira, L.M. 1994. Characterization of enterococci isolated from human and nonhuman sources in Brazil. Diagn Microbiol Infect Dis, 20, 61–7.
76. Tannock, G.W. and Cook, G. 2002. Enterococci as members of the intestinal microflora of humans. In: Gilmore, M.S., Clewell, D.B., et al. (eds), The enterococci: pathogenesis, molecular biology, and antibiotic resistance. Washington, DC: ASM Press, 101–32.

77. Teixeira, L.M., Facklam, R.R., et al. 1995. Correlation between phenotypic characteristics and DNA relatedness with Enterococcus faecium strains. J Clin Microbiol, 33, 1520–3.
78. Tripathi A, Shukla SK, Singh A, Prasad KN. A new approach of real time polymerase chain reaction in detection of vancomycin-resistant enterococci and its comparison with other methods. *Indian J Med Microbiol* 2013; *31* : 47-52.
79. Tornieporth, N. G., Roberts, R. B., John, J., Hafnier, A., and Riley, L. W.,1996. Risk factors associated with vancomycin-resistant, *Enterococcus faecium* infection or colonization in 145 matched case patients and control patients. Clin. Infect. Dis. 23:765-772.
80. Tyrrell, G.J., Turnbull, L., et al. 2002. Enterococcus gilvus sp. nov. and Enterococcus pallens sp. nov. isolated from human clinical specimens. J Clin Microbiol, 40, 1140–5.
81. V. Gupta,Singla. N,et al,Speciation and antimicrobial susceptibility pattern of Enterococci from tertary heath care centre in north India,*J Clin Diag Res2007;1*: (5) ,32-5.
82. Walsh, Christopher., 2001. "Molecular Mechanisms that confer antibacterial drug resistance." Nature17 Aug. 406: 775-781. 113.Wegener, H. C., Madsen, M., Nielsen, N., and Aarestrup, F. M., 1997. Isolation of vancomycin resistant *enterococcus faecium* from food. Int. J. Food Microbiol. 35:57-66
83. Woodford, N., Johnson, A. P., and Morrison, D., 1995. Current perspectives on glycopeptide resistance. Clin. Microbial. Rev. 8:585-61
84. Xu X, Lin D, Yan G, et al. *vanM*, a new glycopeptide resistance gene cluster found in *Enterococcus faecium*. Antimicrob Agents Chemother 2010;54:4643–4647.
85. Zarrilli R, Tripodi MF, Di Popolo A, Fortunato R, Bagattini M, Crispino M, *et al*. Molecular epidemiology of high-level aminoglycoside-resistant enterococci isolated from patients in a university hospital in southern Italy. J Antimicrob Chemother 2005; 56:827-35.

Appendix

CLINICAL PROFORMA

PROFORMA

Name : IP No.:

Age :

Sex :

Address :

Investigation

Lab. No. :

Gram Stain :

Catalase :

Culture Plate findings :

i) Mac Conkey Agar-

ii) 5% Sheep Blood Agar-

Phenotypic Identification :

i) Bile Esculin Hydrolysis-

ii) Growth on 6.5 % NaCl-

iii) Heat Tolerance (60 º C for 30 minutes)

iv) Arginine Hydrolysis

Antibiotic Susceptibilty

Penicillin G (10 µg)- S/R ; Ciprofloxacin (5 µg)- S/R ;

Chloramphenicol(30 µg) – S/R ; Tetracycline (30µg) – S/R

Doxycycline(30 µg)- S/R ; Vancomycin (30µg) – S/R

Teicoplanin(30µg) – S/R ; Linezolid(30µg) – S/R

High Level Gentamicin (120µg) – S/R

Vancomycin Screen Agar-

E strip –

i) MIC of Vancomycin

ii) Mic of Teicoplanin

PCR for vanA gene

MEDIA PREPARATION

Blood agar:

Ingredients

Sterile sheep blood -5 ml

Nutrient agar -100 ml

Autoclave the nutrient agar base at 121º C for 15 minutes. Cool to 45-50º C and add blood with sterile precautions and pour into Petri dish plates.

MacConkey Agar

Ingredients Grams/litre

Peptic digest of animal tissue - 17

Proteose peptone - 3

Lactose - 10

Bile salts - 1.5

Sodium chloride - 5

Neutral red - 0.03

Agar -15

Final pH at (25º C) 7.1±0.2.

Suspend 51.53 grams in 1000 ml of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121ºC) for 15 minutes. Mix well and pour into petri dish plates.

Mueller Hinton Agar:

Ingredients

Beef infusion - 300 g/l

Casein acid hydrolysate - 17.50 g/l

Starch -1.50 g/l

Agar -17.00 g/l

Final pH at 25º C 7.4.

Suspend 38 gms in 1000 ml of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs

pressure (121ºC) for 15 minutes. Mix well and pour 20-25 ml of it into petri dishes of 90 mm diameter to a depth of 4 mm of medium.

