Study on Methicillin-Resistant *Staphylococcus aureus* and Vancomycin-Resistant Enterococci Co-colonization in Patients of Intensive Care Units of Tertiary Health Care Facilities (Hospitals).

by

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DECLARATION

The material contained in this thesis is my original work and I have not presented any part of this thesis/work elsewhere for any other degree.

Muhammad Arfat Yameen

CERTIFICATE

This thesis by Muhammad Arfat Yameen is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, and is satisfying the thesis requirements for the award of degree of

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Table of Contents

Chapter 4: Results

LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
BAA	Bile Aesculin Agar
BEAA	Bile Esculin Azide Agar
BHI	Brain Heart Infusion
BSAC	British Society for Antimicrobial Chemotherapy
BSIs	Blood Stream Infections
CA-MRSA	Community Acquired *Staphylococcus aureus*
CDC	Center for Disease Control
Chi sq.	Chi Square Test
CoNS	Coagulase-Negative Staphylococci
DNA	Deoxyribonucleic Acid
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
HA-MRSA	Hospital Acquired *Staphylococcus aureus*
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MICU	Medical Intensive Care Unit
MLST	Multilocus Sequence Typing
MRCoNS	Methicillin-Resistant Coagulase-Negative Staphylococci
MRS	Methicillin-Resistant Staphylococci
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MRSE	Methicillin-Resistant *Staphylococcus epidermidis*
MSA	Mannitol Salt Agar
MSSA	Methicillin-Sensitive *Staphylococcus aureus*
NCCLS	National Committee for Clinical Laboratory Standards
PBP	Penicillin Binding Proteins
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
PICU	Paeds Intensive Care Unit
PYR	L-pyrrolidonyl-b-napthylamide

QD	Quinupristin/Dalfopristin
REAP	Restriction Endonuclease Analysis of Plasmid
S. aureus	*Staphylococcus aureus*
SBA	Sheep Blood Agar
TBE	Tris Borate EDTA Buffer
Tm	Melting Temperature
VISA	Vancomycin-Intermediate *Staphylococcus aureus*
VISA	Vancomycin-Intermediate *Staphylococcus aureus*
VRE	Vancomycin-Resistant enterococci
VRSA	Vancomycin-Resistant *Staphylococcus aureus*
VSE	Vancomycin-Sensitive enterococci

LIST OF TABLES

LIST OF FIGURES

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Muhammad Arfat Yameen

SUMMARY

Aim of this study was to isolate, identify and characterize methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) from ICUs of three Allied Hospitals of Rawalpindi Medical College, Rawalpindi, Pakistan. The study involved the investigation of co-colonization/ co-existence, antibiotic resistance and molecular identification. Samples from 216 cases were taken from Paeds intensive care units (PICUs) and Medical intensive care units (MCUs) of three hospitals over the period of 16 months. A total of 432 samples (2 sample/patients) were taken from nasal cavity and peri-rectum area of each patient. Each sample was processed for isolation of staphylococci and enterococci. The bacterial isolates were selectively enumerated on Mannitol salt agar (MSA) for *S. aureus* and Bile aesculin agar (BAA) for enterococci revealed 82 as *Staphylococcus aureus* and 159 as enterococci. Nasal cavity was the best site for the isolation of staphylococci and peri-rectum was the best area for the isolation of enterococci. Standard morphological, phenotypic and biochemical tests were done for identification of *S. aureus* and enterococci. Furthermore API Staph (bioMérieux, Germany) was used to confirm identification of 82 isolates of *S. aureus*. Molecular identification was done by amplification of 16S rRNA, staph 16S, *nuc* gene, *coagulase* gene, *mecA* gene for *S. aureus* to discriminate it as MRSA and Methicillin-sensitive *S. aureus* (MSSA) whereas *ddl E. faecalis*, *ddl E. faecium*, *vanA* and *vanB* were used to identify enterococci as *Enterococcus faecalis (E. faecalis)* and *Enterococcus faecium (E. faecium)* and as vancomycin-resistant *E. faecalis* and vancomycin-resistant *E. faecium*. Both the simple and multiplex PCR techniques were used for the identification of these two types of isolates.

Antibiotic susceptibility tests revealed that all of the MSSA and MRSA isolates were sensitive to vancomycin, teicoplanin, quinupristin/dalfopristin and linezolid but MRSA were found to be resistant to, amoxicillin/clavulanic acid, cephradine, ciprofloxacin, levofloxacin, gentamicin, erythromycin, imipenem and tetracycline. For enterococci, VRE were resistant to all tested antibiotics except linezolid, quinupristin/dalfopristin and teicoplanin. Whereas vancomycin-susceptible

enterococci (VSE) were resistant to cephalexin, cefoxitin, cephalothin, cephradine, ciprofloxacin, erythromycin, gentamicin, levofloxacin and tetracycline. MIC was performed to determine the sensitivity and resistance of all isolates using a selection of commonly used antibiotics such as ciprofloxacin, oxacillin, tetracycline and vancomycin. Vancomycin was in the range of 1-4 mg/L for all isolates whereas for VRE it ranged from 64-512 mg/L. Absence of known virulence determinants along with antibiotics resistance was confirmed by amplification through PCR for critical virulence genes mainly, methicillin-resistant gene, *mecA* and vancomycin-resistance types-A (*vanA*) and type-B (*vanB*). The isolated MRSA, MSSA, VSE and VRE are promising candidate for further investigation of their resistance characterization, clonality and molecular typing.

In this study patient stayed an average 6.72 (SD ± 9.11) days in ICUs department. *S. aureus* was isolated more frequently from nasal samples and majority of the patients were from two age groups <1 year and >35 years in which *S. aureus* were isolated more from age group >35 years. Patients of MICU were more colonized with *S. aureus* and dominant category of colonization was male patients. Patients from rural areas were colonized more with nasal MRSA while MSSA were more from urban patients. Majority patients were from lower social class with equal isolation of nasal MRSA from this and middles class patients. There were no specific association found among clinical outcomes and isolation of *S. aureus*.

Enterococci were more frequent from peri-rectal samples than from nasal and there were only few cases where both nasal and peri-rectal samples of the same patient had VRE isolates. Nasal VRE isolation was random from all age groups while majority of nasal VSE isolates were from age group >35 years and age group <1 year. Among gender enterococci isolation was random and from both ICU (MICU and PICU) it was almost equal. Urban patients had the major portion of nasal and peri-rectal VRE and VSE than rural patients. Lower and middle class patients were more colonized with enterococci in both the sites. In disease categories, patients with pneumonia and miscellaneous disease group were more colonized with enterococci than other categories.

Combination therapy of antibiotics was more frequently used for treatment of admitted patients. No particular association was seen with the treatment of antibiotics and isolation of MRSA and VRE. There was some relation seen in case of vancomycin treatment and VRE isolation which need further experiments for confirmation.

INTRODUCTION

INTRODUCTION

The main objective of all infection control programs is preventing the spread of infections within the health-care environment. Infection control concerns with prevention (hand hygiene, disinfection, sterilization, vaccination, and surveillance), investigation and management of spread of infection within a particular healthcare setups. Routinely collected statistical data from these health care institutes is essential for describing the occurrence and magnitude of disease, monitoring morbidity and mortality, and provide a data base for highlighting risk factors that can then be avoided.

Colonization of pathogenic and antibiotic resistant organisms proceeds to infections and in hospital settings nosocomial infection results from these pathogens. Nosocomial infections are the commonest type of infection increasing worldwide. It is the main contributor to morbidity and mortality and becoming more important due to increasing economic crises.

The key factors of increased nosocomial rates are inadequate medical standards and practice, poor hygiene of health care personals and overcrowding of patients, excessive use of invasive medical devices mainly urine catheters, central intravenous lines and ventilators, unsafe and unnecessary use of injectable, lack of immunity due to different factors, antibiotic resistance because of excessive and irrational use of antibiotics and emergence of new resistant microorganisms to antibiotics. (Sibbald et al., 2006; Ducel, 1995.). Additionally, nosocomial infections rates are high in intensive care units because of prolonged hospital stay of patient, severity of underlying disease, invasive procedures, invasive devices and extensive use of different broad-spectrum antibiotics. Patients which are already colonized with these drug-resistant pathogens, become reservoir of nosocomial infections by horizontal transmission in healthcare facilities (Puzniak et al., 2001).

MRSA and VRE are the most common nosocomial pathogens in health care facilities (Crossley, 2001). The increasing infection rate of MRSA and VRE is becoming problematic because of the limited treatment and therapy and ultimately it leads to prolonged patient hospitalization, increased mortality and treatment costs. Another emerging threat regarding VRE is in the possibility of transfer of resistance

genes to other species such as MRSA (Cetinkaya, Falk and Mayhall, 2000; Perl, 1999; Puzniak et al., 2001).

1.1: Co-infection and co-colonization

Co-infection and co-colonization with VRE and MRSA among patients of intensive care units are very common (Richards et al., 1999a and 1999b; Ostrowsky et al., 2001). Co-colonization can be explained as VRE-positive peri-rectal culture with MRSA-positive anterior nares culture collected concurrently (Furuno, et al., 2005). There are few data indicating the frequency with which concomitant colonization or co-infection with MRSA and VRE occurs within the same patient. Co-colonization and co-infection may increase the chances of transfer of vancomycin resistance from enterococci to staphylococci and ultimately leads to emergence of vancomycin-resistant *Staphylococcus aureus* (VRSA). Studies showed that both of these isolates contained the *vanA* gene. And it is suggested that the *vanA* gene was acquired by *S. aureus* from VRE in these two patients having prior co-colonization with MRSA and VRE (Chang et al., 2003; Noble et al., 1992).

Proper hand hygiene after patient contacts decreases the spread of staphylococci from patient to patient. Incidentally, older children and adults are more resistant than neonates to colonization. Treatment with an antibiotic to which *S. aureus* is resistant favours colonization and the development of infection.

1.2: *Staphylococcus aureus* as a human pathogen

Staphylococci belong to phylum Firmicutes and class cocci. These are identified as Gram positive cocci of uniform size; it appears as grape-like clusters when viewed through a microscope and as large, round, golden-yellow colonies, often with β-haemolysis, when grown on blood agar plates. Pathogenic staphylococci can be distinguished from other staphylococci as small colonies surrounded by bright yellow zones on Mannitol salt agar medium. While other non-pathogenic staphylococci forms reddish purple zone.

S. aureus is catalase positive as it has ability to convert hydrogen peroxide in to water and oxygen thus on the basis of this test it can be distinguished as staphylococci from enterococci and streptococci which are catalase negative. *S.*

aureus isolates can be further identified by positive bound coagulase test (both slide and tube coagulase test) and DNAse test which distinguishing it from other staphylococci.

1.2.1: Colonization and infections with *S. aureus*

S. aureus is having a vast range of virulence factors. This gives it versatile ability to establish infections in various organs of human body causing skin infections to deep rooted systemic infections (Lowy, 1998; Sibbald et al., 2006). Humans are natural reservoir for *S. aureus* as commensal. Most commonly it colonizes the anterior nares (the nostrils), although the respiratory tract, opened wounds, intravenous catheters, and urinary tract are also potential sites for infection. Healthy individuals may carry MRSA asymptomatically for periods ranging from a few weeks to many years.

Carriage rate of *S. aureus is* approximately 30% in healthy adults in their anterior nares, nasal cavity, nasopharynx and on different area of the skin. Newborn may become colonized after direct contact of the adult carrier. There is no disease symptom in majority of the carriers. (Ryan KJ, 2004), but the immune-compromised patients or individuals during and after hospitalization due to serious disease are more susceptible to *S. aureus* infection (Foster, 2005; van Belkum, 2006). *S. aureus* can cause a range of illnesses from minor skin infections and abscesses, to life-threatening diseases such as meningitis, pneumonia, endocarditis, toxic shock syndrome, and septicaemia (Waldvogel, 2000). *S. aureus* has established itself as multidrug resistant organism and become the causative agent of different infections. This all is the outcome of using great number of available antibiotics initially to treat this organism.

Resistance spread among *S. aureus* clones and emergence of multi-resistant isolates is alarming and it is the need of the time to counteract the effects of antibiotic resistance development by paying attention on hospital antibiotic prescription regimens (Appelbaum, 2006; Johnson et al., 2005). During the last decade, MRSA became dominant pathogen due to high rate of infection in both inpatients and outpatients, and especially among infants admitted in neonatal intensive care units. Systemic MRSA infections have become a major concern

because of the higher mortality. Antimicrobial therapy becoming more and more critical as organism is gradually evolving resistance to most classes of antibiotics, thus serious staphylococcus infections may lead to direful consequences. Already more than 95% of patients with staphylococcal infections, worldwide, do not respond to therapy with antibiotics such as penicillin or ampicillin (de Sousa and Conceição, 2005).

The prevalence of MRSA among hospitalized patients varies geographically. It is generally high in Japan, southern Europe and United States but is very low in Scandinavia and Switzerland (Boyce, 1990; Voss et al., 1994; Heininger, 2007; Sader, 2006; Raymond and Aujard, 2000; Wisplinghoff et al., 2003, 2004). Similarly the prevalence of antibiotic resistant of nosocomial *S. aureus* has been increasing since 1960,s (Wisplinghoff et al., 2004; NNIS Report, 2004; Styers et al., 2006). In a United States Surveillance report more than 24,000 cases of nosocomial *S. aureus* bacteremia, there was 22 to 57% increase in methicillin resistant isolates between 1995 and 2001 (Wisplinghoff et al., 2004). The National Health and Nutrition Examination Survey (NHANES) noted that the prevalence of MRSA nasal colonization in United States in 2003-2004 was 1.5%, up from 0.8% in 2001-2002 (Gorwitz et al., 2008).

1.2.2: Genomic makeup of MRSA

MRSA was first identified in the 1960s. All MRSA isolates have in their genome staphylococcal chromosomal cassette (SCC) *mec* genetic element, which carries mobile *mecA* gene. This gene codes for an altered penicillin-binding protein (PBP) that has low affinity for binding with β-lactams (e.g. penicillins, cephalosporins and carbapenems). There is horizontal transfer of *mecA* gene among different staphylococcal species (Layer and Ghebremedhin, 2006) and between different Gram positive bacteria but the mechanism responsible for this transfer is not know. There are five different SCC*mec* types have been described for *S. aureus* which are different in size and structure (Fitzgerald and Sturdevant, 2001).

Five major types of SCC*mec* elements (I–V) have been defined based on *mec* gene complex and ccr gene allotypes (Ito et al., 1999, 2001, 2004). Types I, II, and III are commonly found in hospital-acquired isolates, whereas type IV is mostly found

among community isolates (Regev et al., 2003; Lu et al., 2005; Ma et al., 2002). However, certain isolates from health care facility also have SCC*mec* type IV (McDougal et al., 2003). SCC*mec* type V caring isolates are rare. Community MRSA isolates from Australia (O'Brien et al., 2004; Coombs et al., 2006) and Taiwan (Boyle-Vavra et al., 2005) harbour SCC*mec* type V.

1.2.3: Risk with MRSA colonization

MRSA colonization increased the risk of subsequent MRSA infection as compared with MSSA colonization (Davis et al., 2004). Patients in intensive care units and surgical wards are on high risk of developing MRSA infection.

MRSA infection mainly develops in hospitalized patients with risk factors related to health care and these are the major cause of both HAI especially intensive care units (ICUs) and CAI. Often these organisms are resistant to multiple antibiotics, especially to β-lactamase-resistant penicillins i.e. methicillin, oxacillin, cloxacillin and flucloxacillin, so the infections caused by these are difficult to treat and patient need more prolonged hospitalization, and ultimately the cost of medical care also increases. *S. aureus* was the cause of 16% of all nosocomial infections in the U.S between 1995 and 1998 (Rice, 2006).

1.3: Enterococcus and its pathogenesis

Enterococcus is a genus of lactic acid bacteria of the phylum Firmicutes. Members of this genus were classified under Group D streptococcus and were not accepted as a separate genus until genetic evidence fully distinguished the enterococci from streptococci in 1984 (Schliefer and Killper-Baltz, 1984). The genus enterococcus is comprised of facultative anaerobic Gram positive cocci, non motile, exhibit gamma-haemolysis on sheep blood agar and occur in short chains or pairs (Diplococci). Enterococci are difficult to distinguish from streptococci on physical characteristics alone. Two species are common commensal organisms in the intestines of humans: *Enterococcus faecalis (E. faecalis)* 90-95% and *Enterococcus faecium (E. faecium)* 5-10%. There are rare clusters of infections with other species including *E. casseliflavus, E. raffinosus* (Gilmore et al., 2002). The optimum growth temperature for enterococci is 35°C and most isolates can grow at 10 to 45°C. All isolates grow in media containing 6.5% NaCl and can hydrolyze aesculin. Enterococci are usually

catalase negative and produce a cell wall antigen that is identified as the streptococcal group D antigen. They are considered strict fermenters because they lack a Kreb's cycle respiratory chain (Willett, 1992).

1.3.1: Colonization and infections with enterococci

Enterococci are widespread in nature, have ability to grow and survive in harsh environments. They are most commonly detected in the fecal flora of most animals and humans. They are also identified and recovered from foods such as milk and meat products and from various environmental sources (Aarestrup, 2002). They are part of the normal bacterial flora of the human bowel. Two species *E. faecalis* and *E. faecium* are most common and frequent commensal organisms in the intestines of humans. *E. faecalis* have been one of the most common bacteria isolated from feces of healthy individuals (90-95%) while *E. faecium* have less probability (5-10%).

Enterococci are common cause of nosocomial infections of which most dominating species isolated from infection sites is *E. faecalis* which accounts for 80–90% and *E. faecium* for 5–10% being isolated from most of the rest. The pathogenicity of enterococci was recognized long ago by MacCallum and Hastings who isolated an organism which they named *Micrococcus zymogenes* with properties consistent with those of enterococci from a case of acute endocarditis (McCallum and Hastings, 1899). Enterococci are usually associated with urinary tract and wound infections, commonly caused by *E. faecalis*. It was recognized very early that enterococci were able to cause bacteremia and endocarditis and accounts for 5-20% of all endocarditis (Murray, 1990). Enterococci were found to be the second most commonly isolated organism (15.8%) in 1999 during a prevalence study of urinary tract infections (Bouza, 2001). Commonly the emerging nosocomial enterococcal infections are bacteremia, surgical site and intra-abdominal infections more rarely causes nervous system infections (Richards, 2000; Sitges-Serra, 2002; Dettenkofer, 1999; Nachman, 1995). *E. faecalis* and *E. faecium* are resistant to many commonly used antimicrobial agents (aminoglycosides, aztreonam, cephalosporins, clindamycin, penicillins, nafcillin, oxacillin and trimethoprim-sulfamethoxazole). Exposure to cephalosporins is particularly an important risk factor for colonization and infection with enterococci. These isolates have acquired

resistance towards vancomycin and ampicillin which increased alarmingly over the past few years (Iwen, 1997). The main concern is prevalence of VRE in hospitalized patients. In the United States, the prevalence of hospital outbreaks of VRE have been reported as high as 47% in some studies (Cetinkaya, et al., 2000). In Europe, a high prevalence has also been observed in the United Kingdom (10.4%) and Italy (19.6%) (Goossens et al., 2003). In France, the prevalence remains low (<2%) (Jones, et al., 2004; Schouten et al., 2000).

The National Nosocomial Infections Surveillance (NNIS) System of the Centres for Disease Control and Prevention reported 28.5% vancomycin resistance rate among enterococci causing infections in intensive care units in 2003. There is 12% increase in VRE infections since 1991(NNIS Report, 2004). Moreover, VRE caring *vanA* gene for vancomycin resistance can transfer to pathogens such as *S. aureus* both in vitro and in vivo (MMWR, 2002).

1.3.2: Genomic makeup of VRE

There are the two types of vancomycin resistance in enterococci, one called intrinsic resistance as found in isolates of *E. gallinarum* and *E. casseliflavus/E. flavescens*. This is a low-level resistance to vancomycin and having *vanC* gene in genome. The second type of resistance is acquired resistance in which enterococci can become resistant to vancomycin by acquisition of genetic information from another organism. Commonly, this resistance is seen in *E. faecium* and *E. faecalis*, but also has been recognized in *E. raffinosus*, *E. avium*, *E. durans*, and several other enterococcal species. All of these isolates mostly have *vanA* and *vanB* genes. There are six known phenotypes of glycopeptides resistant genes in enterococci have been identified designated as vanA, vanB, vanC, vanD, vanE and vanG. They can usually be distinguished on the basis of the level, inducibility and transferability of resistance to vancomycin and teicoplanin. vanA and vanB are the most clinically relevant. Mode of action of vancomycin is formation of complexes with the D-alanyl-D-alanine termini of normal peptidoglycan cell wall precursors, thereby inhibiting cell wall synthesis. But these resistant genes encode a ligase responsible for the synthesis of the depsipeptide D-alanyl-D-lactate. This depsipeptide is incorporated into the terminal portion of the peptidoglycan cell wall precursor, thus limiting vancomycin-peptidoglycan precursor binding (Zirakzadeh and Patel, 2006).

VanA-type resistance is mediated by transposon Tn*1546,* which contains the *vanA* gene cluster, encodes eight polypeptides and are characterized by resistance to both vancomycin and teicoplanin. Transposon Tn*1546* may be located either on plasmid or genomic chromosome and this is responsible for the transfer of *vanA* gene to *S. aureus* from *E. faecalis* (Weigel et al., 2003). Characteristically vanB-type glycopeptide resistance is acquired inducible resistance to vancomycin only not the teicoplanin. The *vanB* sequence varies among different enterococcal isolates (Zirakzadeh and Patel, 2006).

1.3.3: Risk with VRE colonization

Patients colonized with VRE are common source of transferring infection with in health care facility and previous hospitalization is a risk factor for harbouring VRE at the time of hospital admission (Ostrowsky et al., 1999; Weinstein et al., 1996a). VRE colonization leads to VRE infections. In children, risk factors for VRE infections include young age, use of invasive devices, antimicrobial drug administration, immunosuppression, low birth weight, and underlying malignancy (Singh-Naz et al., 1999). Similarly in adults the risk factors includes critical illness, immune suppression, surgical procedures (e.g. Cardiac surgery), invasive devices, prolong hospital stay, broad spectrum antimicrobial therapy and intravenous vancomycin administration.

1.4: Biochemical and molecular identification and characterization of *S. aureus* and enterococci

Different biochemical and molecular techniques are in use for identification and characterization of *S. aureus*, enterococci and their resistant isolates. Gram staining, differential and selective growth medium and different biochemical test are used for identifying these microbes.

Most commonly used molecular technique is polymerase chain reaction (PCR) which is employed for rapid identification of these organisms up to species level by amplification of 16S rRNA of the genome of the particular organism and by targeting genes regulating production of specific proteins.

1.4.1: Biochemical identification of *S. aureus*

Traditionally, identification of staphylococci based on different biochemical characteristics and tests. Staphylococci are Gram-positive cocci and on nutrient agar show white, circular colonies. On blood agar *S. aureus* may show β-haemolysis around colonies. Other biochemical tests used to separate staphylococcal species include catalase test, coagulase test, growth and fermentation of mannitol salt, DNase test and novobiocin sensitivity test. *S. aureus* can be separated from other staphylococcal species by positive catalase, coagulase and DNase tests. *S. aureus* are novobiocin-sensitive and ferment mannitol, giving characteristic yellow zone around colonies. Staph API kit is also used for species identification of staphylococci which is based on series of different biochemical test.

1.4.2: Molecular identification and characterization of *S. aureus*

In case of *S. aureus*, *nucA* gene which encodes thermonuclease was used as a target DNA to identify *S. aureus* (Brakstad et al., 1992) and similarly *mecA* gene was targeted and amplified to identify methicillin resistance (Murakami et al., 1991). Nowadays, for rapid identification of MRSA in blood specimen or other clinical samples, multiplex PCR with both the *nucA* and *mecA* primers is most reliable method (Louie et al., 2002). Multiplex PCR with *mecA* and coagulase gene can also be used for simultaneous detection and identification of *S. aureus* and MRSA (Rallapalli et al., 2008).

The most widely used molecular typing techniques for *S. aureus* are pulse field gel electrophoresis (PFGE) (Cookson et al., 2007), Multilocus sequence typing (MLST) (Cookson et al., 2007, Enright et al., 2000) and *spa*-sequence typing (Harmsen et al., 2003). In PFGE, restriction enzyme *Sma*I is used to obtain fragments of DNA which are separated on a special agarose gel. This analysis is extremely helpful in studying population structures and in the identification of *S. aureus* outbreaks. MLST is based on the DNA sequence analysis of 7 house keeping genes, which gave typing results that are compared with internet accessible database. Most widely used typing technique is *spa*-sequence typing. In this technique genotyping of *S. aureus* is performed by targeting the variation in a tandem repeat region of the protein A encoding *spa* gene which vary both in number and in sequence. By

determining the sequence of the repeats, a profile is constructed which can be used for clustering and making internet accessible database (Schouls et al., 2009).

1.4.3: Biochemical identification of enterococci

Current methods for identification of enterococcus depends upon physiological and biochemical methods. These include Gram stain, catalase test, bile esculin and salt tolerance reactions, or by PYR tests (Facklam and Collins, 1989). Specie identification may require as many as 15 other biochemical tests (Facklam and Collins, 1985). Colonies are transparent or translucent, showing haemolysis after incubation for 18-24 hours on blood agar (Facklam and Collins, 1989). *E. faecalis* and *E. faecium* exhibit Gama or no haemolytic action on sheep blood agar. MHA with 6% NaCl is used as selective medium for the isolation of enterococci. The high salt concentration and facultative aerobic growth at 45°C distinguish this from other organisms.

1.4.4: Molecular identification and characterization of enterococci

In case of enterococci, D-Ala:D-Ala ligase catalyses the dimerization of D-Ala before its incorporation in late peptidoglycan precursors (Neuhaus 1960; Walsh, 1989). Vancomycin binds the terminal D-Ala:D-Ala structure, preventing formation of crosslinks and pentapeptide structures from extending during synthesis. vanA and vanB resistances are due to incorporation of D-alanyl-D-lactate (D-Ala- D-Lac) into peptidoglycan precursors that have reduced affinity for glycopeptides (Bugg et al., 1991, Dutka-Malen et al., 1990-1). D-Ala-D-Lac is synthesized by D-Ala: D-Ala ligase-related proteins VanA (Devriese et al., 1993.) and VanB (Evers et al., 1994), respectively. In VRE isolates, these enzymes are present in addition to the chromosomally encoded D-Ala:D-Ala ligase, *ddl* in *E. faecium* (Sylvie et al., 1995) or *ddl* in *E. faecalis* (Evers et al., 1994). Thus genes encoding D-alanine-D-alanine ligases *ddl E. faecalis* and *ddl E. faecium* are specific for *E. faecalis* (Dutka-Malen et al., 1990-1) and *E. faecium* respectively (Sylvie et al., 1995), whereas the *vanA* and *vanB* genes are associated with VanA (Dutka-Malen et al., 1990-2) and VanB types (Quintiliani et al., 1994) of glycopeptides resistance. Thus these both *ddl* E. *faecalis* and *ddl E. faecium* and *vanA* and *vanB genes* were targeted for

identification of *E. faecalis* and *E. faecium* and for vancomycin resistance either by simple or multiplex PC (Rahimi, 2007).

Genetic methods, such as PFGE which have been the methods of choice when investigating clonal relationships among enterococci. (Torell, E. 2003). There were shortcomings in PFGE, to overcome this more sophisticated genetic typing systems such as amplified fragment length polymorphism (AFLP) and MLST have been developed (Willems et al., 2000: Homan et al. 2002).

1.5: Antibiotic resistance in *S. aureus* and enterococci

S. aureus have remarkable versatility in their behaviour towards antibiotics (Grassi, 1988). Therefore effective management of the clinical conditions antibiotic susceptibility profile of clinical isolates is very imperative and desirable. MRSA isolates are known to be one of the problem nosocomial pathogen in term of its resistance to β-lactams and other antimicrobial. MRSA is multidrug resistant pathogen. Antimicrobial susceptibility pattern can be accessed either by Kirby-Bauer modified disc diffusion or MICs by broth and agar dilution methods. The National Committee for Clinical Laboratory Standards (NCCLS), now called the Clinical and Laboratory Standards Institute (CLSI), recommends the cefoxitin disk screen test, the latex agglutination test for PBP2a, or a plate containing 6 µg/ml of oxacillin in MHA supplemented with NaCl (4% w/v; 0.68 mol/L) as alternative methods of testing for MRSA than simple disc diffusion with oxacillin antibiotic disc. The cefoxitin disk diffusion test is preferred for testing *S. aureus, S. lugdunensis*, and coagulase-negative staphylococci for resistance to the penicillinase-stable penicillins. Cefoxitin is used as a surrogate for detecting oxacillin resistance and now any report for oxacillin as susceptible or resistant based on the cefoxitin result (CLSI, 2007). MIC is defined as the lowest concentration of antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. There are standard guidelines available for testing MICs including British society for antimicrobial chemotherapy (BSAC) and CLSI guide for antimicrobial susceptibility testing.

Enterococci shows intrinsic resistance to a number of commonly used antibiotics, particularly the cephalosporins and also have ability to acquire resistance to many

broad-spectrum antibiotics (Cetinkaya et al., 2000; Jones et al., 1995a; Liassine et al., 1998). During the last few years, enterococci have acquired resistance to a number of important antibiotics such as glycopeptides (Sader et al., 1994). Enterococci are resistant to major three antimicrobial agents i.e. penicillin, aminoglycosides and vancomycin thus creating a problem for clinicians to treat patients in health care institutions. Antimicrobial susceptibility testing of clinical enterococcal isolates, rational use of antibiotics, systematic surveillance programs and control of faecal colonization of resistant enterococci in hospital staff are some of the measures to be adopted for control of the drug resistance in enterococci (Pai and Kim, 1998).

In 1992 different *in vitro and in vivo* experiments revealed that vancomycin resistance genes from *Enterococcus faecalis* could be transferred by horizontal gene transfer to *S. aureus*, which resulted in high-level vancomycin resistance to *S. aureus* (Nobel et al. 1992). In 2002, a VRSA strain was isolated from the catheter tip of a diabetic, renal dialysis patient in Michigan (Chang et al., 2003).The isolate contained the *mecA* gene for methicillin resistance as well. Vancomycin MICs of the VRSA isolate and VanA phenotype of *Enterococcus* species were consistent and the presence of the *vanA* gene was confirmed by PCR. This was further confirmed by DNA sequencing of the VRSA in which *vanA* gene which was identical to that of a *vanA* gene of *Enterococcus faecalis* was recovered. This *vanA* gene was later found to be encoded within a transposon located on a plasmid carried by the VRSA isolate (Weigel et al., 2003).

1.6: Aims and objectives of the research project

Following are the specific aims and objectives of present research project.

1. Isolation of *S. aureus* and enterococcus spp. from the nasal and peri-rectum area of the patients admitted in MICU and PICU of tertiary health care facilities.
2. Identification of MSSA, MRSA, Multidrug resistant enterococci and VRE isolates by different biochemical techniques including API-Kit, coagulase test, catalase test etc. using MRSA252 and SH1000 as indicator organism for staphylococci spp. and OG1RF Liv59 and TX0016 Liv66 as indicator organisms for enterococcus spp.
3. Screening for co-colonization of MRSA and VRE.
4. Antibiotic susceptibility profiling of *S. aureus* and enterococcus isolates by disc diffusion method MICs for selected antibiotics.
5. Precise identification of the bacteria by molecular techniques i.e. PCR.
6. Determine the relationship of MRSA and VRE colonization with the use of antibiotics.
7. Analysing rational and irrational use of antimicrobials in the health care facilities.

LITERATURE REVIEW

LITERATURE REVIEW

2.1: *S. aureus*

2.1.1: *S. aureus* as commensal and pathogen

S. aureus is a commensal on human skin and most prevalent in nasal cavity. It is one of the most virulent organisms and is considered as the frequent cause of infection both in hospital and community (Lowy, 1998). The widespread use of antimicrobial agents to treat staphylococcal infections has resulted in the emergence of resistant forms of these organisms. To date most MRSA have become resistant to number of antibiotics like β-lactams (Kim et al., 2004). Similarly like MRSA large number of coagulase-negative *staphylococci* (CoNS) not only have a high rate of methicillin resistance but also resistant to other antibiotics (Petinaki et al., 2001; Spencer 1996; Tacconelli et al., 2001). Presently MRSA and methicillin-resistant *Staphylococcus epidermidis* (MRSE) isolates have been uniformly susceptible only to glycopeptides. But recently, numbers of isolates resistant to glycopeptides have been reported (Hanaki et al., 1998; Hiramatsu et al., 1997; Sieradzki and Tomasz, 1997).

S. aureus has the characteristic ability to acquire antimicrobial resistance. It is a pathogen of greater concern because of its virulence (Chambers, 2005) and has the ability to cause life threatening infections. It also has the capacity to adopt to different environmental conditions (Lowy, 1998, 2003). The increasing resistance of this pathogen to various antibiotics complicates treatment of infections. Effective measures to prevent its infections are therefore urgently needed. Overcrowding or situations where persons are in close proximity to others, such as in prisons and in sporting teams is a true risk of increased prevalence (Kazakova et al., 2005). It has been shown that increased nasal colonization of *S. aureus* also increased the risk of acquiring an infection with this pathogen. In humans, nose is the main ecological niche where it resides. Eradication of *S. aureus* from nasal carriers may prevent infection in specific patient categories like patients on haemodialysis and general surgery patients. However, recent clinical trials in orthopaedic and non-surgical patients failed to prevent subsequent infections even after eliminating *S. aureus* from the nose (Heiman et al., 2005).

2.1.2: MRSA and antibiotic resistance

MRSA is an important problem in many parts of the world. It causes nosocomial infections in hospitals especially in ICUs, nursing and residential homes. One of important emerging concern nowadays is community-onset of MRSA transmission and infections. There are many serious infections in humans caused by *S. aureus*, including endocarditis, deep-seated abscesses, and osteomyelitis.

MRSA is now endemic worldwide, and vancomycin is the terminal antibiotic of choice for treatment of infections by these strains. Vancomycin binds with terminal dipeptide of the peptidoglycan monomer thus inhibiting cell wall formation in the organism. However, vancomycin-resistant strains possess an altered thickened cell wall with many free monomers capable of binding with the drug. Emergence of vancomycin-resistant *S. aureus* in the hospital settings are occurring faster than expected (Hiramatsu et al., 2004). Several new antibiotics including linezolid, quinupristin/dalfopristin and daptomycin have been developed and are reported to be potential hope for treatment of infection caused by multi drug resistant MRSA and vancomycin-intermediate *S. aureus* (VISA) (Akins and Rybak, 2001).

MRSA may be sensitive to some other antibiotics, like clindamycin, macrolides, tetracycline, trimethoprim-sulfamethoxazole and quinolones, or it may be resistant to all antibiotics except vancomycin. Vancomycin remained the only predictable active antibiotic against all strains of *S. aureus*, and MRSA in particular (Rubeena et al., 2001).

Some strains of *S. aureus* over express β-lactamase and thus appear to be resistant to oxacillin and methicillin despite being *mecA*-negative. Beta lactamase is an enzyme that cleaves the penicillin molecule at its cyclic ring, and second generation penicillin like methicillins were specifically designed to resist β-lactamase activity (Blot et al., 2002).

A study conducted in India during 2006, in which out of 783 *S. aureus,* two strains were found to be vancomycin and teicoplanin-resistant with MIC 32 µg/ml and 64 µg/ml respectively. Six strains were VISA (two strains with MIC 16 µg/ml and four strains with MIC 8 µg/ml) and two strains with teicoplanin-intermediate (MIC 16 µg/ml). All VRSA and VISA had shown growth on BHI vancomycin screen agar

(vancomycin 6 µg/ml) and were *mecA* PCR-positive. None of these isolates have amplified *vanA*/*vanB* gene by PCR (Tiwari and San, 2006).

In Pakistan, Hafiz et al., (2002) conducted a study in major cities to determine the frequency of MRSA. The study concluded that there were 42% MRSA isolated out of 792 isolates from eight different laboratories and no VRSA was isolated. Another study on mode of infection, incidence of MRSA and their susceptibility against glycopeptides and fucidic acid was conducted by Bukhari *et al.* (2004). They isolated 350 S. *aureus* strains out of 1800 random clinical specimens. Of these 135 (38.5%) were found to be MRSA and were selected for determining susceptibility against vancomycin, teicoplanin & fucidic acid. Susceptibility study showed 96%, 94% and 86% resistance against vancomycin, teicoplanin and fucidic acid respectively.

Anwar et al., (2004) studied the prevalence of S. *aureus* and MRSA nasal carriage in general population of Lahore-Pakistan. Out of total 1660 nasal swabs of healthy peoples (1024 urban and 636 rural) 246 (14.82%) were positive for S. *aureus*. Of these 246 isolates, 48 (19.51%) isolates were MRSA. Prevalence was high in males (15.47%) as compared to females (13.26%) and in urban areas (16.99%) as compared to rural areas (11.32%). Maximum nasal carriage was found in the age group up to 9 years (20.23%) with decrease in the age groups 10-19, 20-29 and 30-39 years followed by small rise in the elderly subjects. They finally concluded that nasal S. *aureus* carriage as well as methicillin resistance between these isolates were more common in urban community.

2.1.3: Molecular aspects of MRSA

The probes, constructed from the *nuc* gene are employed to detect all *S. aureus* genomes present and thus provide easy and rapid approach to identify *S. aureus* directly from clinical specimens (Palomares et al., 2003). Standard bacterial identification and susceptibility testing frequently required as long as 72 hours to report results. There may be difficulty in rapid and accurate identification of methicillin resistant strains. The use of the PCR is a rapid and simple process for the amplification of target DNA sequences, which can be used to identify and test bacteria for antimicrobial resistance. However, many sample preparation methods

are unsuitable for PCR utilization in the clinical laboratory because they either are not cost-effective, take too long to perform, or do not provide a satisfactory DNA template for PCR (Jaffe et al., 2000).

Detection of microorganism was previously difficult or impossible by traditional microbiological methods. Introduction of PCR techniques opened the way into new era by allowing rapid detection of microorganisms. In clinical microbiology laboratories, molecular detection mostly performed by PCR technology. Initially PCR was involving single round and procedures like detection by gel electrophoresis. However, with the introduction of automation various steps and stages of PCR like DNA or RNA extraction, amplification and product detection is merged like in Real time PCR and molecular laboratories becomes more efficient and cost-effective. Broad-range PCR is used for the identification of diseases like infective endocarditis and bacterial meningitis. (Speers, D.J. 2006). Similarly PCR can be used for detection of the *mecA* gene multiplexed with the *nuc* gene to allow rapid molecular detection of *S. aureus* and confirmation of MRSA from positive blood culture bottles (Louie et al. 2002).