Bile Esculin Agar:

Ingredients :

Peptone - 5 gm

Beef extract -3gm

Oxgall(bile) -40gm

Esculin -1gm

Ferric citrate -0.5gm

Agar -15gm

Distilled water -1 L

pH 7.0

heat to dissolve the contents completely, sterilize at autoclave at 121ºC for10 minutes, pour into slants/ petri plates.

6.5% NaCl broth:

Nutrient broth - 1L

NaCl - 6.5gm

Dissolve the contents completely , autoclave at 121ºc for15 min and distribute in tubes.

Brain -Heart infusion agar:

Ingredients :

Agar - 15gm

Bran heart infusion broth -1L

pH 7.4

Dissolve the agar completely by boiling . autoclave at 121ºc for 15 min. cool to about 50ºC and pour into petri dish plates.

Vancomycin Screen agar:

Ingredients :

Agar - 15gm

Brain heart infusion broth - 1 L

Vancomycin - 6mg/L

Prepare Brain heart infusion agar as described above , cool to 50ºC and Add Vancomycin 6µg/ml , mix well and pour into petri dish plates.

Cation Adjusted Mueller –Hinton broth: (MHA broth 2) (Himedia lab).

Cation adjusted Mueller- Hinton broth base - 21 gm

Distilled water -1L

Dissolve the contents by boiling and sterilize by autoclaving at 121ºC for 15 min

TABLE. 1.ZONE DIAMETER INTERPETIVE STANDARDS FOR *ENTEROCOCCUS SPP.*

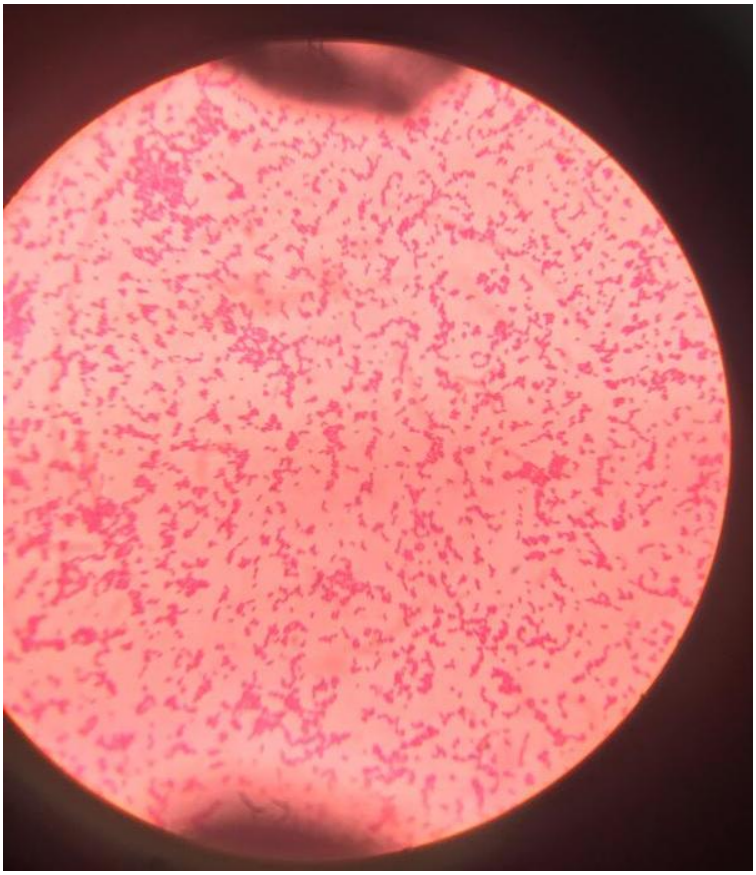
Antimicrobial agent	Disk content	Zone diameter		
		R	IM	R
Penicillin G	10 units	≤14	-	≥15
Ciprofloxacin	5µg	≤16	16-20	≥17
Doxycycline	30µg	≤12	13-15	≥16
HLS-high level gentamicin	120µg	6	7-9	≥10
Vancomycin	30µg	≤14	15-16	≥17
Teicoplanin	30µg	≤10	11-13	≥14
Chloramphenicol	30µg	≤12	-	≥18
Linezolid	30µg	≤20	-	≥23
Quinupristin	15µg	≤15	16-18	≥19
Tetracycline	30µg	≤14	15-18	≥19