MRSA produces a penicillin binding protein (PBP), PBP 2a or PBP 29 in addition to the usual PBPs. This is the primary mechanism of methicillin resistance in staphylococci and is referred to as intrinsic resistance. PBP 2a has a low affinity for β-lactams. Resistance in *S. aureus* can be heterogeneous, because factors other than PBP 2a influence the degree to which it is expressed (Berger et al., 1989). In addition, bacterial strains with low-level resistance to methicillin may produce large amounts of β-lactamase and therefore not exhibit intrinsic resistance. Methicillin resistant organism can be recognized by the presence of *mecA* gene. *mecA* is a chromosomally derived gene that has been cloned and sequenced (Murakami and Tomasz 1989). It has a very high level of homology in MRSA and methicillin-resistant coagulase-negative staphylococci (MRCoNS) and is absent from methicillin-susceptible staphylococci isolates (Predari, et al., 1991; Suzuki et al., 1995). Additionally, the *mecA* gene is similar and identical in all staphylococcal strains and thus it is a useful molecular marker for identification of methicillin resistance (Archer and Niemeyer 1994; Vannuffel et al., 1995). Automated systems have excellent specificity but often lack sensitivity in detecting MRS, particularly

coagulase-negative strains. Many biochemical methods are used for identification of methicillin resistance in isolates from clinical laboratories. This includes agar dilution, disk diffusion, and broth dilution. These methods based on phenotypic expression of strains rather than the presence of the *mecA* gene, and their results depend on various variables, particularly needed isolated colonies from an overnight subculture on solid agar from clinical sample (Chambers 1997).

Tayfour et al., (2005) suggested that no single technique was clearly superior to others for typing MRSA strains and the availability of plasmid analysis with antibiotic sensitivity tests on a routine bases may be helpful in characterizing isolates that cause outbreaks of MRSA. Three methods, antibiotic sensitivity tests, plasmid profile and restriction endonuclease analysis of plasmid (REAP) are used for typing and gathering of epidemiological data. These methods are cheap, practical and can easily interpret and employed together in clinical laboratories.

2.2: Enterococci and vancomycin resistance

2.2.1: Enterococci prevalence and virulence

Enterococci are widely distributed in nature. Intestinal tract of humans and animals is the natural habitat of these organisms. They gain entry into raw material and foodstuffs through the water supply, food animals, unhygienic conditions of the production and handling.

Enterococci are not considered to be highly virulent, but their intrinsic resistance and ability to acquire resistance to many broad-spectrum antibiotics allows them to cause super infections in patients already receiving antimicrobial therapy (Cetinkaya et al., 2000; Jones et al., 1995a; Liassine et al., 1998). The specie of high concern in human infections is *E. faecalis* and the other one of increasing concern is *E. faecium* due to high resistance to antibiotics especially in nosocomial settings (Nelson et al., 2000; Sahm et al., 1997; Mathai et al., 1994). Enterococcus species are most commonly involved in urinary, gastrointestinal tract and pelvic infections (Noble et al., 1992; Desai et al., 2001; Murray et al., 1990).

2.2.2: VRE and vancomycin resistance

Enterococci have been known to be resistant to most antibiotics used in clinical practice. Multidrug-resistant enterococci and VRE are commonly isolated from humans, animal sources, aquatic habitats, agricultural run-off which indicates their ability to enter the human food chain. VRE are emerging as a global threat to public health (Lukaova et al., 2003).

VRE were first reported in Europe, in 1988 (Uttley et al., 1988), they have since been identified with increasing frequency in many nations (Boyle et al.,1993; Centers for Disease Control and Prevention 1993; CDR Wkly 1995; Boyce et al., 1994; Morrison et al.,1996; von Gottberg et al., 2000). The overall prevalence of VRE isolates collected from hospitals in 6 geographic regions of the United States from July 1988 through April 1989 was very low (0.3%) (Gordon et al., 1992). A national surveillance study evaluated that 4.4% of isolates were VRE in US hospitals in 1992 (Jones et al., 1995b). The percentage of VRE nosocomial infections reported to the CDC-NNIS system increased from 0.3% to 7.9% in 1989 and 1993 respectively (Schaberg et al., 1991). This increase was higher among patients in ICUs, rising from 0.4% to 13.6% in the same time interval. In some hospitals, VRE became established as an endemic nosocomial pathogen (Morris et al., 1995).

Results from the 1997 SENTRY program revealed that 14.1% of enterococcal blood stream infections (BSIs) in the United States were due to VRE (Pfaller et al., 1999). In the SCOPE project on nosocomial BSIs in 49 US hospitals over the period 1995–1998, it was found that 17.7% of enterococcal isolates displayed resistance to vancomycin and the proportion of resistance to vancomycin was higher (50.5%) among *E. faecium* isolates than (3.1%) among *E. faecalis* isolates (Edmond et al., 1999).

The SENTRY program was designed to monitor the spectrum of microbial pathogens along with antimicrobial resistance patterns for both nosocomial and community-acquired infections on a global scale (Pfaller et al., 1998). Many publications have described the results of various aspects of the SENTRY program

(Pfaller et al., 1999; Edmond et al., 1999; Doern et al., 1999; Jones et al., 1999a, 1999b).

2.2.3: Molecular aspects of VRE

The phenotypic identification based on Gram staining of growth on Bile aesculin azide agar (BEAA) and subculturing of cocci on sheep blood agar plates for vancomycin disk diffusion and hydrolysis of L-pyrrolidonyl-b-napthylamide (PYR) testing. The phenotypic method was used for surveillance cultures that yielded growth on BEAA. Enterococcal strains were identified to the species level by conventional biochemical tests. PYR-positive isolates were further characterized into VRE and non-VRE depending upon vancomycin inhibition zone size. The isolates were characterized into vanA, vanB and vanC depending upon minimum inhibitory concentration (MIC) values (Cereda et al., 2002).

Molecular identification of the enterococcal species targets several genes coding heat shock protein 60, elongation factor EF-Tu, D-Ala: D-Ala ligase and manganese-dependent superoxide dismutase. The use of PCR for identification of genus and species of enterococci has been reported previously (Jackson et al., 2004; Goh et al., 2000; Ozawa et al., 2000).

From different geographic regions of Brazil, 51 VRE isolates were studied. PCR analysis demonstrated that all the isolates harbored the *vanA* gene and in a majority of strains the gene was associated with a transferable plasmid of 70 kb. The *vanA* element integrity in these enterococci strains and the different pulsed-field gel electrophoresis patterns suggest horizontal transmission of the vancomycin resistance transposon in Brazilian strains (Palazzo et al., 2006).

Enterococci are very important nosocomial pathogens because of its natural and acquired resistance to antimicrobial agents, including glycopeptides, vancomycin and teicoplanin (Cetinkaya et al., 2000; Gold 2001). There are 06 phenotypes of VRE including vanA, vanB, vanC, vanD, vanE and vanG (Cetinkaya et al., 2000; McKessar et al., 2000). These phenotypes correspond to the genotypes *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG*. Most common phenotypes among VRE isolates are vanA and vanB. Generally, VRE with *vanA* genotype shows vanA phenotype with high-level resistance to both vancomycin and teicoplanin, whereas

VRE with *vanB* genotype shows vanB phenotype characterized by various levels of resistance to vancomycin but susceptibility to teicoplanin (Cetinkaya et al., 2000).

Transposon Tn*1546,* which contains the *vanA* gene cluster, encodes eight polypeptides and are characterized by resistance to both vancomycin and teicoplanin. This is located either on plasmid or genomic chromosome and is responsible for the transfer of *vanA* gene to *S. aureus* from *E. faecalis* (Weigel et al., 2003). The Tn1546-like elements or *vanA* elements are produced by several modifications in different positions of the transposons causing their polymorphism and are often carried out by conjugative plasmids (Cetinkaya et al., 2000; Arthur and Courvalin 1993). As a member of the Tn3 family, Tn1546 preferred plasmid DNA for insertion, but this mobile element has also been identified in chromosomal DNA (Handwerger and Skoble 1995).

Transfer of VanB-type resistance to glycopeptides among enterococci has been reported to be associated with the movement of large chromosomal genetic elements or of plasmids. Transposon Tn*1549* is conferring vancomycin resistance in clinical isolates of enterococcus species (Garnier et al., 2000).

Friederike et al., (2001) confirmed the type of vancomycin resistance (*vanA*, *vanB*, *vanC1*, *-C2* or *-C3*) by polymerase chain reaction (PCR). A ratio of 50 samples out of 1026 (4.9%) was found VRE-positive. All the isolated strains carried the *vanA* resistance gene.

2.3: Antibiotic resistance in bacteria

Rapid development of antibiotic resistance is a great problem in the therapeutic use of antimicrobial agents and there is marked increase in the resistance of bacteria to antimicrobial agents during the last decades. Microorganisms somehow developed altered receptors for the antimicrobial agents and become resistant. The wide spread and irrational use of antibiotics are responsible for the development of multidrug resistant bacteria, increased number of nosocomial and community-acquired infections and thus increased the health care costs (Snyder et al., 2000).

There are several studies reporting the influence of antibiotic selective pressure on the frequency of VRE occurrences in hospitals mainly by using glycopeptides, third-

generation cephalosporins, quinolones and lincosamides. A study was conducted in the Department of Hemato-Oncology of the Teaching Hospital in Olomouc (DHO), Czech Republic to evaluate the relationship between VRE occurrence and antibiotic use. The study was of 6 years from 1998 to 2003 and during this period hygienic and epidemiological conditions were controlled and unchanged. It was concluded by this study that is effect of use of glycopeptides and third-generation cephalosporins on occurrence of VRE and no influence of quinolones and lincosamides over the 6-year period (Kolar et al., 2006).

In many countries antibiotics are easily available from pharmacies and market without a proper prescription of a physician. Non-compliant patients are another major problem. They forget to take medication, interrupt their treatment when they begin to feel better, or unaffordable to buy a full course of medication, all these provide an idea environment for microbes to adapt rather than be killed. Similarly low quality antibiotic formulations and self-medication also aggravate the situation (Kondo et al., 1999).

Resistance is either chromosomal or extra-chromosomal or plasmids mediated. Plasmids are transmissible and are present in many bacteria and also in *S. aureus* and enterococci. In addition to plasmid bacteria can also contain transposons also called as jumping genes. They have the ability to enter the plasmid as well as chromosomes. The genetic basis for the antimicrobial resistance may be acquisition and expression of new DNA by horizontal gene transfer or mutation in cellular genes or acquired genes that alter antimicrobial target sites or affect gene expression (Normark and Normark, 2002).

Certain resistant genes are involved in the formation of enzymes which inactivate antibiotics by chemically modification or degradation and ultimately inactivate the drug. Some resistant genes modified or replaced the molecules which normally bound by antibiotic, thus effectively removes the drug's target. Resistant bacteria may also possess genes that are expressed by blocking the entry of drug molecule into the cell, or causes efficient efflux pump to export the antibiotic from the cell before it has met its target (Levy, 1998).

2.3.1: *S. aureus* and antibiotic resistance

The first reported isolation of VISA occurred in Japan in 1997 (Hiramatsu et al., 1997) and more than 100 VISA isolates have since been reported (Appelbaum 2006). These strains were generally in the intermediate level of resistance to vancomycin but with moderately raised minimum inhibitory concentration (MICs. However, they are frequently also resistant to the other glycopeptides used in clinical practice (teicoplanin). They appear to have developed from strains of MRSA (Waldvogel 1999).

In September 2002, VRSA isolate was obtained from a patient in Pennsylvania. Species identification was confirmed by standard biochemical tests and analysis of 16S ribosomal DNA, gyrA and gyrB sequences. All the results were consistent with *S. aureus* identification. The isolate was resistant to vancomycin (MIC 32 µg/ml), aminoglycosides, β-lactams, fluoroquinolones, macrolides, quinupristin/dalfopristin, rifampin, teicoplanin and trimethoprim-sulfamethoxazole (Tenover et al., 2004).

S. aureus showed vancomycin hetero-resistance which results low response of MRSA to vancomycin in spite of susceptibility to vancomycin in the laboratory. This increased the mortality, seen in patients with MRSA infection (Sakoulas and Moellering, 2008). There are several similarities between VRSA and VISA infections. Fridkin (2001) studied that all the isolates from patients were susceptible to chloramphenicol, linezolid, quinupristin/dalfopristin and trimethoprim-sulfamethoxazole where as the VRSA isolate was highly resistant to vancomycin with MIC 1024 µg/ml and VISA isolates were only moderately resistant to vancomycin with MIC 8 µg/ml (Fridkin, 2001; Hageman et al., 2001).

Deng et al., (2007) isolated bacteria from the wound excretions of 306 burn patients hospitalized during 2001 to 2006 for analyzing their strains and their antibiotic resistance. They concluded that drug resistance to antibiotics in the burn ward may be related to the β-lactamases from Acinetobacter baumannii and multiple-drug-resistance of MRSA.

2.3.2: Enterococci and antibiotic resistance

Enterococcal resistance includes β-lactamase-mediated resistance, ampicillin resistance based on altered penicillin-binding proteins (PBP), and high-level aminoglycoside resistance (Malathum et al., 1999). Enterococci have been known to be resistant to most antibiotics used in clinical practice. They are naturally resistant to cephalosporins, aminoglycosides and clindamycin and may also be resistant to tetracyclines and erythromycin. They are intermediate sensitive to penicillin and ampicillin and glycopeptides. The strains that produce β-lactamase are rare (Urbášková, 1999). The important resistance is to vancomycin which made enterococci a global threat to public health. VRE threatens to compromise effective treatment of infections caused by these multiresistant bacteria particularly in seriously ill patients who may need treatment with vancomycin where other antibiotics have failed.

Since their initial isolation from patients in the United Kingdom and France, VRE infections have been increasingly detected throughout the world (Woodford, 1998). These bacteria are often resistant to multiple antibiotics, thus limiting the number of therapeutic options available to the physician (Gold, 2001).

2.3.3: New antibiotics for MRSA and VRE

Linezolid and quinupristin/dalfopristin are two newly approved antibiotics which are active against MRSA and VRE. These both are now available for clinical use (Fey et al., 2003). Before these new antibiotics were developed, antimicrobial therapy for these infections was limited. These two antibiotics can be used to promote the heterogeneous treatment of infections and therapy with these agents may decrease overall resistance to vancomycin by reducing its use. However, vancomycin, linezolid and quinupristin/dalfopristin should be used only for serious infections due to antibiotic-resistant strains, such as MRSA, VRE-resistant, coagulase-negative staphylococci and penicillin-resistant pneumococci (Quale et al., 1996; Montecalvo et al., 1999).

Linezolid is a treatment option for joint infections following arthroplasty that are caused by resistant bacterial strains, such as MRSA, MRSE or VRE. The treatment cost with linezolid is high, but it markedly reduces hospital stay costs and increases

patients' comfort during treatment (Jahoda et al., 2006). It is not yet recognized as a standard therapy for infective endocarditis but its use becomes a necessity when infection is due to multidrug-resistant microorganisms (Mancino et al., 2008). Linezolid has unique mechanism of action which involves inhibition of bacterial protein synthesis through binding to the domain V regions of the 23S rRNA gene (Meka et al., 2004). Resistance to linezolid requires mutations of multiple gene copies. Linezolid is 100% bioavailable when given by either oral or intravenous route. In two controlled trials of hospital-acquired pneumonia, a trend was seen for linezolid superiority over vancomycin (Rubinstein et al., 2001; Wunderink et al., 2003).

Quinupristin/dalfopristin is active against *S. aureus*, including MRSA, *S. pneumoniae*, and Gram-positive anaerobes such as Clostridium spp. It is effective against vancomycin-sensitive as well as vancomycin-resistant *E. faecium* but has little in vitro activity against *E. faecalis*. The clinical success rate of quinupristin/dalfopristin was comparable (68%) to the comparator agents (71%). (Meka et al., 2004). Quinupristin/dalfopristin has been used to treat patients infected by *S. aureus* intolerant of or failing standard therapies (Drew et al., 2000).

2.4: Nosocomial infection and co-colonization with MRSA and VRE

Nosocomial bacterial infections are a major cause of morbidity in hospitalized patients. Patients in ICUs have 5-10 times greater rate if nosocomial infection than those in general wards (Finkelstein et al., 2000). The re-emergence of infectious diseases and the continuous development of antibiotic resistance among a variety of pathogenic bacteria poses a serious threat to public health worldwide (Desselberger, 2000). Among these pathogenic microorganisms, enterococcus, staphylococcus and streptococcus are common closely related species that cause a wide variety of infections and diseases (Boyce, 1997; Lowy, 1998; Hancook & Gilmore, 2000).

It has been studied that chances of nosocomial infections increases with prolonged stay of patient in health care facility. Longer the stay, greater is the chances of contact of the patient with the health care personnel, greater exposure to environmental microorganisms and more frequent are the invasive procedures. All

this leads to nosocomial infections. Delagado et al., and Sing-Naz et al., had shown the mean duration of stay to be longer in patients with nosocomial infection (9.8 vs 1.8 days) (Delagado et al., 1990 and Singh-Naz et al.,1996).

2.4.1: Nosocomial infections and MRSA

Since the 1980s, MRSA has been commonly linked with hospital acquired nosocomial infections, but recently community acquired strain, has also been emerged (Vandeneschet al., 2003).

Today MRSA is the major nosocomial pathogen worldwide. Recent surveillance studies in hospitals in various parts of the world indicate a varying incidence of MRSA strains depending on the country and the hospital. In the USA, the National Nosocomial Infections Surveillance System (NNISS) recorded an increase of MRSA in large USA hospitals, from 4% in the 1980s to 50% in the late 1990s. In some hospitals the resistance frequencies as high as 80% have been recorded (Campos, Cipriano and Freitas, 2001).

2.4.2: Nosocomial infection and VRE

Enterococci have emerged as important causes of nosocomial (Cetinkaya et al., 2000; Me´ndez-Alvarez et al., 2000; Murray 2000; Rice 2001). They were reported as the second most common cause of nosocomial infections in the United States (Schaberg et al., 1991). The most frequent enterococcal infections include urinary tract infection, surgical site infection and bacteremia. Enterococci represent the second leading cause of nosocomial urinary tract infections (Moellering, 2000) and the third leading cause of nosocomial bacteremia. Other infections caused with low frequency are CNS and neonatal infections. It rarely causes respiratory tract infections, osteomyelitis or cellulitis (Murray, 1998). They include 20 species, but most human enterococcal infections are caused by *E. faecalis* and *E. faecium.* A few cases of human infections caused by other enterococci spp. such as *E. durans, E. gallinarum* and *E. casseliflavus* have also been reported (Liassine et al.,1998; Leclercq and Courvalin 1997). Other infections caused with lower frequency are CNS and neonatal infections. Enterococci rarely cause respiratory tract infections, osteomyelitis, or cellulitis (Murray 1998).

Enterococci have been documented to be the third most prevalent pathogens in nosocomial bloodstream infections (BSIs) in the United States and are associated with 5%–15% of cases of bacterial endocarditis (Emori, and Gaynes 1993). In 1996, data from the Surveillance and Control of Pathogens of Epidemiologic Importance (SCOPE) program monitoring nosocomial BSIs revealed that enterococci accounted for 11.7% of all isolates (Jones et al., 1997), an increase in incidence of 3% over that reported in 1993 by the National Nosocomial Infection Surveillance (NNIS) system of the Centers for Disease Control and Prevention (CDC) (Centers for Disease Control and Prevention 1993).

2.4.3: Co-colonization with MRSA and VRE

Colonization with VRE and MRSA are more common among patients requiring intensive care (Richards 1999a and 1999b; Ostrowsky 2001). MRSA has become the predominant form of clinically significant *S. aureus* within ICUs and increasingly within some community settings as well (Lucet et al., 2003; Crowcroft 2002; Salgado 2003; Naimi 2001).

As both VRE and MRSA are widespread in hospitals so it is not uncommon for a patient to be colonized or infected with both and treated with vancomycin (Courvalin, 2006; Gould, 2005). Co-colonization results in transfer of resistant gene for vancomycin from VRE to MRSA thus resulting emergence of VRSA. Six cases of VRSA in U.S. hospitals have been described since 2002 (Centres for Disease Control and Prevention, 2003; Weigel et al., 2003) VRSA strains have acquired vancomycin resistance genes *vanA* from VRE (Weigel et al., 2003; Flannagan et al., 2003).

There are many recommended measures which are promoted to control the colonization and spread of MRSA and VRE in hospitals but, surveillance data suggest that in spite of adopting these measures there was no appreciable decrease in rate of infection or colonization with either of these organisms in the United States. The reasons for this failure are unclear but it might be because of ineffectiveness or poor implementation of adopted measures (Centres for Disease Control and Prevention, 1997).

2.5: Identification methodology for MRSA and VRE

2.5.1: Identification of MRSA

Traditionally *S. aureus* is confirmed by slide coagulase test (clumping factor) and the tube coagulase test (free coagulase). The positives on the slide coagulase test should be confirmed with the tube coagulase test. DNase media plates can also be used as additional confirmation. A good correlation was shown between DNase production and coagulase activity when tested on *S. aureus* isolates from clinical samples (Jeffries et al., 1957; DiSalvo ,1958; Blair et al., 1967). Both *S. aureus* and *Staphylococcus epidermidis* can produce extracellular DNase (Baird-Parker, 1965; Raymond and Traub, 1970; Zierdt and Gold, 1970) but *S. aureus* can do in greater quantities (Weckman and Catlin, 1957; Zierdt and Gold, 1970). However several alternative methods including broth based methods (broth with high salt concentration along with methicillin, oxacillin and cefoxitin), solid agar media (Mannitol salt agar, Mueller Hinton Agar with 4% NaCl and 6 mg/L oxacillin), chromogenic media, rapid screening kits, molecular assays and automated systems are increasingly being used. Isolation from screening swabs can be a lengthy procedure, due to the number of 'contaminating' organisms that are present in swabs from non-sterile sites. A wide range of commercial biochemical kits are available, both manual and automated. These are based on an array of biochemical tests giving a profile assessed against databases/tables. Example for this type of kit is Staph API kit. Many automated systems combine biochemical identification of *S. aureus* with antibiotic sensitivity panels for the confirmation of MRSA like AutoScan-4®, BD Phoenix™ and Vitek® 2 (Ishii et al., 2006).

Most of molecular methods used for the detection of MRSA relying on simple and multiplexed PCR. Primers are used detecting genes specific for *S. aureus* including *nuc* and *fem,* and *mecA* detecting methicillin resistance (Louie et al., 2002). Antibiotic sensitivity testing using disc diffusion methods and MICs remain the most widely used but results are influenced by a range of factors including medium, NaCl concentration, temperature, inoculum and test agent. several recent studies using cefoxitin disc diffusion method suggest greater reliability than with oxacillin (Broekema et al., 2009; Felten et al., 2002).

2.5.2: Identification of VRE

Current methods for identification of enterococci depend upon physiological and biochemical methods (Facklam et al., 1989). These include Gram stain, catalase reaction, specific growth media including brain infusion agar, BAA, Slanetz-Bartley agar (Doming et al., 2003; Ramotar et al., 2006), salt tolerance reactions, PYR tests, Characterization of morphology and identification by appropriate biotyping methods (En-coccus test, API 20 Strep, Merlin system) based on determination of enzyme profile (Delmans et al., 2008). Speciation of enterococci may require as many as 15 other biochemical tests. Enterococcus exhibit alpha, beta or no hemolytic action on sheep blood agar (Facklam et al., 1985).

For VRE identification specimens are directly inoculated on BEAA plates containing 6µg/ml of vancomycin. Black colonies are identified as enterococci. Vancomycin resistance can also be determined by inoculating a suspension of the organism onto a commercially available brain heart infusion (BHI) agar plate containing 6µg/ml vancomycin. The National Committee for Clinical Laboratory Standards (NCCLS) recommends performing a vancomycin MIC test and also motility and pigment production tests to distinguish species with acquired resistance (*vanA* and *vanB*) from those with *vanC* intrinsic resistance (NCCLS, 1999). Identification of enterococci by phenotypic methods may take 1-2 days (Drahovska et al., 2002). For this reason, molecular techniques, randomly amplified polymorphic DNA analysis, ribotyping (Price et al., 1999), intergenic ribosomal PCR (Tyrell et al., 1997) have been developed to identify enterococci at the species level. The *ddl*- PCR (D-alanin-D-alanin ligase PCR) (Dutka-Malen et al., 1995) and *vanC* (*vanC* operon coding for intrinsic resistant to a low level of glycopeptides), *vanB* and *vanA* PCR are for are useful in routine practice for identification of VRE (Dutka-Malen et al., 1995).

MATERIALS & METHODS

MATERIALS AND METHODS

3.1: Isolation and identification of *S. aureus* and enterococci

3.1.1: Specimen collection and inoculation

Routine cultures of the anterior nares and peri-rectal area were obtained for MRSA and VRE from patients within 48 hours of admission to both PICU and MICU. Specimens were processed within two hours of collection by the standard microbiology technique. All the patients admitted in ICUs were included in the study.

Specimens were collected on sterile cotton wool swabs socked in sterile normal saline. The swabs were inserted in both the nares to ensure proper collection of nasal microflora and from peri-rectal area for isolation of staphylococci. Both types of specimens were processed within two hours of collection by the standard microbiology technique (Cheesbrough, 2006). The samples were left for overnight growth in BHI broth before inoculation on selective or differential medium. For staphylococci BHI (Oxoid, UK) and sheep blood agar (SBA) (Oxoid, UK) were used as enriched nonselective medium and mannitol salt agar (MSA) (Oxoid, UK) was used as selective medium. The agar plates were then incubated at 35°C for 18-24 hours in aerobic atmosphere (CLSI, 2007).

For isolation of enterococci same nasal and peri-rectal swabs were inoculated onto bile-aesculin agar (BAA) (Oxoid, UK) plates and were incubated at 45°C for 24–72 h. The isolated colonies were sub-cultured on mueller hinton agar (MHA) (Oxoid, UK) with 6% NaCl at 45°C.

3.1.2: Isolation and identification of isolates

3.1.2.1: Staphylococci

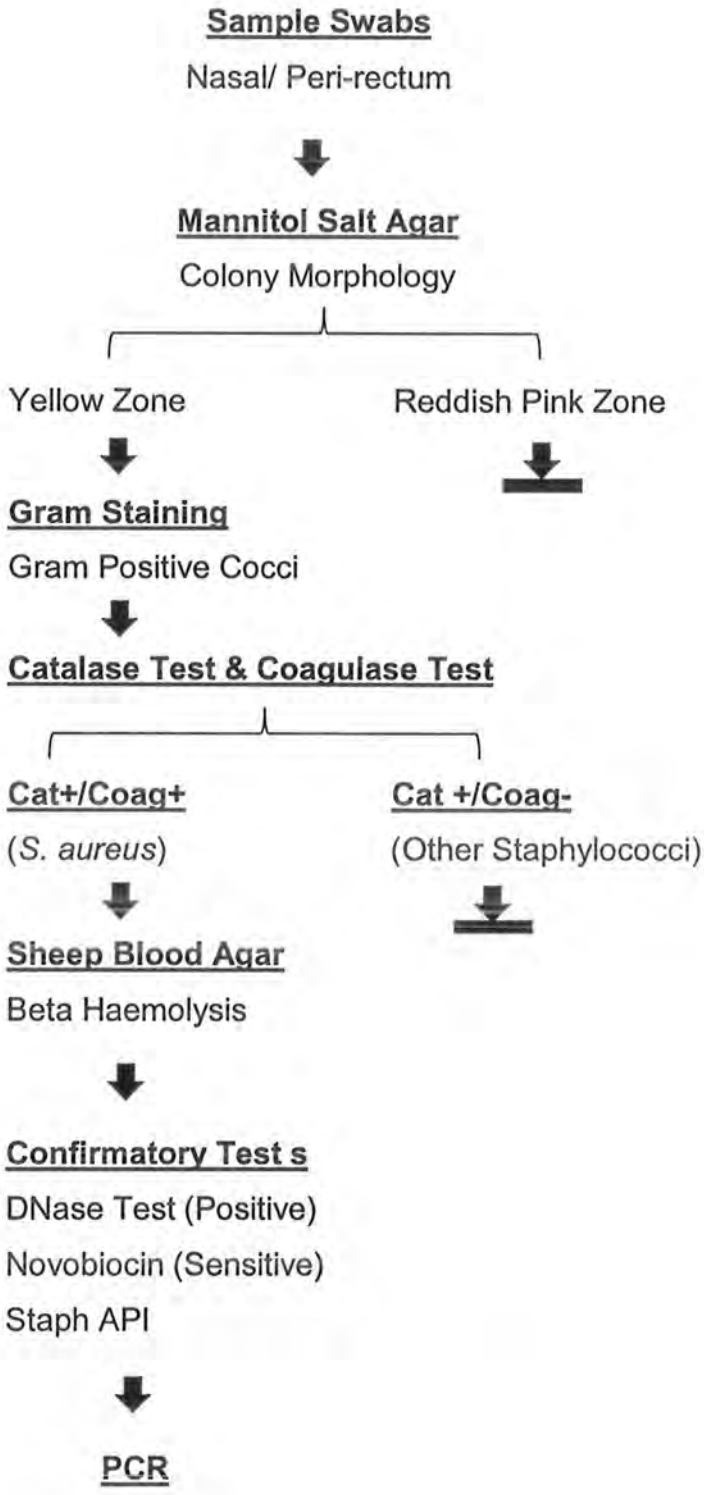
The plates were examined for staphylococci by colony morphology- yellow to cream or occasionally white 1-2 mm diameter, slightly raised colonies after overnight incubation on BHI agar. Some isolates showed beta-hemolysis on sheep blood agar plates. Staphylococci were identified as Gram-positive cocci of uniform size, appeared characteristically in groups mostly but also seen singly and in pairs in few slides.

Gram staining was performed on all MSA colonies. Gram-positive colonies with characteristic microscopic morphology were subjected to Catalase test. It was performed to differentiate staphylococci from non-catalase producing bacteria. This test detects the presence of catalase enzyme by the decomposition of hydrogen peroxide to release oxygen and water. Catalase enzyme is present mostly in cytochrom-containing aerobic and facultative bacteria (staphylococci and enterococci).

S. aureus isolates were further identified by positive bound coagulase test which converts fibrinogens directly to fibrin without requiring coagulase reacting factor. Lyophilized rabbit plasma (IMVS Veterinary Service Division, Australia) as well as fresh rabbit plasma was used for slide coagulase test. Few samples were subjected to tube coagulase test for confirmation. All coagulase positive isolates were confirmed by identification of deoxyribonuclease (DNase) enzyme by performing DNase test on DNase agar (Oxoid, UK). Isolates were streaked on to the surface of the agar medium and incubated at 35-37°C for 18-24 hours (Jeffries et al., 1957). 1N hydrochloric acid was flooded on the surface of the ager plate containing growth. The DNA in the media precipitated due to the effect of 1N HCl and medium became opaque. Clear zones formed around the colonies which produced DNase enzymes in sufficient quantity to hydrolyse the DNA. All expected *S. aureus* were grown on SBA to check the haemolysis. Most of the *S. aureus* showed β-haemolysis on SBA.

Sensitivity against novobiocin was performed to distinguish between *S. aureus* and *S. saprophyticus*. Novobiocin (MP Biomedicals, US) 1 mg/ml solution was prepared and serially diluted to make the desire concentration. The universal blank antibiotic discs were socked to make 5 µg disc of Novobiocin alternatively novobiocin disc (Oxoid) 30 µg were also used for susceptibility profile. Susceptibility was performed according to CLSI guidelines for susceptibility testing (CLSI, 2007). Isolates which gave <16 mm zone of inhibition were considered resistant. Intrinsic novobiocin resistance in *S. saprophyticus* (Vickers et al., 2007) distinguishes *S. aureus* as it is sensitive to novobiocin. Identification protocol for staphylococci is shown in scheme Fig. 3.1.

Fig 3.1: Staphylococci identification scheme



3.1.2.2: Enterococci

Enterococci showed black zones around the colonies due to the formation of black iron phenolic compounds derived from aesculin-hydrolyis products and ferrous iron on Bile Aesculin Agar plate (BAA) (Oxoid). BAA was used to differentiate between enterococci/Group D streptococci and non Group D streptococci. Enterococci/Group D streptococci hydrolyze aesculin to form aesculetin and dextrose. Aesculetin combines with ferric citrate in the medium to form a dark brown or black complex which is indicative of a positive result. While the Bile salts in the medium inhibits Gram-positive bacteria other than enterococci/Group D streptococci. Further confirmation was achieved by growing enterococci on KF streptococci agar (Oxoid, UK) on which they showed pink or red colonies. KF streptococci agar used to detect Group D streptococci/enterococci from fecal samples. Catalase and coagulase test were performed on all expected enterococci colonies, both were negative. On SBA, all enterococci showed β-haemolysis after overnight growth at 37°C.

3.1.3: Confirmation of isolates

3.1.3.1: Confirmation of *S. aureus*

The confirmation of *S. aureus* was performed by API Staph (bioMérieux Germany) according to user's instructions manual. Biochemical tests in API Staph are voges proskauer (VP), alkaline phosphatase (PAL), Nitrate reduction (NIT), Urase (URE), arginine hydrolase (ADH), along with sugar fermentation tests like glucose (GLU), fructose (FRU), mannose (MNE), trehalose (TRE), mannitol (MAN), xylitol (XLT), melibiose (MEL), raffinose (RAF), xylose (XYL), sucrose (SAC), α-methyl-D-glucoside (MDG) and N-acetyl-glucosamine (NAG). The biochemical and sugar fermentation tests of this system allowed presumptive identification of staphylococci up to species level.

The incubation box (provided in the kit, the tray and lid) was prepared by distributing 5 ml of sterilized distilled water or demineralized water to create a humid atmosphere. Overnight growth of the organisms on BHI medium was taken to make homogeneous suspension of the bacterial isolates in API Staph medium with a turbidity equal to 0.5 Mc Farland. Microtubes of the API kit are filled according to the

instruction and ADH and URE tests were overlaid by sterilized mineral oil to create anaerobic condition.

Incubation boxes were closed by lids and were incubated at 35-37°C for 18-24 hours. After this the VP test, NIT test and PAL test was made with VP1 and VP2 agents, NIT1 and NIT2 reagents and Zyme A and ZYME B reagents respectively. The last 21st test was performed by flooding the bacterial suspension on agar plate and after it dried, a drop of lysostaphin solution 200 mg/ml was put on the surface of each plate. These plates were incubated at 37°C for 24 hours. The resistant isolates were considered positive for this test. Results from biochemical and sugar fermentation test were recorded after 24 hours. The 8 digit numerical code was derived and was interpreted with the help of API web software.

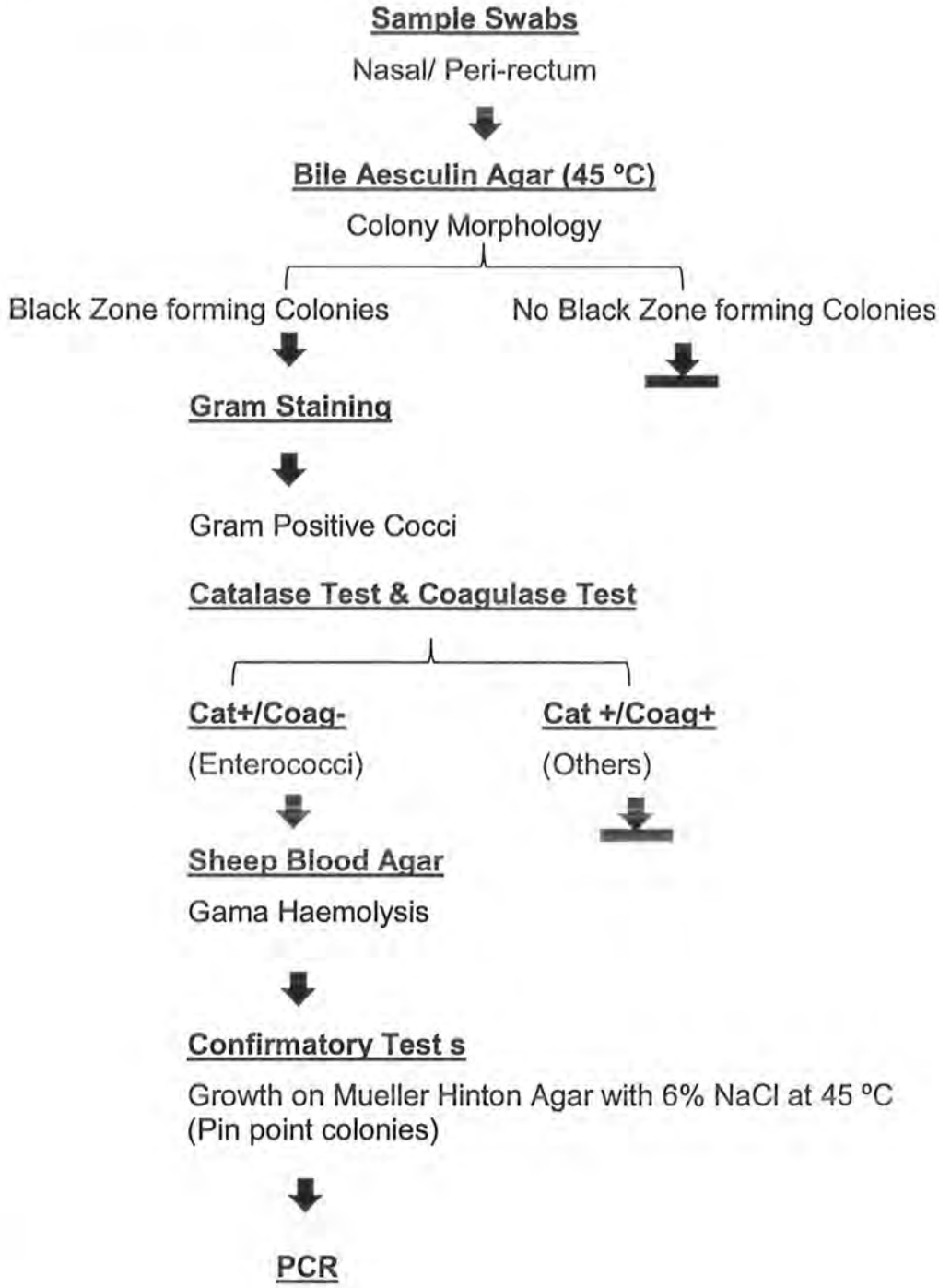
The species level identification was confirmed by amplifying *nuc* gene by PCR which is most commonly targeted for identifying *S. aureus* or MSSA. The MRSA was confirmed by searching methicillin resistance gene with the help of *mecA* primer.

3.1.3.2: Confirmation of enterococci

MHA with 6% NaCl was used as selective medium for the isolation of enterococci. The high salt concentration and facultative aerobic growth at 45°C distinguish this from other organisms.

The specie identification of enterococci (*E. faecalis* and *E. faecium)* was performed by targeting *ddl E. faecalis and E. faecium* genes and VRE was identified with the help of *vanA* and *vanB* primers. The enterococci identification protocols are mentioned in scheme Fig. 3.2.