R-resistant, IM-intermediate S-sensitive

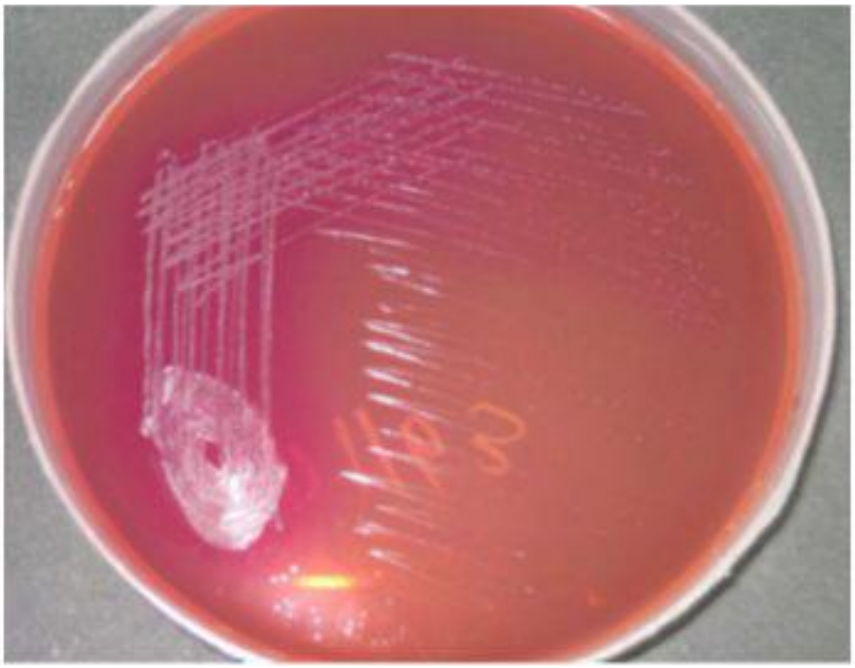
Table 2 E-Strip MIC Interpretive standards

ANTIMICROBIAL AGENT	E strip MIC level in µg		
	S	I	R
Vancomycin	≤4	8-16	≥32
Teicoplanin	≤8	16-32	≥32