Fig. 3.2: Enterococci identification scheme



3.2: Polymerase chain reaction (PCR)

3.2.1: Extraction of genomic DNA

Pure culture of *S. aureus* and enterococci were used for molecular analysis of the isolates. Nonviable and mixed cultures were not processed for molecular characterization study. The control strain used for MSSA was SH1000 and for MRSA was MRSA252 while for *E. faecalis* was OG1RF Liv 59 and for *E. faecium* was TX0016 Liv66. All these controlled strains were taken from Lab-H, School of Biological Sciences, University of Liverpool, Liverpool, UK, for research purpose.

DNeasy blood and tissue kit (Qiagen, Germany) was used for the isolation of DNA. The selected isolates were grown over night in BHI broth. Maximum 2×10^9 cells were harvested in eppendrof tubes by centrifuge at 7500rpm for 10 minutes. Bacterial pellets were resuspended in 180 µl of enzymatic lysis buffer (20mM Tris-HCl (Fluka, Germany, pH 8.0), 2 mM Sodium EDTA (BDH Chemicals, UK) 1.2% Tritone X-100 (Sigma)). Freshly prepared lysozyme solution (Sigma) (200 mg/ml) was added to lysis buffer just before use. Rest of the procedure was executed according to manufacturer's instructions. Eppendrof tubes containing extracted DNA were stored at -20°C.

Wizard® Genomic DNA purification kit (Promega Corporation, USA) was also used for DNA extraction. 1 ml of overnight culture was added to a 1.5 ml microcentrifuge tube and centrifuge at 16,000 × g for 2 minutes to pellet the cells and was subjected to series of reactions according to manufacturer's instructions. The isolated DNA was stored at 2-8°C.

Manually, DNA was extracted by using Triton X lysis buffer by the method mentioned by Louie et al. (2002) for DNA extraction from bacterial colonies. In this method for DNA isolation, 1 µl loop full of organisms which were grown on BHI agar was inoculated into 100 µl aliquot of Triton X-100 lysis buffer (100 mM NaCl, 10mM Tris-HCl (Fluka, Germany, pH 8), 1 mM EDTA (BDH Chemicals, UK, pH 9), and 1% Triton X-100 (Sigma) containing 2 µl of a 1 mg/ml solution of Lysostaphin (Sigma) in case of *S. aureus* and Lysozyme (Sigma) 10 mg/ml for enterococci. The suspension

was incubated in a 37°C water bath for 10 minutes and boiled for an additional 10 minutes.

The suspension was cooled at room temperature for 5 minutes and centrifuge at 16,000 X g for 1 minute. Supernatant was separated which is used as template for PCR. Isolated DNA was stored at -4 to -20°C (Louie et al., 2002)

3.2.2: Determination of the quality of extracted DNA

A 10 µl aliquot of each extracted DNA was mixed with 6X DNA loading dye (Fermentas, Canada) and loaded onto the ethidium bromide (final concentration 0.5 µg/ml Sigma Ltd. USA) stained 1% agarose gel (Sigma) in 0.5X Tris-Borate-EDTA (TBE) buffer and run at 100 V for about one hour prior to viewing under Molecular Imager Gel Doc XR+ System, Bio-Rad Laboratories, USA.

3.2.3: Reagents used in PCR

BioMix™, Bioline, UK (BIOTAQ™ DNA Polymerase, 2 mM dNTPs, 32 mM $(NH_4)_2SO_4$, 125 mM Tris-HCl (pH 8.8), 0.02% Tween 20, 3 mM $MgCl_2$, Inert Dye) and Go Tag® Green Master Mix, Promega Corporation, USA (GoTag® DNA Polymerase, Green GoTag® Reaction Buffer (pH 8.5), 400µM dNTPs and 3mM $MgCl_2$) were used as master mix in PCR reaction.

Primers were purchased from Sigma Geno§ys (Sigma-Aldriche, USA), Alpha DNA (Alpha DNA, Germany) and e-Oligo (Gene Link, USA) and dissolved in sterile ultra pure water or nuclease free water provided by the supplier to the required concentration before storing at -20°C. Stock solution of 1000 pmoles/µl of primers was prepared by using nuclease free water. Working solution was diluted 50 times to gave final concentration of 20 pmoles/µl. 0.5 µl of working primer solution was taken to achieved 10 µM (equal to 10 pmoles/µl) concentration of primers required in PCR reaction.

HyperLadder™1 (Bioline, UK) with separation range 200-10000 bp and leading dye color is blue and the high intensity bands are on 1000 and 10000 bp. O'GeneRuler™ 1 Kb and GeneRuler™ 100 bp DNA Ladder (Fermentas, Canada) were also used in the study. O'GeneRuler™ 1 Kb DNA ladder is prepared with six

different plasmids containing pUC, λ phage and yeast genome sequence. Its separation range is 250-10000 bp with orange dye solution. The high intensity bands are on 2000 and 3000 bp. GeneRuler™ 100 bp DNA ladder is a mixture of chromatography-purified individual DNA fragments. Its separation range is 100-1000 bp with orange dye solution. The high intensity bands are on 500 bp. Agarose (Sigma-Aldrich Co. USA) was used for the interpretation and detection of amplified PCR product for gel electrophoresis.

3.2.4: Identification of *S. aureus* (MSSA and MRSA) with PCR

All PCRs were carried out by keeping standard precautions to avoid contamination. These included preparation of reaction mixtures and clinical specimens in two separate places (DNA-free PCR cabinet and DNA-preparation PCR room) and the use of gloves, laboratory coats, face masks, and a negative control (sterilized distilled water/nuclease free water).

All those isolates which showed morphological and biochemical characteristics of *S. aureus* were subjected to PCR for confirmation. The primers used for identification of *S. aureus* were *nucA* for detecting *nuc* gene, coagulase and *coag* primers for detecting coagulase gene and for MRSA, *mecA* primers were used to detect methicillin resistant *mec* gene (Louie et. al., 2000, 2002; Brakstad et al., 1992; Murakami et al., 1991). Each primers pair was tested for amplification specificity annealing temperatures between 50°C to 60°C.

3.2.4.1: PCR with 16S rRNA, staph 16S, *coagulase* gene and *nucA* primer

Brakstad et al., (1992) demonstrated the identification of *S. aureus* by PCR amplification of *nuc* gene. In our study same gene was targeted for identification of *S. aureus.* The PCR reaction mixture was prepared according to concentration mentioned in Table 2 for *mecA* primer. The thermocycling conditions were 94°C for 5 minutes, followed by 30 cycles each of 94°C for 15 seconds, 50°C for 30 seconds, and 72°C for 30 seconds with a final extension at 72°C for 5 minutes (final extension). The PCR tubes were held at 4°C until removed from thermocycler. The thermocyling took almost an hour. The primers along with their product sizes are mentioned in Table 3.1. Similar PCR conditions were applied on the coagulase primers, *coag* primers and staph 16S primers. Genus specific primers 16S rRNA

and staph 16S amplified at 290 bp and 750 bp respectively and species identification were performed by targeting *nuc* and coagulase gene. The *nucA* amplified at 270 bp and two primers used to target coagulase gene were *coag* primer and coagulase primer amplified at 810 bp and 230 bp respectively.

Table 3.1: Oligonucleotides primers used for detection of *S. aureus*

Primer Names	Sequences (5 —▶3)	Company	Product Size (bp)	Reference
16S rRNA (1)	GCGGATCCTGACTGCAGTGCCAGCAGCCGCGGTAA	Alpha DNA/Sigma	292	Relman et al., 1992
16S rRNA (2)	GCGGATCCGCGGCCGCGGACTACCAGGGTATCTAA T			
Staph 16S (1)	GTTATTAGGGAAGAACATATGTG	Alpha DNA/Sigma	750	Relman et al., 1992
Staph 16S (2)	CCACCTTCCTCCGGTTTGTCACC			
***mecA* (1)**	AAA ATCGATGGTAAAGGTTGGC	Sigma/e-Oligo	533	Murakami et al., 1991
***mecA* (2)**	AGT TCTGCAGTACCGGATTTGC			
***nucA* (1)**	GCGATTGATGGTGATACGGTT	Sigma/e-Oligo	270	Brakstad et al., 1992
***nucA* (2)**	AGCCAAGCCTTGACGAACTAAAGC			
***Coag* gene (1)**	CGAGACCAAGATTCAACAAG	Alpha DNA	810	Rallapalli et al., 2008
***Coag* gene (2)**	AAAGAAAACCACTCACATCAGT			
***Coagulase* sense**	GACGACACCGAACCCTATTT	e-Oligo	230	Yanagihara et al., (2006)
***Coagulase* antisense**	CACGGATACCTGTACCAGCA			

bp: base pair

Table 3.2: Composition of PCR reaction mixture

Components	Volume (µL)	Final concentration
Template	2.5	>100 ng
Primers (10 µM) each	1	0.5 µM
PCR Master Mix (2X)	12.5	1X
Nuclease free water	9.0	N.A

Total reaction volume was 25 µL.

3.2.4.2: PCR with *mecA*

Primer sequences are given in Table 3.1. Reaction mixture was prepared by adding 0.5 µl of each two PCR primers *mecA* (1) and *mecA* (2) (0.2 µM final concentration). PCR reaction mixture composition is given in the Table 3.2.

Thermocycling conditions in Biometra T1 Thermocycler, Biometra, Gerrmany was 94°C for 5 minutes followed by 30 cycles each of 94°C for 15 seconds (denaturation step), 52°C for 30 seconds (annealing step), 72°C for 30 seconds (elongation step) and finally 72°C for 7 minutes (final extension). Samples were held at 4°C until run on agarose gel.

3.2.4.3: Multiplex PCR for rapid identification of MRSA

Both the primer *nucA* and *mecA* mentioned in the Table 3.2 was added simultaneously for the rapid identification of MRSA. Conditions for multiplex PCR was the same as that for *mecA* primer while the reaction mixture composition changed accordingly. Total volume of reaction mixture used was 25 µl.

3.2.4.4: Electrophoretic detection and result interpretation of PCR product for MSSA and MRSA

Aliquot (10 µl) of each PCR product was loaded onto 1% agrose (Sigma) stained with ethidium bromide (final concentration 0.5 µg/ml; Sigma Ltd USA). 0.5X Tris-Borate-EDTA (TBE) buffer was used as running buffer in electrophoresis tank. Samples were loaded with marker GeneRuler™ 1 kb DNA Ladder (fermentas) and run at 100 V for about one hour prior to viewing under Molecular Imager Gel Doc

XR+ System, Bio-Rad Laboratories, USA, to check for the presence of PCR product. The product sizes of PCR product are mentioned in the table 3.1, which were obtained by the amplification of the target gene.

Numerous reports for the detection of MRSA by PCR indicate high sensitivity and rapid turnaround time. When interpreting the results of the test, a positive outcome indicates the presence of the *mec* gene. Presence of this gene indicates resistance to methicillin and all ß-lactam antibiotics (Wielders et al., 2001). All the MRSA gave product at 533 bp with *mecA* primers. The expected amplified DNA products were *nucA* (270 bp), *Coag* gene (810 bp) and Coagulase gene (230 bp) from *S. aureus* isolates only (designated as MSSA), while staphylococcal (750 bp) 16S rRNA primer sets gave bands with every staphylococcus isolates. Multiplex PCR using *mecA* and *nucA* primers giving rapid identification of MRSA in clinical samples (Louie et al., 2002).

3.2.5: PCR for identification of *E. faecium* and *E. faecalis* and VRE

3.2.5.1: Amplification with *ddl* primer to identify *E. faecium* and *E. faecalis*

Molecular identification of isolated were performed with *ddl* primers to a detect gene encoding D-alanine-D-alanine ligase specific for *E. faecium* and *E. faecalis* The Primers used for *ddl* gene and vancomycin resistance gene are listed in the Table 3.3, along with their product sizes (Kariyama et al., 2000). All the oligos for enterococci were purchased from Sigma Geno§ys (Sigma Alrdrich, USA), Alpha DNA (Alpha DNA, Germany) and e-Oligo (Gene Link, USA).

3.2.5.2: Amplification with *vanA* and *vanB* to identify VRE

Vancomycin resistance genes were detected by using *vanA* and *vanB* primers. Primers sequences are mentioned in Table 3.3.

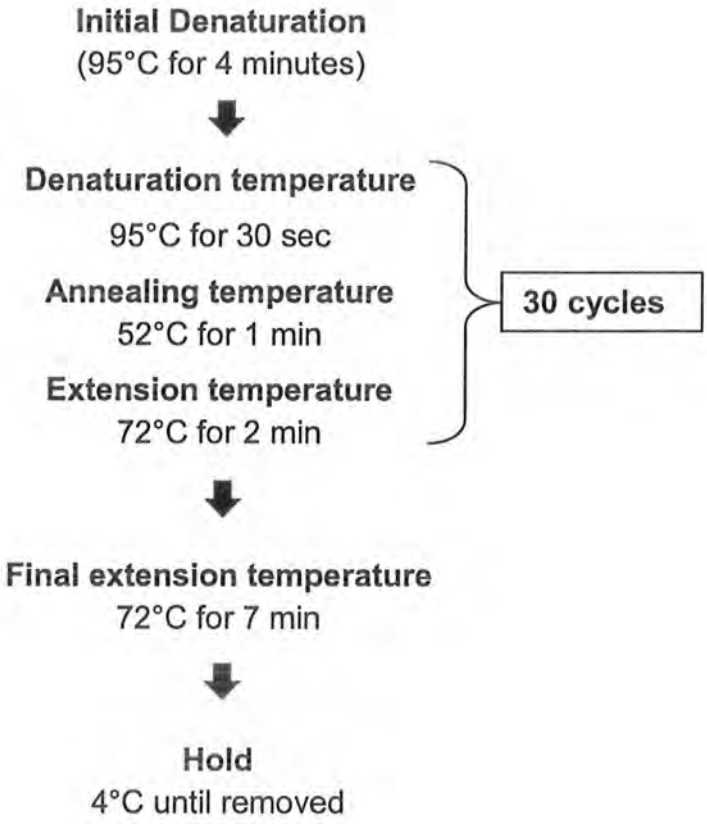
Table 3.3: Oligonucleotides primers used for the detection of enterococci

Primer Designation	Sequences (5 ⟶3)	Company	Product Size (bp)	Reference
E. faecium				
ddl E. faecium(1)	TTGAGGCAGACCAGATTGACG	Alpha DNA/ Sigma	658	Kariyama et al., 2000
ddl E. faecium(2)	TATGACAGCGACTCCGATTCC			
E. faecalis				
ddl E. faecalis(1)	ATCAAGTACAGTTAGTCT	Alpha DNA/ Sigma	941	Kariyama et al., 2000
ddl E. faecalis(2)	ACGATTCAAAGCTAACTG			
vanA (1)	GGGAAAACGACAATTGC	Alpha DNA/ e-Oligo	732	Kariyama et al., 2000
vanA (2)	GTACAATGCGGCCGTTA			
vanB (1)	GTGCTGCGAGATACCACAGA	Alpha DNA/ e-Oligo	635	Kariyama et al., 2000
vanB (2)	CGAACACCATGCAACATTTC			

bp: base pair

Amplification was carried out in Biometra T1 Thermocycler (Biometra, Germany) with conditions mentioned in the amplification protocol in Fig. 3.3.

Fig. 3.3: Amplification protocol



3.2.5.3: Electrophoretic detection and result interpretation of PCR product for VSE and VRE

The PCR products were analyzed on 1% agarose gel. The amplification sizes are mentioned in the Table 6. DNA ladder (Hyperladder 1 and O'Gene Ruler) was used to compare the size of PCR amplified fragments. 1% agarose gel stained with ethidium bromide in a 0.5X Tris-Borate-EDTA buffer in Gel Box (Gibco Brl, Life Technologies USA). Electrophoresis was performed at 100V for one hour and gel was viewed under Molecular Imager Gel Doc XR+ System, Bio-Rad Laboratories, US.

Presence of *ddl E. faecalis* (941 bp) *and E. faecium* (658 bp) confirmed the specie identification of enterococci. The *vanA* gene (732 bp) or *vanB* (635 bp) gene indicates the presence of resistance to vancomycin.

3.2.6: Colony PCR

Colony PCR Protocol contributed by Hancock et al., (2003) was used. Reasonable sized colony (approx. 2-3 mm in diameter) was chosen and resuspended in 100 µl sterilized distilled water. PCR reaction mixture for colony PCR is mentioned in Table 3.4.

Table 3.4: Reaction mixture for colony PCR

Components	Volume (µL)
Colony Suspension	2.5
Primers 1 (10 µM)	0.5
Primers 2 (10 µM)	0.5
Go Taq® Green Master mix (2X conc.)	12.5
Nuclease free water	9.0

Total reaction volume was 25 µL. Sample was overlaid with sterile mineral oil.

Thermocycling conditions in Biometra T1 Thermocycler (Biometra, Germany) for Colony PCR was 96°C for 3 minutes followed by 30 cycles each of 94°C for 40 seconds, 55°C for 40 seconds, 72°C for 1 minute and finally 72°C for 10 minutes.

Samples were held at 4°C until run on agarose gel. The result was seen under Molecular Imager Gel Doc XR+ System, Bio-Rad Laboratories, USA.

3.3: Antimicrobial susceptibility testing by disc diffusion

For all the isolates the antimicrobial susceptibility testing was performed by Kirby-Bauer modified disc diffusion method (Mulder and Farnham, 2001). The direct colony suspension method is the most convenient and recommended method by CLSI for inoculum preparation. Inoculum was prepared by making a direct broth or saline suspension of 24-hours old colonies from sheep blood ager to. The colonies were picked up with the help of sterile wire loop from the surface of agar plate and dipped into sterilized 0.9% normal saline (Otsuka, Pakistan) or sterilized distilled water to make direct-colony suspension of the isolates. Turbidity was matched with 0.5 McFarland standard against a card with a white background and contrasting black lines. Suspensions were then streaked onto MHA plates with the help of sterilized cotton wool swab.

MHA considered best by CLSI for routine susceptibility testing of non-fastidious bacteria because it gave acceptable reproducibility for susceptibility testing, low in sulfonamide, trimethoprim, and tetracycline inhibitors, satisfactory growth of most non-fastidious pathogens on this media and large data collected for this medium concerning susceptibility tests (CLSI, 2006).

Fifteen minutes after adjusting the turbidity, sterile cotton swab was dipped into the inoculum suspension. Swab were rotated several times and press firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. MHA plates were inoculated by streaking the swab over the entire sterile agar surface. Streaking was repeated two or three time approximately 60° each time for even distribution of inoculum. After inoculation the lid was left ajar for few minutes before applying the antibiotics disks.

The antimicrobial susceptibility testing was performed by using the following antibiotics: Amoxicillin/clavulanic acid, ampicillin, cephalexin, cefoxitin, cephalothin, cephradine, ciprofloxacin, erythromycin, gentamicin, imipenem, levofloxacin,

linezolid, methicillin, oxacillin, penicillin g, quinupristin/dalfopristin, teicoplanin, tetracycline and vancomycin (Table 3.5).

Table 3.5: List of antimicrobial discs (Oxoid, UK) used for Disc diffusion assay

S/N	Antimicrobial Agents	Disc Code	Disc Potency (µg)
1	Amoxicillin/clavulanic acid	AMC30	30
2	Ampicillin	AMP25	25
3	Penicillin G	P10	10 units
4	Methicillin	MET10	10
5	Oxacillin	OX1	1
6	Cephalexin	CL30	30
7	Cefoxitin	FOX30	30
8	Cephalothin	KF30	30
9	Cephradine	CE30	30
10	Ciprofloxacin	CIP5	5
11	Levofloxacin	LEV5	5
12	Erythromycin	E15	15
13	Gentamicin	CN30	30
14	Tetracycline	TE30	30
15	Imipenem	IPM10	10
16	Linezolid	LZD30	30
17	Quinupristin/Dalfopristin	QD15	15
18	Teicoplanin	TEC30	30
19	Vancomycin	VA30	30

Table 3.5 describes the name of antimicrobial discs used along with codes and potencies used for antimicrobial susceptibility testing. The plated were then incubated at 35°C for 24 hours. The disc diffusion technique and zone interpretation of each antimicrobial agent was used in accordance with CLSI guidelines (CLSI, 2007).

All plates screening oxacillin/methicillin resistance were examined after complete 24 hours to avoid the hetero-resistance, in this cells expressing resistance may grow more slowly than the susceptible population and may be missed at temperatures above 35°C. This phenomenon occurs in staphylococci resistant to penicillinase-stable penicillins, such as oxacillin. All cells in a culture may carry the genetic information for resistance, but only few may express the resistance *in vitro*. Thus accurate detection of oxacillin/methicillin resistance is difficult due to coexistence of both susceptible and resistant colonies within a culture of staphylococci (Bannerman, 2003). That is why CLSI recommends incubation of isolates being tested against oxacillin, methicillin, or nafcillin at 33-35°C (maximum of 35°C) for a full 24 hours before reading (CLSI, 2007). The results were interpreted according to NCCLS guidelines for oxacillin susceptibility testing: a zone size of ≤10 mm was considered resistant; a zone size of ≥13 mm was considered susceptible. Methicillin resistant *S. aureus 252* (MRSA252) was used as control for the antimicrobial susceptibility pattern.

3.3.1 Turbidity standard equivalent to 0.5 McFarland

McFarland is a turbidity standard against which the turbidity of the test and control inocula was compared. McFarland 0.5 standard is comparable to a bacterial suspension of 10^8 cfu/ml. The 0.5 McFarland was prepared by adding 0.5 ml of a 1.175% (w/v) barium chloride dihydrate ($Bacl_2.2H_2O$) solution to 99.5 ml of 1% (v/v) sulfuric acid. The accuracy of the density of a prepared McFarland standard was checked by using a spectrophotometer with a 1 cm light path.

A small volume of the turbid solution was transferred to a screw-cap bottles of the same type as used for preparing the test and control inocula. Later it was stored in a well-sealed container in a dark place at room temperature (20-28°C). The standard

was kept up to 6 months. The standard was always used after shaking immediately before use against a card with a white background and contrasting black lines.

3.4. Antimicrobial susceptibility testing by minimum inhibitory concentrations (MIC)

BSAC, (2009) methods for antimicrobial susceptibility testing was followed for determining MICs. Standard antibiotic powders of ciprofloxacin, oxacillin, tetracycline and vancomycin were obtained from MP Biomedicals UK. Stock solutions were prepared by following the manufacturer's recommendations. Stock solutions were freezed and thawed only once and then discarded.

Range of antibiotic concentrations used was from 0 mg/L to 512 mg/L. The calculation for the stock solution was made by the following equation:

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

Where P = potency given by the manufacturer (µg/mg), V = volume required (mL), C = final concentration of solution (multiples of 1000) (mg/L), and W = weight of antibiotic in mg to be dissolved in volume V (mL).

For example, $\frac{1000}{980} \times 10 \times 10 = 102.04$ mg Powder

If a powder with a potency 980 µg/mg, dissolved in 10 mL of solvent will give 10,000 mg/L stock solution. As Microbial contaminations of powders are extremely rare so this can be ignored. All stock solutions were prepared from the initial 10,000 mg/L solution. For 1000 mg/L, 1 mL of 10,000 mg/L solution was added in 9 mL solvent. Similarly 100 µl of 10,000 mg/L solution when added in 9.9 mL solvent give 100 mg/L.

Dilution range 0- 512 mg/L was prepared for all antibiotics. All dilution was prepared in universal containers having labels of the appropriate dilutions as 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0 mg/L. From the 10,000 mg/L stock, dispense the following amounts with a micropipette: 1024 µl for container labelled as 512, 512 µl into the container labelled as 256, 256 µl into the container labelled 128, 128 µl into the container labelled 64, 64 µl into the container labelled 32, 32 µl into the

container labelled 16. From the 1000 mg/L stock, 160 µl was dispensed into the container labelled 8, 80 µl into the container labelled 4, 40 µl into the container labelled 2. From the stock 100 mg/L 200 µl was dispensed into the bottle labelled 1, 100 µl into the container labelled 0.5, 50 µl into the container labelled 0.25. No antibiotic is added to the bottle labelled 0 mg/L (antibiotic free growth control).

MHA was prepared following the manufacturer's instructions. Measured volume of 20 ml of cooled molten agar was added to each container, including antibiotic free control container. It was ensured that the medium was cooled to 50°C before adding the antibiotic. Medium was mixed well before pouring into Petri dishes, marked with each concentration. Petri dishes were allowed to set and then let the surface dry for 10-15 minutes in drying cabinet. Plates were than stored at 4-8°C protected from light if not inoculated. Preferably the plates were used on the day of preparation.

The inoculum was adjusted so that 10^4 cfu/spot should apply to the plates. Desired inoculum was prepared in suspension by picking few morphologically similar colonies with the help of sterile loop and suspended in sterilized distilled water. Turbidity was compared equal to or greater than the 0.5 McFarland standard. The suspensions were used within 30 min of preparation.

With the help of micropipette, 1-2 µl of suspension was dropped on to the surface of the agar and allowed absorbed into the agar before incubation. Staphylococci (other than tests on methicillin/oxacillin) was incubated at 35-37°C in air for 18-20 hours while staphylococci tests on methicillin/oxacillin was incubated at 30°C in air for 24 hours. Enterococci were given 35-37°C in air for 18-20 hours.

3.4.1. Reading and interpretation

Control isolates were grown on antibiotic-free plate. As MIC is the lowest concentration of antibiotic at which there is no visible growth of the organism so the growth of one or two colonies or a fine film of growth was disregarded (Andrews, 2001).

3.5. Storage of *S. aureus* and enterococci isolates

3.5.1. 16% Glycerol Broth

All confirmed *S. aureus* and enterococci isolates were stored in 16% v/v glycerol broth at -70°C. The glycerol broth was prepared by adding 16 ml glycerol in 84 ml nutrient broth and dispensed 5 ml amount in screw-cap bottles. These bottles were then sterilized by autoclaving at 120°C for 15 minutes. The pH of the medium ranged from 7.2-7.6 at room temperature. Using a sterile swab, the entire growth of an overnight pure culture of was sub-cultured in 5 ml of sterile glycerol broth and immediately stored in freezer at -70°C. After 24 hours, the viability of the organism was checked by thawing the suspension at 35°C and inoculated on Sheep Blood agar plates.

3.5.2. Microbank

S. aureus and enterococci isolates were also stored in Microbank at -70°C. Microbank® Bacterial and Fungal Preservation System (Pro-Lab Diagnostics, US) is a sterile vial containing porous beads which serve as carriers to support microorganisms. Individual colored beads are packaged approximately 25 beads in a cryovial containing cryopreservative liquid. The beads are washed and are of a porous nature allowing microorganisms to readily adhere onto the bead surface. After inoculation the cryovials were kept at -70°C for extended storage. After 24 hours, viability was checked by taking out a single bead out of the vial and directly inoculated on MHA for enterococci and sheep blood agar for *S. aureus* and incubated at 35°C for 24 hours.

3.6. Clinical data

Patient's data including age, gender, marital status, date of admission, ward, residence and socioeconomic status, clinical diagnosis, history of vancomycin intake, surgical interventions, invasive procedure and devices, current medication profile was collected from the hospital record. Age of the patients was grouped into six categories <1 year, 1 to < 6 years, 6 to <12 years, 12 to < 18 years, 18 to < 35 years and > 35 years. Gender was grouped into male, female, male child and female child according to the age categories. The patients with age more than 12

years were considered adult. On the bases of specimen collected from ward they were assigned reference numbers. The specimens were collected from patients admitted in medical intensive care units (MICU) and paeds intensive care units (PICU).

The patients admitted reasons or diagnosis were grouped into seven categories. Disease which appeared in more than five patients was given individual category while all other reasons or diagnosis were included in category named "Others". Factors including invasive devices, surgical interventions and poor hygiene were focused during the study. Invasive devices and antibiotic medication were grouped into 7 and 14 categories respectively according to their usage frequency. Increased and irrational use of antibiotics especially vancomycin for the treatment of common infection was strongly focused for MRSA and VRE study. Low socioeconomic status and poor living was highlighted in the study. Cross questioning was made from the patients as well as from the attendants of the patients regarding family history.

3.7. Statistical analysis

The statistical package for social sciences (SPSS) version 13.0 for windows system on personal computer (PC) was used for data interpretation and statistical analysis. The statistical analysis was made by average, ±standard deviation, chi-square, and one way ANOVA was applied. The p value ≤ 0.05 was considered as "statistical significant".

RESULTS

RESULTS

4.1: Isolation and identification of *S. aureus* and enterococci

4.1.1: Specimen collection and inoculation

A total of 432 specimens were collected from 216 patients (02 samples/patient) admitted during Dec. 2007 to March 2009 in MICUs and PICUs of RMC and Allied Teaching Hospitals, Rawalpindi, Pakistan and processed for identification of staphylococci and enterococci.

4.1.2: Isolation and identification of isolates

4.1.2.1: Staphylococci

The Result on MSA media brought out that 117 nasal and 68 peri-rectal isolates gave yellow zone forming colonies. Of these 117 nasal staphylococci, 80 (68.4%) showed β-haemolysis on SBA and 68 (58.1%) gave positive coagulase and DNase test. All these nasal staphylococci were Gram positive and catalase positive. The other 68 peri-rectal staphylococci demonstrated β-haemolysis in 28 (41.2%) isolates, 14 (20.6%) isolates showed positive coagulase and DNase test. All these peri-rectal staphylococci were Gram positive and catalase positive. Table 4.1 shows the number and percentages of MSA positive nasal and peri-rectal staphylococci along with different biochemical test.

Novobiocin susceptibility was done with all yellow zone forming colonies which were also positive for coagulase test. 68 (100%) nasal and 14 (100%) peri-rectal isolates showed sensitivity with novobiocin disc. The susceptibility of novobiocin helps in discriminating between *S. aureus* and *S. saprophyticus*. Sensitivity with novobiocin predicts presence of *S. aureus*. All nasal and peri-rectal *S. aureus* were confirmed further with API kit (Fig. 4.1).

Table 4.1: Biochemical characteristics of MSA-positive staphylococci isolates

			Nasal samples		Peri-rectal samples	
Biochemical Tests			Y-Zone (n = 117)	RP-Zone (n = 53)	Y- Zone (n = 68)	RP-Zone (n = 52)
Hae-SBA	NBH	N (%)	37 (31.6)	42 (79.2)	40 (58.8)	45 (86.5)
	BH	N (%)	80 (68.4)	11 (20.8)	28 (41.2)	7 (13.5)
Gram-positive cocci in clusters	Yes	N (%)	117 (100)	53 (100)	68 (100)	52 (100)
Catalase Test	Positive	N (%)	117 (100)	53 (100)	68 (100)	52 (100)
Coagulase Test	Positive	N (%)	68 (58.1)	_____	14 (20.6)	_____
	Negative	N (%)	49 (41.9)	53 (100)	54 (79.4)	52 (100)
DNAse Test	Positive	N (%)	68 (58.1)	_____	14 (20.6)	_____
	Week Positive/ Negative	N (%)	49 (41.9)	53 (100)	54 (79.4)	52 (100)
NS	Sensitive	N (%)	68 (100)	_____	14 (100)	_____
API test	Confirmed *S. aureus*	N (%)	68 (100)	_____	14 (100)	_____

BH: Beta Haemolysis, Hae-SBA: Haemolysis on Sheep Blood Agar, MSA: Mannitol Salt Agar, NBH: No Beta Haemolysis, N: Numbers, NS: Novobiocin Susceptibility, RP-Zone: Reddish pink Zone, Y-Zone: Yellow Zone.

Fig. 4.1: API-Staph test

1: Control strain MRSA 252 with API code 6736113.

2 to 5: Isolate # N6, N22, N44 and N85 with API code 6736153.

4.1.2.2: Enterococci

Of the total 432 samples from 216 patients admitted in RMC and Allied Teaching Hospitals, Rawalpindi, were screened for nasal and peri-rectal enterococci. Out of 216 nasal samples 56 (25.9%) showed growth of enterococci on BAA plate while 160 (74.1%) gave negative result or no enterococcal growth. Of 56 nasal isolates, 05 were VRE (03 *E. faecium* and 02 *E. faecalis)* and 51 were VSE (28 *E. faecium* and 23 *E. faecalis*). All 56 nasal isolates gave same negative result for catalase test and coagulase test whereas all were Gram-positive with gama haemolysis on SBA and gave growth on MHA with 6% NaCl at 45°C.

Similarly out of 216 peri-rectal samples, 103 (47.7%) showed typical enterococci morphology on BAA plates and 113 (52.3%) gave negative result or no enterococcal growth. Of these 103 isolates, 10 were VRE (05 *E. faecium* and 05 *E. faecalis*) and 93 were VSE (43 *E. faecium* and 50 *E. faecalis*). All 103 peri-rectal isolates were Gram positive, catalase negative, coagulase negative and all gave same distinct growth on MHA with 6% NaCl at 45 °C with gama haemolysis on SBA (Table 4.2). Appendix Fig. A-1a, A-1b and A-1c show the colony morphology of staphylococci on SBA, MSA and enterococci on BAA media.

Table 4.2: Biochemical characteristics of BAA positive *Enterococci* from Nasal and peri-rectum

			Enterococci from Nasal cavity (n=56)				*Enterococci* from Peri-Rectum (n=103)			
			VRE-FM	VRE-FS	VSE-FS	VSE-FM	VRE-FM	VRE-FS	VSE-FS	VSE-FM
Biochemical Tests										
Gram Staining	G+	N	3	2	23	28	5	5	50	43
		%	5.4	3.6	41.1	50	4.9	4.9	48.5	41.7
Cat- Test	Neg	N	3	2	23	28	5	5	50	43
		%	5.4	3.6	41.1	50	4.9	4.9	48.5	41.7
Coag. Test	Neg	N	3	2	23	28	5	5	50	43
		%	5.4	3.6	41.1	50	4.9	4.9	48.5	41.7
CT-MHA	PPC	N	3	2	23	28	5	5	50	43
		%	5.4	3.6	41.1	50	4.9	4.9	48.5	41.7

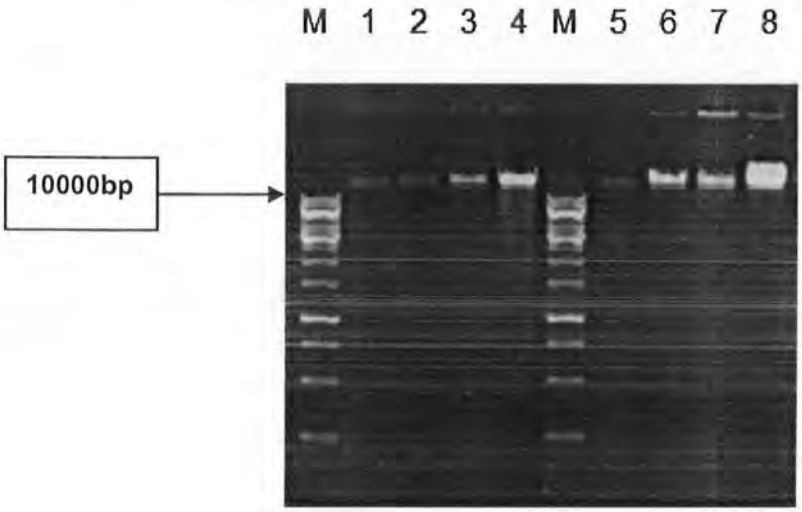
Cat: Catalase, Coag: Coagulase, CT-MHA: Confirmatory test on Muller Hinton Ager with 6% NaCl, G+: Gram Positive, PPC: Pin Point Colonies, N: Numbers, Neg: Negative, VRE-FM: VRE- faecium, VRE-FS: VRE- faecalis, VSE-FM: VSE- faecium, VSE-FS: VSE- faecalis.

4.2: Polymerase chain reaction (PCR)

4.2.1: Genomic DNA

The size of the purified extracted genomic DNA was in range of 10000 bp to 150000 bp when visualized on 1% agarose gel, mixed with 6 X DNA loading dye in 0.5X Tris-Borate-EDTA (TBE) buffer and run at 100 V (Fig. 4.2). HyperLadder-1 (Bioline) (separation range 200-10000 bp) and GeneRule 1 Kb (Fermentas) (separation range 250-10000 bp) were used to measure the size of PCR amplified fragments (Fig. 4.3).

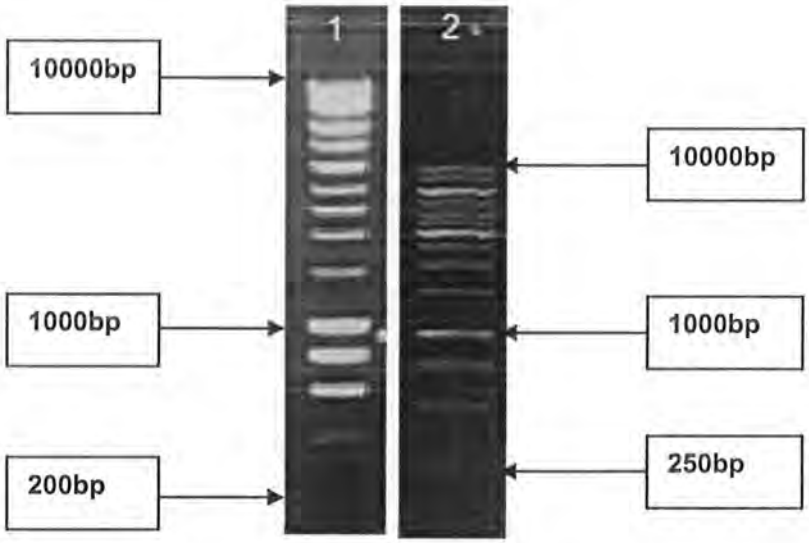
Fig. 4.2: Agarose gel showing isolated genomic DNA by genomic DNA purification kit



M: 1 kb GeneRuler, L1-L4: *S. aureus* isolatess (L1: MRSA 252, L2: N6S, L3: N10S, L4: N22S), L5-L8: Enterococci isolatess (L5: P18E, L6: N40E, L7: N82E, L8: N139E).

M: DNA Marker, **L**: Lane

Fig. 4.3: DNA markers used in PCR



L1: Hyperledder-1, L2: GeneRuer 1Kb.

4.2.2: Identification of *S. aureus* (MSSA and MRSA) with PCR

PCR was conducted under standard working precautions. 16S rRNA, staph 16S, coagulase, *coag* and *nucA*, primers were used for detection of *S. aureu* or MSSA while *mecA* primers were used for detecting methicillin resistant gene. The SH1000 was used as control strain for MSSA and MRSA 252 was used as control strain for MRSA.

Genotypic analysis confirmed 82 isolates of *S. aureus*, 68 (31.5%) found out of 216 nasal samples and similarly 14 (6.5%) out of 216 peri-rectal samples (Table 4.2). Out of these 68 nasal *S. aureus,* 36 (16.7%) gave amplification with *mecA* primer and 32 (14.8%) did not. Thus total 36 isolates from nasal cavity were MRSA and 32 were MSSA. Similarly out of 14 peri-rectal *S. aureus*, 11 (5.1%) were MRSA and 03 (1.4%) were MSSA (Table 4.3).