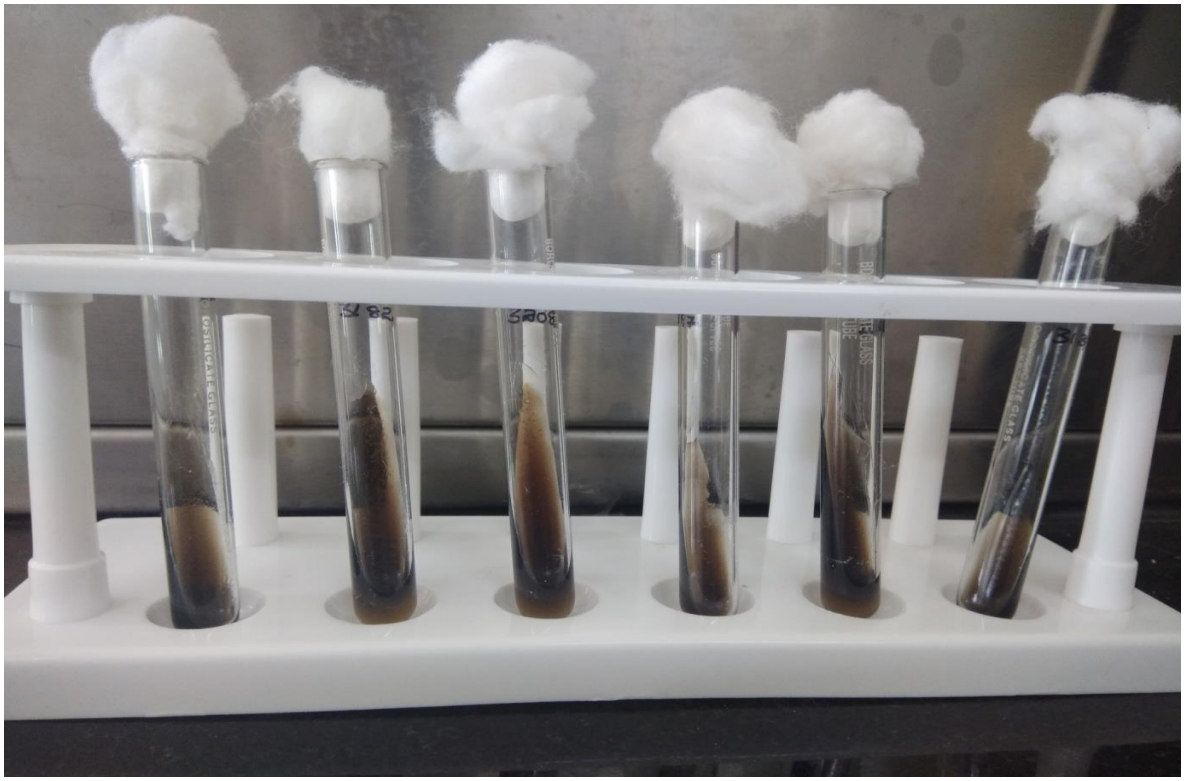
COLOUR PLATES



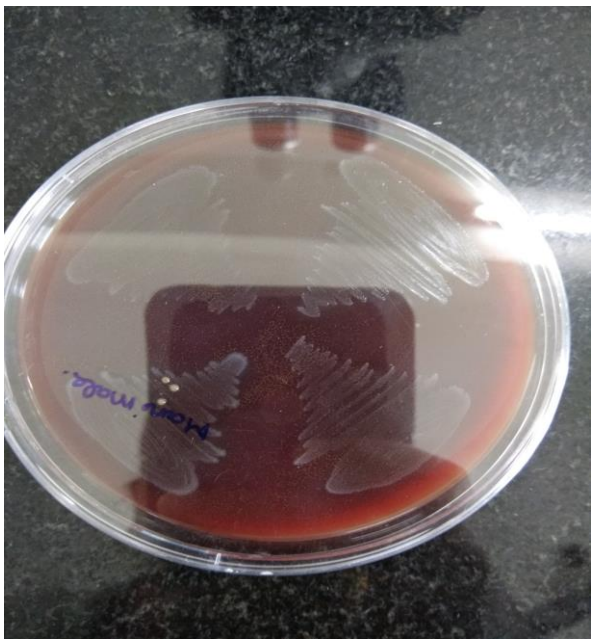
Gram Stain showing *Enterococcus faecalis* on Mac Conkey Agar



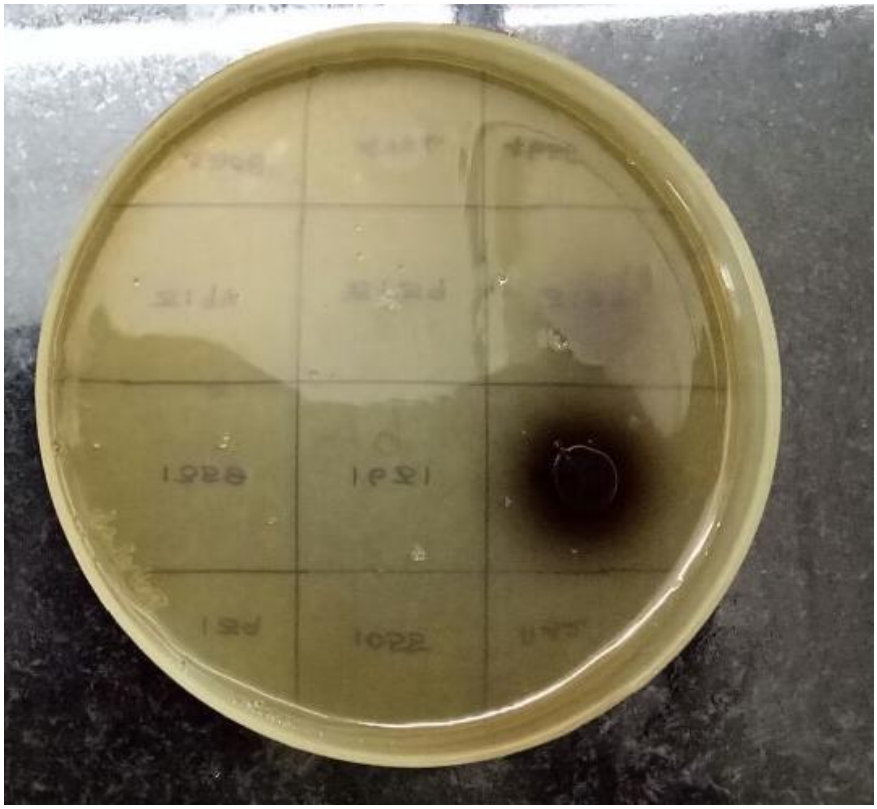
Showing colony of *Enterococcus faecalis* on Mac Conkey Agar