As primer length and sequence is important in determining the parameters for successful amplification so primers were optimized by varying the annealing temperature 5°C below the lowest Tm of pair of primers (Innis et al., 1990). Fig. 4.4a and 4.4b shows the optimization of primers with control strains MRSA 252 and SH1000. Fig. 4.5, 4.6 and 4.7 shows the PCR amplification of different isolates of

staphylococci with 16S rRNA, staph 16S, coagulase and *nucA* primers. Isolates giving bands with *nucA* primer are shown in appendix-B (Fig. B-1 to B-10).

Table 4.3: PCR results for nasal and peri-rectal isolates of staphylococci

	Positive	Negative
	N (%)	N (%)
Isolates type and primers		
Nasal staphylococci		
*mec*A	36 (52.9)	32 (47.1)
*nuc*A	68 (100)	_____
Coag	68 (100)	_____
Peri-rectal staphylococci		
*mec*A	11 (78.6)	3 (21.4)
*nuc*A	14 (100)	_____
Coag	14 (100)	_____

N: Numbers, PCR: Polymerase Chain Reaction

Fig. 4.4a: Agarose gel electrophoresis patterns showing PCR amplification products of SH1000 with 16S rRNA and staph 16S primers

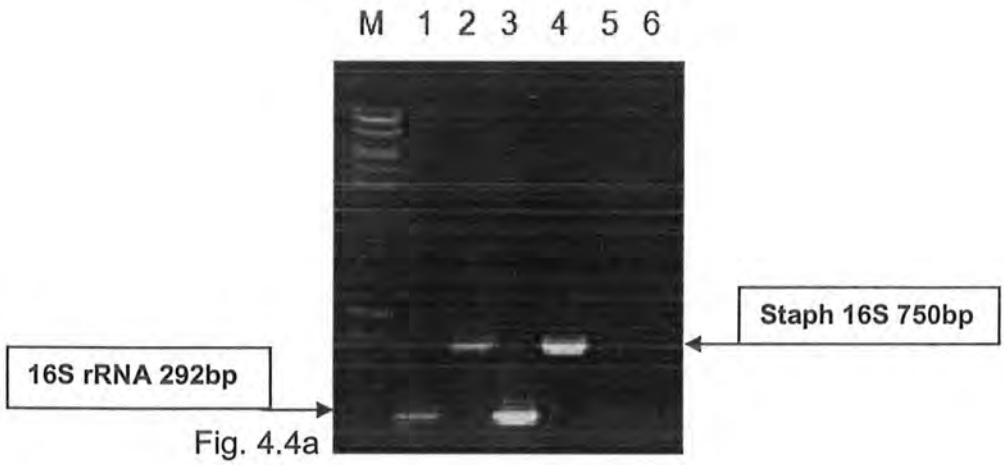


Fig. 4.4a

M: Hyper ladder 1, L1: SH1000+16S rRNA, L2: SH1000+Staph 16S, L3: N6S+16S rRNA, L4: N6S+ Staph 16S, L5: Negative Control (H2O)+16S rRNA, L6: Negative Control (H2O)+ Staph 16S.

Fig. 4.4b: **Agarose gel electrophoresis patterns showing PCR amplification products of MRSA 252 with *nucA* and *mecA* primer**

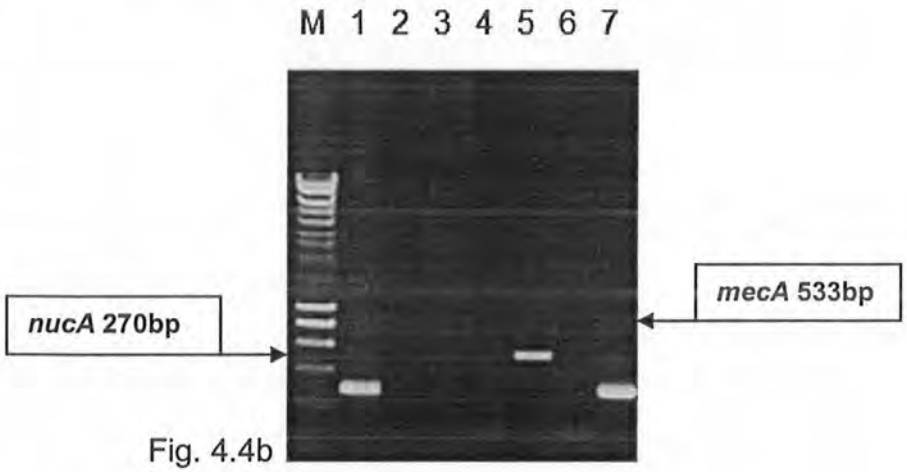
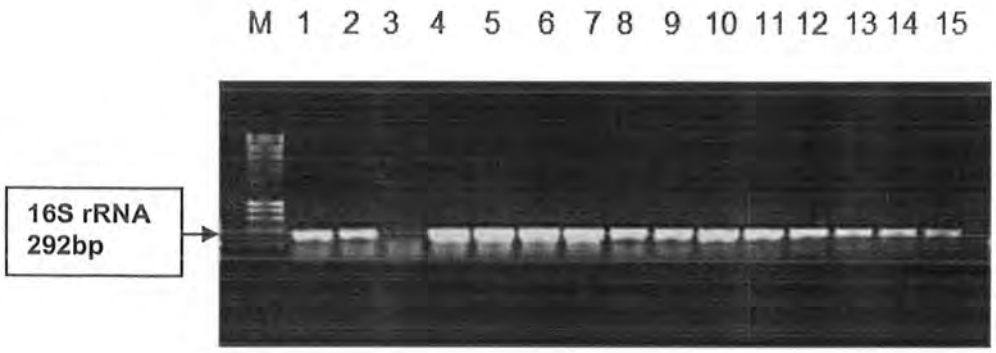


Fig. 4.4b

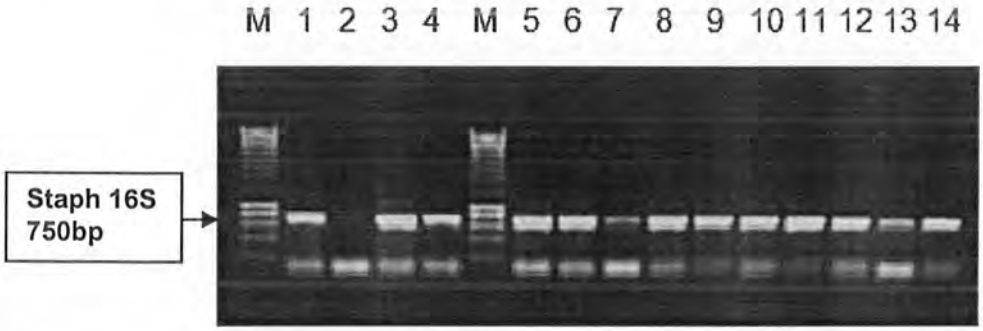
M: Hyper ladder 1, L1: SH1000+nucA, L2: Negative Control (H2O)+nucA, L3: SH1000+mecA, L4: Negative Control (H2O)+mecA, L5: MRSA 252+mecA, L6: Negative Control (H2O)+mecA, L7: MRSA 252+nucA.

Fig. 4.5: Agarose gel analysis of the 292-bp 16S rRNA amplicons from various *S. aureus* isolates



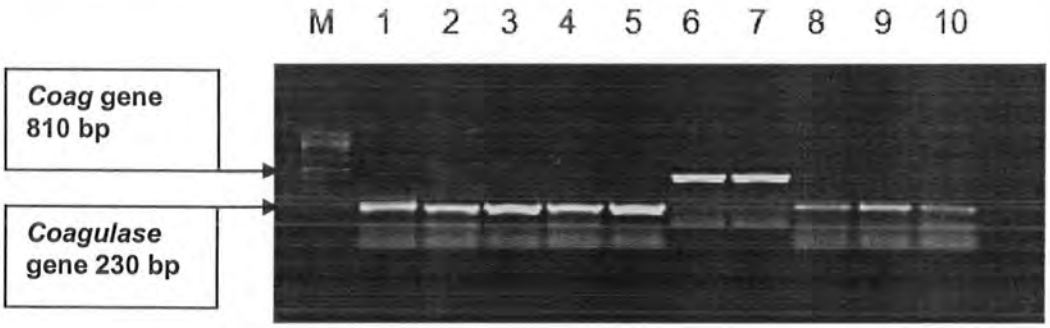
M: 1 Kb DNA Marker (Hyper ladder 1), L1: SH1000, L2: MRSA 252, L3: Negative Control (SD water), L4: N1S, L5: N3S, L6: N6S, L7: N10S, L8: N22S, L9: P22S, L10: P26S, L11: P27S, L12: N38S, L14: P40S, L15: N44S.

Fig. 4.6: Agarose gel analysis of 750-bp Staph 16S amplicons from various *S. aureus* isolates



M: 1 Kb DNA Marker (Hyper ladder 1), L1: SH1000, L2: Negative Control (SD Water), L3: N6S, L4: N10S, L5: N51S, L6: N52S, L7: N54S L8: N57S, L9: N60S, L10: N67S, L11: N70S, L12: N71S, L13: P71S, L14: N73S.

Fig. 4.7: Detection of *S. aureus* by coag and coagulase PCR



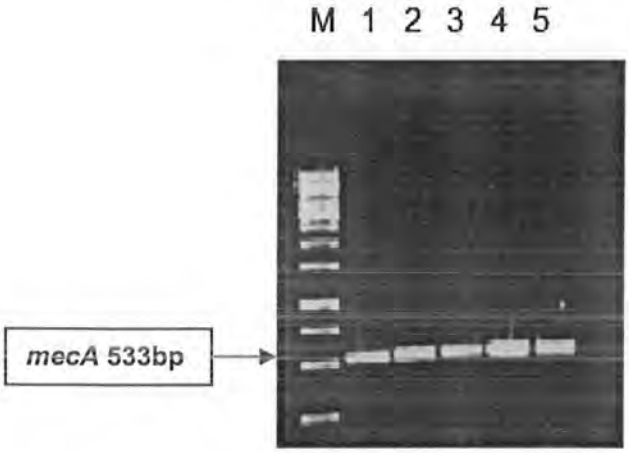
M: 1 Kb DNA Marker (GeneRuler), Coag Primer: L1-5 and L8-10 (L1: SH100, L2: N6S, L3: N10S, L4: N22S, L5: P22S, L8: P99S, L9: N101S, L10: P109S), Coagulase primer: L6-7 (L6: P40S, L7: N44S).

4.2.3: PCR with *mecA*

The *mecA* primers gave amplification at 533 bp and all those isolates of *S. aureus* which amplified with *mecA* primer, were confirmed as MRSA. MRSA 252 was used as control in this experiment (Fig. 4.8, 4.9, appendix Fig. C-1 to C-3).

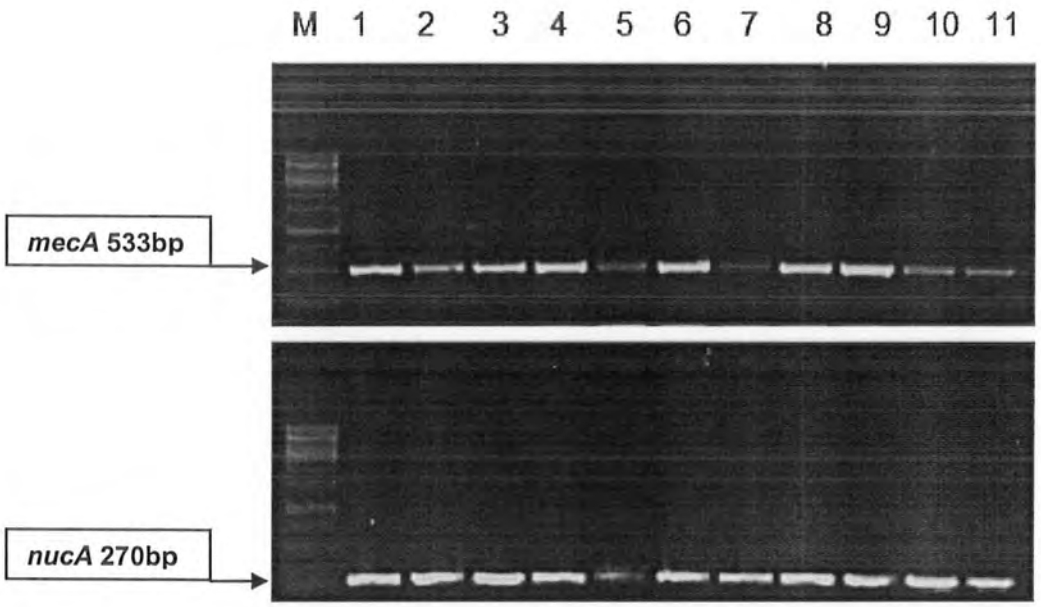
Fig. 4.9 showing same isolates amplified for both *mecA* and *nuc*A primers, while Fig. 4.8 and Fig. C-1, C-2 and C-3 shows all confirmed MRSA isolates which gave band with *mecA* primer. Fig. C-4 shows the PCR with different isolates for identification of MRSA.

Fig. 4.8: Detection of MRSA by *mecA* PCR



M: 1 Kb DNA Marker (GeneRuler), L1: MRSA 252, L2: N5S, L3: N6S, L4: N10S, L5: N22S.

Fig. 4.9: Detection of MRSA by *mecA* and *nucA* PCR

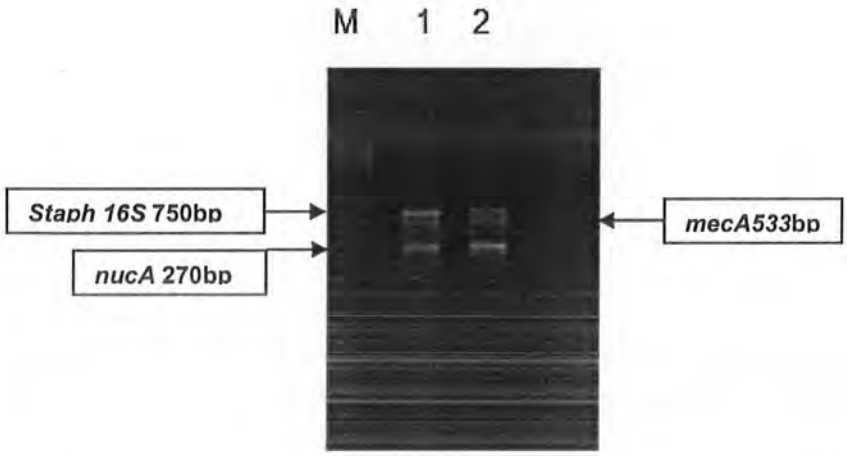


M: 1 Kb DNA Marker (GeneRuler), L1: MRSA 252, L2: P40S, L3: N44S, L4: N51S, L5: N52S, L6: N54S, L7: N57S, L8: N60S, L9: P67S, L10: N71S, L11: P71S.

4.2.4: Multiplex PCR for rapid identification of MRSA

All confirmed *S. aureus* isolates were subjected to multiplex PCR with three primers staph 16S, *nuc*A, *mec*A and MRSA 252 was used as control strain. Result of multiplex PCR was correlated with simple PCR result. Fig. 4.10, 4.11b and 4.12 shows result of multiplex PCR with different primer combinations.

Fig. 4.10: Agarose gel showing PCR-amplified products of the staph 16S, *nucA* and *mecA* genes



M: GeneRuler 1 Kb, L1: MRSA 252 (46 °C Annealing Temp.), L2: MRSA 252 (48 °C Annealing Temp.).

Fig. 4.11a&b: Agarose gel showing PCR-amplified products of *mecA* and *nucA* genes

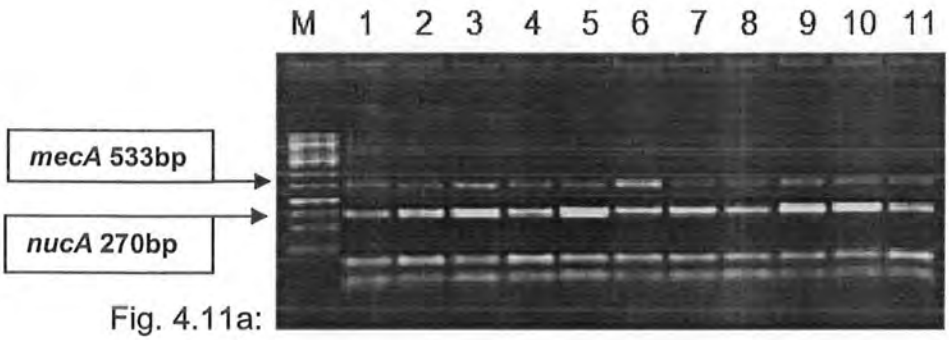


Fig. 4.11a:

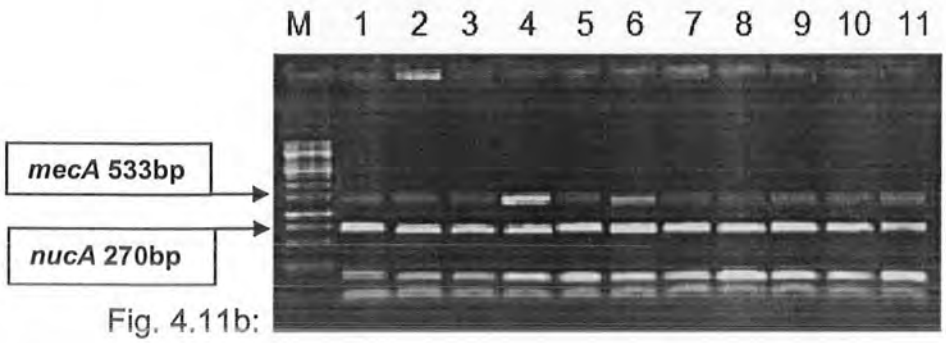
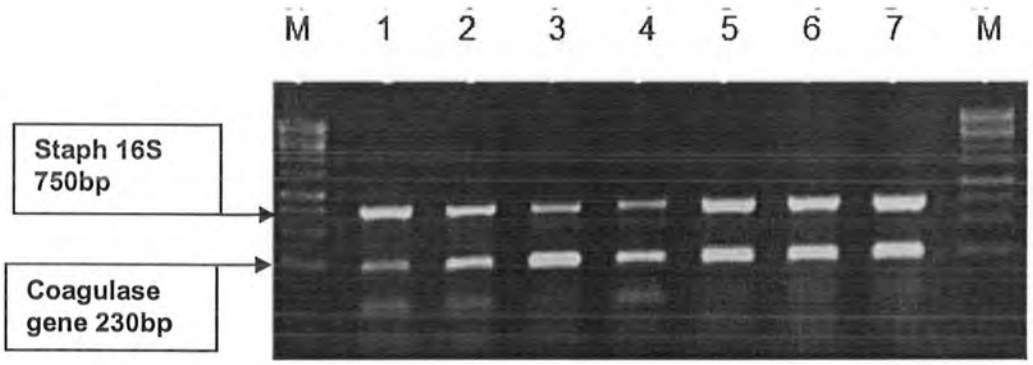


Fig. 4.11b:

4.11a: M: 1 Kb DNA Marker (GeneRuler), L1: MRSA 252, L2: N6S, L3: N22S, L4: P40S, L5: N44S, L6: N83S, L7: N85S, L8: N86S, L9: P90S, L10: N91S, L11: N93S.