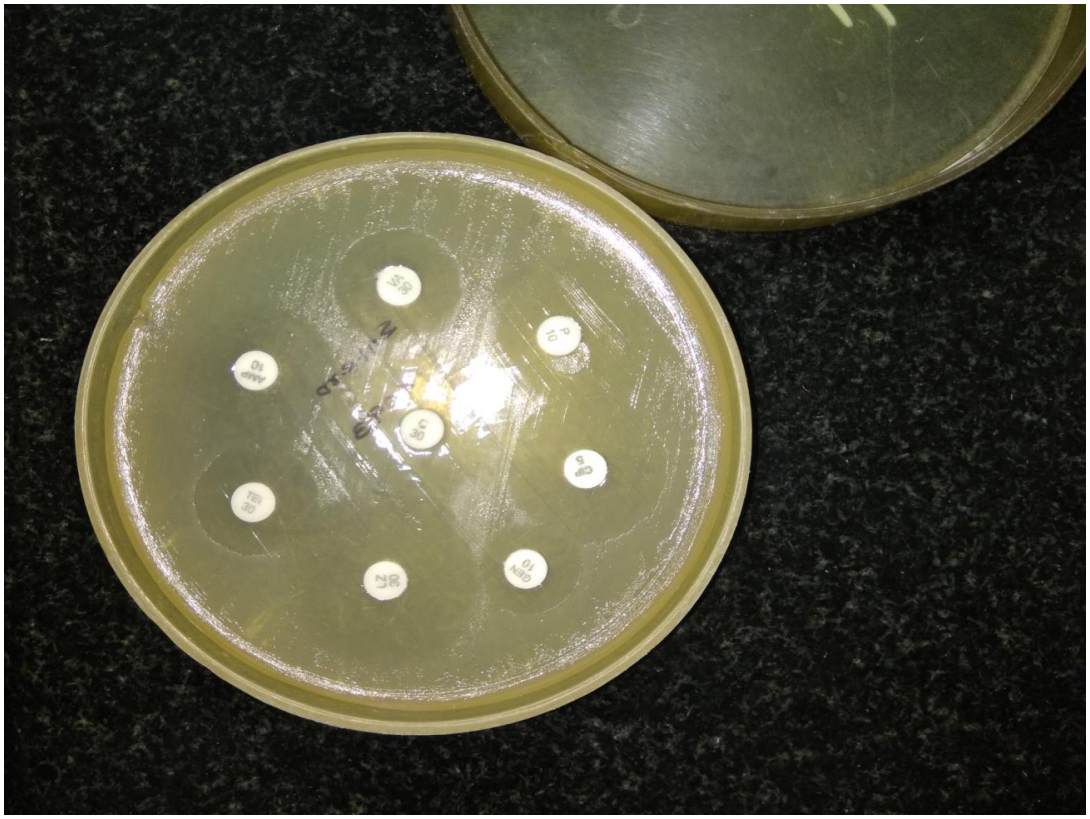
BILE – ESCULIN POSITIVE ISOLATES



BILE ESCULIN **5% SHEEP BLOOD AGAR SHOWING NON-HAEMOLYTIC COLONY**

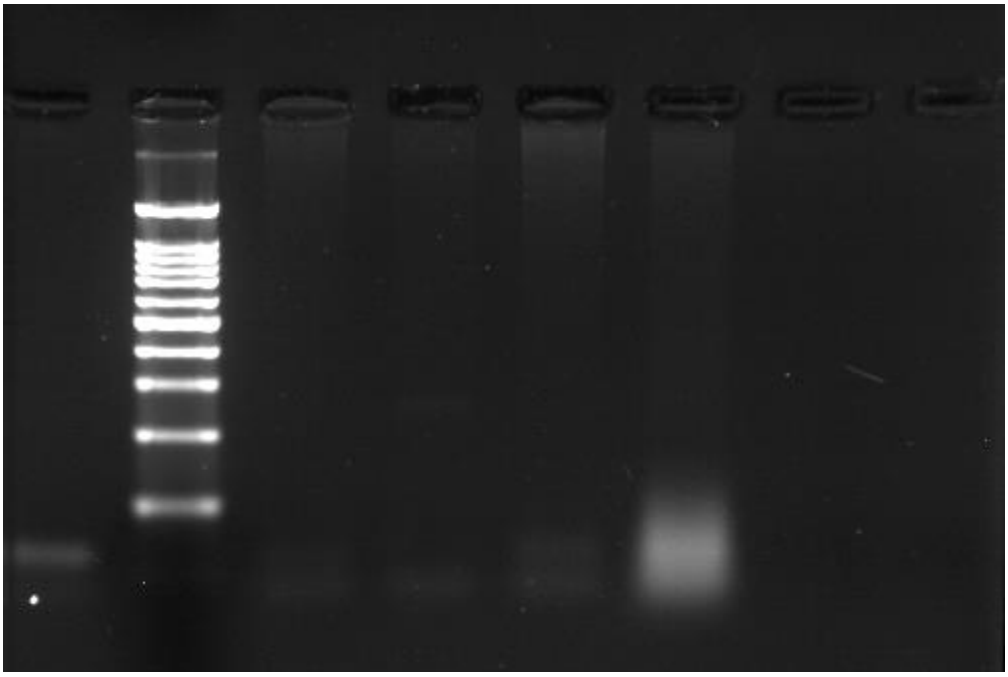


Vancomycin Screen Agar



ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *ENTEROCOCCUS FAECALIS*

Vancomycin E Strip



Polymerase Chain reaction with Van A Gene

S. NO	AGE	SEX	OP/IP	WARD	CLINICAL DIAGNOSIS	SPECIMENS	ISOLATE	ANTIBIOTIC SUSCEPTIBILITY									VRE SCREEN	MIC of Vancomycin	MIC of Teicoplanin	VRE GENOTYPE van A
								Penicillin	Tetracycline	Vancomycin	Linezolid	Ciprofloxacin	chloramphenicol	Teicoplanin	Doxycycline	High Level Gentamycin				
1	24	Female	IP	Urology	UTI	Urine	*E.Fecalis*	S	S	S	S	R	S	S	S	S	Negative	S		-
2	21	Female	IP	CMCHIS	Fever	Urine	*E.Fecalis*	S	S	S	S	S	S	S	S	S	Negative	S		-
3	45	Male	IP	Medicine	UTI	Urine	*E.Fecalis*	S	S	S	S	R	R	R	S	S	Negative	S		-
4	42	Female	IP	Urology	PUO	Urine	*E.Fecalis*	S	S	S	S	S	S	S	S	s	Negative	S		-
5	11	MCH	IP	Medicine	UTI	Urine	*E.Fecalis*	R	S	S	S	R	S	R	S	S	Negative	S		-
6	24	Male	IP	Medicine	UTI	Urine	*E.Fecalis*	R	S	S	s	R	R	S	S	R	Negative	S		-
7	65	Male	IP	Pediatric	Fever	Urine	*E.Fecalis*	R	S	R	S	S	S	S	S	S	Negative	S		-
8	2	MCH	IP	Pediatric	Fever	Urine	*E.Fecalis*	R	S	R	S	S	S	S	S	S	Negative	S		-
9	25	Male	IP	Urology	UTI	Urine	*E.Faeciu m*	R	S	S	R	R	R	S	R	S	Negative	S		-
10	5	Male	IP	Pediatric	Fever	Urine	*E.Fecalis*	R	S	S	R	S	R	S	S	S	Negative	S		-
11	25	Male	IP	Urology	UTI	Urine	*E.Fecalis*	R	S	S	S	R	s	R	S	R	Negative	S		-
12	52	Male	IP	Medicine	UTI	Urine	*E.Fecalis*	R	S	S	S	S	R	S	S	R	Negative	S		-
13	62	Male	IP	Surgery	UTI	Urine	*E.Fecalis*	R	S	R	S	R	S	S	S	R	Negative	S		-
14	37	Female	IP	Labour	POSTLSCS	Urine	*E.Fecalis*	R	R	S	S	R	R	R	S	R	Negative	S		-
15	65	Female	IP	Thoracic	URI	Sputum	*E.Fecalis*	S	R	S	S	R	S	R	S	R	Negative	S		-
16	24	Female	IP	Labour	PID	vaginal swab	*E.Faeciu m*	S	S	R	S	R	S	R	S	R	Negative	S		-
17	16	Male	IP	Medicine	URI	Sputum	*E.Fecalis*	S	R	S	R	S	R	R	S	R	Negative	S		-
18	26	Female	IP	Thoracic	LRI	Sputum	*E.Fecalis*	R	S	R	S	R	S	R	S	R	Negative	S		-

19	56	Female	IP	Gynec	URI	Sputum	*E.Fecalis*	R	S	R	S	R	S	R	S	R	Negative	S		-
20	5	MCH	IP	Pediatric	UTI	Urine	*E.Fecalis*	S	S	S	S	R	S	S	S	S	Negative	S		-
21	70	Male	IP	Urology	UTI	Urine	*E.Fecalis*	S	S	S	S	R	S	S	S	R	Negative	S		-
22	5	FCH	OP	Pediatric	Fever	Urine	*E.Fecalis*	S	S	S	S	R	S	S	S	R	Negative	S		-
23	6342	Female	IP	Labour	UTI	Urine	*E.Fecalis*	S	S	S	S	R	S	S	S	R	Negative	S		-
24	65	Male	IP	Trama		Urine	*E.Fecalis*	R	R	S	S	R	R	R	S	R	Negative	S		-
25	58	Male	IP	Urology	UTI	Urine	*E.Faeciu m*	R	S	R	S	R	R	R	R	R	Positive	R	R	Negativ e
26	60	Male	Op	Surgery	UTI	Urine	*E.Fecalis*	R	S	S	R	R	S	S	R	R	Negative	S		-
27	35	Male	IP	Surgery	UTI	Urine	*E.Fecalis*	R	R	R	S	S	S	S	S	R	Negative	S		-
28	57	Male	Op	Surgery	UTI	Urine	*E.Fecalis*	R	R	R	S	R	R	S	R	R	Positive	R	R	Negativ e
29	3139	FCH	IP	Pediatric	Fever	Urine	*E.Fecalis*	R	S	R	S	R	S	R	S	R	Negative	S		-
30	84	Male	Op	Urology	UTI	Urine	*E.Fecalis*	S	R	S	S	R	S	R	R	R	Negative	S		-
31	10	MCH	Op	Pediatric	UTI	Urine	*E.Fecalis*	R	R	R	R	S	R	S	R	R	Negative	S		-
32	55	Female	IP	IMCU	CKD	Urine	*E.Fecalis*	S	S	S	S	S	S	R	R	R	Negative	S		-
33	7	MCH	OP	Pediatric	Fever	Urine	*E.Fecalis*	R	R	R	R	R	S	R	S	S	Negative	S		-
34	45	Male	IP	Urology	UTI	Urine	*E.Fecalis*	R	R	R	R	R	S	R	S	S	Negative	S		-
35	21	Female	IP	Labour	Fever	Urine	*E.Fecalis*	R	S	R	S	S	S	S	S	S	Negative	S		-
36	5	MCH	IP	Pediatric	Fever	Urine	*E.Fecalis*	R	R	S	S	R	S	R	S	S	Negative	S		-
37	25	Male	IP	Urology	UTI	Urine	*E.Faeciu m*	R	S	S	S	R	S	S	S	S	Negative	S		-
38	2	FCH	IP	Pediatric	Fever	Urine	*E.Fecalis*	S	R	S	S	S	S	S	S	S	Negative	S		-
39	65	Male	IP	Medicine	UTI	Urine	*E.Fecalis*	R	R	S	S	S	S	S	S	S	Negative	S		-
40	24	Male	IP	Medicine	UTI	Urine	*E.Fecalis*	R	R	S	S	R	S	S	S	S	Negative	S		-
41	11	MCH	IP	Pediatric	Fever	Urine	*E.Fecalis*	R	S	R	R	R	S	R	R	R	Negative	S		-
42	62	Male	IP	Surgery	UTI	Urine	*E.Fecalis*	R	S	S	R	S	S	R	R	R	Negative	S		-
43	42	Female	IP	Surgery	UTI	Urine	*E.Fecalis*	R	S	S	R	R	S	R	S	S	Negative	S		-
44	45	Male	IP	Medicine	UTI	Urine	*E.Fecalis*	R	S	S	R	R	S	R	R	R	Negative	S		-
45	21	Female	IP	CMCHIS	UTI	Urine	*E.Fecalis*	R	R	S	R	R	S	R	R	R	Negative	S		-
46	24	Female	IP	Urology	UTI	Urine	*E.Fecalis*	S	R	S	S	R	S	S	S	S	Negative	S		-
47	54	Male	IP	Pediatric	Fever	Urine	*E.Fecalis*	R	S	R	S	R	S	S	S	S	Negative	S		-
48	49	Male	IP	Medicine	UTI	Urine	*E.Fecalis*	S	S	R	R	S	S	S	S	S	Negative	S		-

49	55	Female	IP	IMCU		Urine	*E.Fecalis*	S		R	R	S	S	S	S	S	Negative	S		-
50	76	Female	IP	Medicine	UTI	Urine	*E.Faeciu m*	R		R	R	R	S	R	R	R	Negative	S		-
51	43	Male	IP	IMCU	UTI	Urine	*E.Fecalis*	R	R	R	R	R	R	R	R	S	Negative	S		-
52	60	Female	Op	Surgery	UTI	Urine	*E.Fecalis*	R		S	S					S	Negative	S		-
53	58	Male	Op	Medicine	UTI	Urine	*E.Fecalis*	R	R	R	R	S	S	R	R	R	Negative	S		-
54	35	Female	Op	Labour	UTI	Urine	*E.Fecalis*	S	S	S	S	S	S	S	S	S	Negative	S		-
55	73	Male	IP	IMCU	PUD-Fever	Urine	*E.Fecalis*	S	S	R	R	S	S	S	S	S	Negative	S		-
56	6	MCH	OP	Pediatric	Fever	Urine	*E.Fecalis*	R	R	R	R	S	S	S	S	R	Negative	S		-
57	18	Male	IP	IMCU	Epilepsy	Urine	*E.Fecalis*	S	S	R	S	R	S	S	S	S	Negative	S		-
58	28	Female	IP	Labour	LSCS-POD	Urine	*E.Faeciu m*	S	S	R	S	R	S	S	S	S	Negative	S		-
59	25	Male	OP	Medicine	UTI	Urine	*E.Fecalis*	S	S	S	S	S	S	S	S	S	Negative	S		-
60	21	Female	OP	Labour	UTI	Urine	*E.Fecalis*	R	R	S	R	S	S	R	S	S	Negative	S		-
61	4	FCH	OP	Pediatric	UTI	Urine	*E.Fecalis*	R	R	R	S	R	S	R	S	R	Negative	S		-
62	8	MCH	OP	Pediatric	Fever	Urine	*E.Fecalis*	S	S	S	S	S	S	S	S	S	Negative	S		-
63	37	Female	OP	Medicine	UTI	Urine	*E.Fecalis*	R	R	R	S	S	S	S	S	R	Negative	S		-
64	36	Female	OP	Gynec	PID	Urine	*E.Fecalis*	R	S	S	S	R	S	S	S	R	Negative	S		-
65	38	Male	IP	Nephro	CKD	Urine	*E.Fecalis*	R	S	R	R	S	S	S	S	S	Negative	S		-
66	47	Female	IP	Surgery	Ulcerleg	Pus	*E.Fecalis*	R	S	R	R	R	S	S	S	S	Negative	S		-
67	39	Female	OP	Urology	UTI	Urine	*E.Fecalis*	R	S	S	S	S	S	S	S	S	Negative	S		-
68	55	Female	IP	Surgery		Ascitic Fluid	*E.Faeciu m*	R	R	R	R	R	S	S	S	S	Negative	S		-
69	2	FCH	IP	Pediatric	PUO	Urine	*E.Fecalis*	R									Negative	S		-
70	65	Male	IP	Medicine	PUO	Urine	*E.Fecalis*	R	S	S	S	S	S	S	S	S	Negative	S		-
71	37	Male	IP	IMCU	CKD	Urine	*E.Fecalis*	R	S	R	S	S	S	S	S	S	Negative	S		-
72	7	FCH	IP	Pediatric	PUO	Urine	*E.Fecalis*	R	R	R	R	R	R	R	S	S	Positive	R	S	Negativ e
73	60	Male	IP	IMCU	UTI	Urine	*E.Fecalis*	R	s	S	R	S	S	S	S	S	Negative	S		-
74	33	Female	IP	Medicine	UTI	Urine	*E.Fecalis*	R	R	R	R	R	R	R	S	S	Negative	S		-
75	21	Male	IP	Surgery	PUO	Urine	*E.Fecalis*	R	S	R	S	S	R	S	S	S	Negative	S		-
76	55	Male	OP	Medicine	UTI	Urine	*E.Fecalis*	R	R	R	R	R	S	R	S	S	Negative	S		-
77	28	Female	OP	Urology	UTI	Urine	*E.Fecalis*	R	S	R	R	R	R	R	S	S	Negative	S		-

78	19	Male	IP	IMCU	CKD	Urine	*E.Fecalis*	R	S	S	R	S	S	S	S	S	Negative	S		-
79	55	Male	OP	Urology	UTI	Urine	*E.Fecalis*	R	R	R	R	R	R	R	S	S	Negative	S		-
80	28	Female	OP	Medicine	Fever	Urine	*E.Faeciu m*	R	S	S	S	S	S	S	S	S	Negative	S		-
81	79	Male	IP	Medicine	UTI	Urine	*E.Fecalis*	S	R	S	R	S	S	S	S	S	Negative	S		-
82	35	Female	OP	Urology	UTI	Urine	*E.Fecalis*	R	R	R	R	S	S	S	S	S	Negative	S		-
83	4	FCH	IP	Pediatric	PUO	Urine	*E.Fecalis*	S	S	S	R	S	S	S	S	S	Negative	S		-
84	11	FCH	IP	Pediatric	PUO	Urine	*E.Fecalis*	R	S	S	S	S	S	S	S	S	Negative	S		-
85	80	Female	IP	Medicine	UTI	Urine	*E.Fecalis*	R	R	R	S	R	S	R	S	S	Negative	S		-
86	32	Male	OP	Urology	UTI	Urine	*E.Fecalis*	R	S	S	R	S	S	S	S	S	Negative	S		-
87	8	FCH	IP	Pediatric	PUO	Urine	*E.Fecalis*	R	S	S	R	R	S	R	S	S	Negative	S		-
88	47	Male	IP	Medicine	PUO	Urine	*E.Fecalis*	R	R	R	R	R	S	R	S	S	Negative	S		-
89	30	Male	OP	Surgery			*E.Fecalis*	R	R	R	R	R	R	S	R	S	Positive	R	S	Negativ e
90	21	Male	OP	Urology	UTI	Urine	*E.Fecalis*	R	S	S	R	R	S	R	S	S	Negative	S		-
91	28	Female	IP	Obestetric	UTI	Urine	*E.Faeciu m*	R	R	R	S	R	S	R	S	S	Negative	S		-
92	30	Male	OP	Nephrolog y	UTI	Urine	*E.Fecalis*	R	S	S	R	S	R	S	R	R	Negative	S		-
93	4	MCH	IP	Medicine	PUO	Urine	*E.Fecalis*	R	S	S	R	R	S	R	S	S	Negative	S		-
94	27	Female	OP	Surgery	UTI	Urine	*E.Fecalis*	R	R	R	R	R	S	R	S	S	Negative	S		-
95	42	Male	IP	Medicine	DM	Urine	*E.Fecalis*	R	S	R	S	R	R	S	R	S	Negative	S		-
96	62	Female	IP	Surgery	PUO	Urine	*E.Fecalis*	R	S	S	R	S	S	S	S	S	Negative	S		-
97	52	Male	OP	Nephrolog y	CKD	Urine	*E.Fecalis*	R	R	R	R	S	R	S	R	S	Negative	S		-
98	39	Female	OP	Urology	UTI	Urine	*E.Fecalis*	R	S	S	S	S	S	S	S	S	Negative	S		-
99	12	MCH	OP	Pediatric	Fever	Urine	*E.Fecalis*	R	R	R	R	R	S	R	S	S	Negative	S		-
100	22	Female	OP	Labour	UTI	Urine	*E.Faeciu m*	R	S	R	S	R	S	S	R	S	Negative	S		-

ABBREVIATIONS:

ATCC – American Type Culture Collection

CLSI- Clinical Laboratory Standard Institute

MDR- Multi Drug Resistance

IP-Inpatient

M-Male patient

F-Female patient

Mch-Male child

Fch-Female child

UTI- Urinary tract infection

S- Susceptible **R**-Resistant

vanA – van A genotype VRE

VRE- vancomycin resistant Enterococci

HLG –High Level Gentamicin

MIC – Minimum Inhibitory Concentration