4.11b: M: 1 Kb DNA Marker (GeneRuler), L1: N101S, L2: N102S, L3: N105S, L4: P109S, L5: P110S, L6: N110S, L7: N113S, L8: P133S, L9: N158S, L10: N177S, L11: N178S.

Fig. 4.12: Agarose gel showing PCR-amplified product of staph 16S and *coagulase* gene



M: 1 Kb DNA Marker (GeneRuler), L1: SH1000, L2: N6S, L3: N10S, L4: N22S, L5: P22S, L6: P40S, L7: N44S.

4.2.5: Identification of *E. faecium* and *E. faecalis* and VRE with PCR

Total 78 isolates gave amplicon of 941 bp with *ddl E. faecalis,* of which 30 (53.6%) from nasal samples and 48 (46.6%) from peri-rectal samples were identified as *E. faecalis.* 81 enterococci isolates gave amplification product of size 658 bp with *ddl E. faecium*, 26 (46.4%) from nasal and 55 (53.4%) from peri-rectum.

Fig. 4.13 and 4.14 shows the amplicones of *ddl E. faecalis* and *ddl E. faecium* at 941 bp and 658 bp on 1% agarose gel respectively. Fig. D-1 to D-3 (Appendix-D) shows PCR with nasal and peri-rectal isolates of enterococci for identification of *E. faecalis* while Fig. E-1 to E-3 (Appendix-E) shows amplified product of *ddl E. faecium* used to identify *E. faecium.* OG1RF Liv59 was used as control strain for *E. faecalis and* TX00016 Liv66 was the control strain for *E. faecium.*

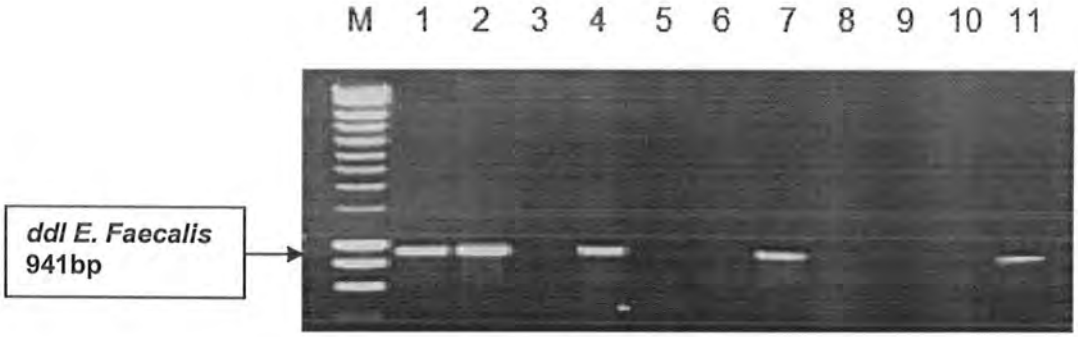
Out of total 159 confirmed *E. faecalis* and *E. faecium,* 15 isolates gave band for *vanA* gene (732 bp), 05 (8.9%) from nasal and 10 (9.7%) from peri-rectum. None of the isolate gave band for *vanB* (635 bp). Over all percentage of VRE were 2.3% and 4.6% while VSE were 23.6% and 43.1% from nasal and peri-rectal samples respectively (Table 4.4). Fig. 4.15 shows amplicon of the confirmed VRE isolates on 1% agarose gel. Fig. F-1 and F-2 (Appendix-F) showing some VRE isolates amplifying vanA gene.

Table 4.4: PCR result for nasal and peri-rectal isolates of enterococci

	Positive N (%)	Negative N (%)
Isolates type and primers		
Nasal enterococci		
ddl E. faecium	26 (46.4)	30 (53.6)
ddl E. faecalis	30 (53.6)	26 (46.4)
vanA	5 (8.9)	51 (91.1)
vanB	———	56 (100)
Peri-rectal enterococci		
ddl E. faecium	55 (53.4)	48 (46.6)
ddl E. faecalis	48 (46.6)	55 (53.4)
vanA	10 (9.7)	93 (90.3)
vanB	———	103 (100)

N: Numbers, PCR: Polymerase chain reaction

Fig. 4.13: Detection of *E. faecalis* by *ddl E. faecalis* PCR

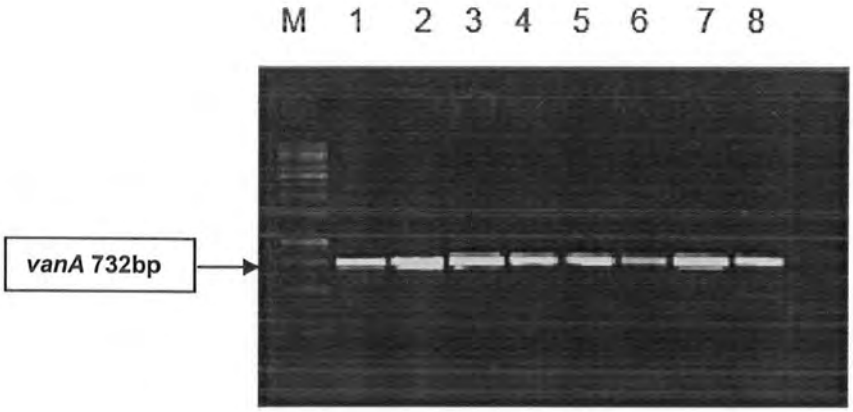


M: 1 Kb DNA Marker (Hyper ladder 1), L1: OG1RF Liv59, L2: P22E, L3: P25E, L4: P26E, L5: N26E: L6: N27E, L7: P27E, L8: P29E, L9: N32E, L10:P31E, L11: OG1RF Liv59

Fig. 4.14: Detection of *E. faecium* by *ddl E. faecium* PCR

M: 1 Kb DNA Marker (GeneRuler), L1: TX00016 Liv66, L2: N169E, L3: P70E, L4: P171E, L5: P174E, L6: P177E, L7: N179E, L8: N180E, L9: P195E, L10: N198E, L11: P198E, L12: P196E, L13: NIL

Fig. 4.15: Detection of VRE by *vanA* gene PCR

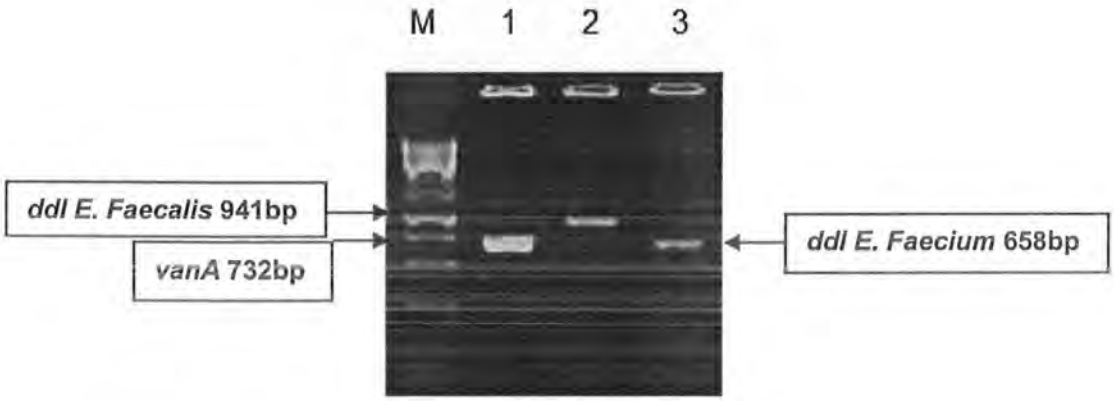


M: 1 Kb DNA Marker (GeneRuler), L1: N40E, L2: N82E, L3: P108E, L4: P128E, L5: N139E, L6: P139E, L7: N145E, L8: P145E, L9: P149E, L10: P130E.

4.2.6: Colony PCR

Colony PCR was applied on all enterococcal isolates positive for simple PCR for quick identification and to bypass the laborious DNA extraction method. All isolates gave positive result with colony PCR. Fig. 4.16 shows amplified products of colony PCR with enterococci isolates. L1 shows peri-rectal isolate of enterococci P18E amplifying *van*A gene, L2 and L3 showed control strain of enterococci, OG1RF Liv59 and TX00016 Liv66 amplifying *ddl* gene respectively.

Fig. 4.16: Agarose gel showing Colony PCR-amplified products of *ddl E. faecalis, ddl E. faecium* and *vanA* gene



M: 1 Kb DNA Marker, L1: P18E+ *vanA*, L2: OG1RF Liv59+*ddl E. faecalis*, L3: TX00016 Liv66+*ddl E. faecium.*

4.3: Antimicrobial susceptibility pattern by disc diffusion

4.3.1: Antibiotic susceptibility pattern of MSSA and MRSA

The comparative study of antibiotic susceptibility pattern of MSSA and MRSA from nasal and peri-rectum, pointed out that MRSA isolates were more resistant to antibiotics than MSSA isolates. All MSSA were resistant to ampicillin and 96.9% were resistant to penicillin G in case of nasal isolates while in peri-rectal MSSA all isolates were resistant to ampicillin, penicillin G and tetracycline. Majority of MRSA were resistant to most of the antibiotics used by disc diffusion except linezolid, quinupristin/dalfopristin and vancomycin both in nasal and peri-rectal isolates. MRSA nasal isolates showed 97.2% resistance to amoxicillin/clavulanic acid, ciprofloxacin and levofloxacin, 94.4% to erythromycin, 91.7% to gentamicin, 86.1% to tetracycline and 77.8% to imipenem. MRSA peri-rectal isolates showed 90.9% to ciprofloxacin, gentamicin and levofloxacin, 81.8% to erythromycin and tetracycline, 63.6% against amoxicillin/clavulanic acid and 45.5% against imipenem. Table 4.5 shows comparison of percentage resistant of MRSA and MSSA.

Table 4.5: Antibiotic resistance profile of MSSA and MRSA (nasal and peri-rectal) isolates

	MSSA in nasal cavity (n=32)		MSSA in peri-rectum (n=3)		MRSA in nasal cavity (n=36)		MRSA in peri-rectum (n=11)	
	% S	% R	% S	% R	% S	% R	% S	% R
AMC30	100	0	66.7	33.3	2.8	97.2	36.4	63.6
AMP25	0	100	0	100	0	100	0	100
CL30	93.8	6.3	66.7	33.3	0	100	0	100
FOX30	100	0	66.7	33.3	0	100	0	100
KF30	100	0	66.7	33.3	0	100	100	100
CE30	100	0	66.7	33.3	0	100	100	100
CIP5	93.8	6.2	66.7	33.3	2.8	97.2	9.1	90.9
E15	78.1	21.9	66.7	33.3	5.6	94.4	18.2	81.8
CN30	100	0	66.7	33.3	8.3	91.7	9.1	90.9
IPM10	100	0	66.7	33.3	22.2	77.8	54.5	45.5
LEV5	100	0	66.7	33.3	2.8	97.2	9.1	90.9
LZD30	100	0	100	0	100	0	100	0
MET10	100	0	33.3	66.6	0	100	0	100
OX1	100	0	66.7	33.3	0	100	0	100
P10	3.1	96.9	0	100	0	100	0	100
QD15	100	0	100	0	100	0	100	0
TEC30	100	0	66.7	33.3	94.4	5.6	72.7	27.3
TE30	65.6	34.4	0	100	13.9	86.1	18.2	81.8
VA30	100	0	100	0	100	0	100	0

AMC30: Amoxicillin/Clavulanic acid 30 µg, AMP25: Ampicillin 25 µg, CL30: Cephalexin 30 µg, FOX30: Cefoxitin 30 µg, CE30: Cephradine 30 µg, CIP5: Ciprofloxacin 5 µg, E15: Erythromycin 15 µg, CN30: Gentamicin 30 µg, IPM10: Imipenem 10 µg, LEV5: Levofloxacin 5 µg, LZD30: Linezolid 30 µg, MET10: Methicillin 10 µg, OX1: Oxacillin 1 µg, P10: Penicillin G 10 IU, QD15: Quinupristin/Dalfopristin 15 µg, TEC30: Teicoplanin 30 µg, TE30: Tetracycline 30 µg, VA30: Vancomycin 30 µg. S: Sensitive, I: Intermediate, R: Resistant

4.3.2: Antibiotic susceptibility pattern of VSE and VRE

The nasal and peri-rectal VSE isolates showed somewhat similar type of resistant pattern. Resistant pattern was divided into three categories, first, in which all isolates (nasal and peri-rectal) were 80 to 100% resistant: cephalexin, cefoxitin, cephradine, ciprofloxacin, erythromycin, gentamicin, methicillin and oxacillin. Second group with resistance in range of 35% to 60%, were including antibiotics amoxicillin/Clavulanic acid, ampicillin, imipenem, levofloxacin, penicillin G, tetracycline and. The last group composed of most susceptible antibiotics against VSE, including linezolid, quinupristin/dalfopristin, teicoplanin and vancomycin (Table 4.5).

The VRE isolates showed almost similar pattern of resistance in isolates of nasal and peri-rectal area. All 05 VRE isolates from nasal cavity showed resistance to cephalexin, cefoxitin, cephradine, gentamicin, methicillin, oxacillin and vancomycin, erythromycin, levofloxacin, ciprofloxacin, 60% against amoxicillin/clavulanic acid, ampicillin, imipenem, penicillin G and tetracycline. All isolates were susceptible to linezolid, teicoplanin and quinupristin/dalfopristin (Table 4.5). While all 10 peri-rectal VRE isolates showed resistance to cephalexin, cefoxitin, cephradine, erythromycin, gentamicin, levofloxacin, methicillin, oxacillin, teicoplanin and vancomycin, 90% to penicillin G and, 80% to imipenem, 70% against amoxicillin/clavulanic acid and ampicillin, 60%, 50% tetracycline and all isolates were susceptible to quinupristin/dalfopristin and linezolid (Table 4.6).

Table 4.6: Antibiotic resistance profile of VSE and VRE (nasal and peri-rectal) isolates

	VSE in nasal cavity (n=51)		VSE in peri-rectum (n=93)		VRE in nasal cavity (n=05)		VRE in peri-rectum (n=10)	
	% S	% R	% S	% R	% S	% R	% S	% R
AMC30	62.7	37.3	62.4	37.6	40	60	30	70
AMP25	60.8	39.2	63.4	36.6	40	60	30	70
CL30	9.8	90.2	1.1	98.9	0	100	0	100
FOX30	0	100	2.2	97.8	0	100	0	100
KF30	15.7	84.4	8.6	91.4	0	100	0	100
CE30	5.9	94.1	0	100	0	100	0	100
CIP5	17.6	82.4	12.9	87.1	0	100	0	100
E15	5.9	94.1	7.5	92.5	0	100	0	100
CN30	13.7	86.3	9.7	90.3	0	100	0	100
IPM10	54.9	45.1	53.8	46.2	40	60	20	80
LEV5	31.4	68.6	17.2	82.8	0	100	0	100
LZD30	100	0	91.4	8.6	100	0	100	0
MET10	0	100	1.1	98.9	0	100	0	100
OX1	0	100	0	100	0	100	0	100
P10	37.3	62.7	37.6	62.4	40	60	10	90
QD15	100	0	98.9	1.1	100	0	100	0
TEC30	100	0	100	0	0	100	0	100
TE30	37.3	62.7	23.7	76.3	40	60	40	60
VA30	100	0	100	0	0	100	0	100

AMC30: Amoxicillin/Clavulanic acid 30 µg, AMP25: Ampicillin 25 µg, CL30: Cephalexin 30 µg, FOX30: Cefoxitin 30 µg, CE30: Cephradine 30 µg, CIP5: Ciprofloxacin 5 µg, E15: Erythromycin 15 µg, CN30: Gentamicin 30 µg, IPM10: Imipenem 10 µg, LEV5: Levofloxacin 5 µg, LZD30: Linezolid 30 µg, MET10: Methicillin 10 µg, OX1: Oxacillin 1 µg, P10: Penicillin G 10 IU, QD15: Quinupristin/Dalfopristin 15 µg, TEC30: Teicoplanin 30 µg, TE30: Tetracycline 30 µg, VA30: Vancomycin 30 µg. S: Sensitive, I: Intermediate, R: Resistant

4.4. MICs of *S. aureus* and enterococci

In the present study, MIC's was determined for each isolate of MSSA, MSSA, VSE and VRE against vancomycin, tetracycline, ciprofloxacin and oxacillin. The susceptibility of each isolate to the drug varied greatly. Antibiotic dilutions were made, ranging from 0.125 mg/L to 512 mg/L for each of these four antibiotics keeping in mind the breakpoint concentration for ciprofloxacin that is 1 mg/L, oxacillin is 2 mg/L, tetracycline is 1mg/L and for vancomycin is 4 mg/L to 8 mg/L both for staphylococci and enterococci.

4.4.1. MICs of MRSA and MSSA isolates

Vancomycin showed susceptibility range from 1 mg/L to 2 mg/L against staphylococcal isolates from nasal cavity. All isolates were susceptible to vancomycin as all inhibited at maximum concentration 2 mg/L. All MRSA and MSSA were susceptible to vancomycin and MICs did not vary greatly among MRSA and MSSA (One way ANOVA, $P>0.05$). In case of tetracycline the MIC range for the susceptibility of isolates ranged from 0.25 mg/L to 128 mg/L. MICs of tetracycline varied greatly among two variables MRSA and MSSA (One way ANOVA, P value <0.05). There were 23.5% susceptible and 76.5% were resistant *S. aureus* to tetracycline. Similarly MICs of ciprofloxacin ranged from 0.125 mg/L to 128 mg/L with which varied among MRSA and MSSA (One way ANOVA, $P<0.05$).

Out of 68 isolates of *S. aureus*, 25 (36.8%) were susceptible to ciprofloxacin while 43 (63.2%) were resistant as the breakpoint was 1 mg/L. For oxacillin the MIC ranges from 0.125 mg/L to 512 mg/L. For oxacillin MIC 2 mg/L is the breakpoint concentration, 32 (47.05%) isolates were varied greatly below breakpoint and were susceptible to oxacillin and considered as MSSA, and 36 (52.94%) isolates were resistant with MIC ranging from 32 mg/L to 512 mg/L. and labeled as MRSA (One way ANOVA, $P< 0.05$) (Table 4.7a).

Table 4.7a: MICs for nasal MRSA (n=36) and MSSA (n=32) isolates

Antibiotic Dilutions (mg/L)	MIC with Tetracycline N (%)	MIC with Ciprofloxacin N (%)	MIC with oxacillin N (%)	MIC with Vancomycin N (%)
0.125	----------	4 (5.9)	1 (1.5)	----------
0.25	2 (2.9)	7 (10.3)	4 (5.9)	----------
0.5	14 (20.6)	14 (20.6)	12 (17.6)	----------
1	2 (2.9) †	4 (5.9) †	15 (22.1)	17 (25)
2	----------	2 (2.9)	---------- †	51 (75)
4	1 (1.5)	----------	----------	----------
8	2 (2.9)	----------	----------	---------- †
16	1 (1.5)	2 (2.9)	----------	----------
32	12 (17.6)	2 (2.9)	1 (1.5)	----------
64	23 (33.8)	----------	4 (5.9)	----------
128	11 (16.2)	33 (48.5)	1 (1.5)	----------
512	----------	----------	30 (44.1)	----------
ANOVA°	F=9.734 (p< 0.05)	F=476.686 (p< 0.05)	F=217.366 (p<0.05)	F=0.307 (p=0.581)

† Breakpoint concentration, °One way ANOVA

Total 14 isolates of *S. aureus* (MRSA and MSSA) from peri-rectal area were subjected for MICs of four antibiotics. Vancomycin showed similar inhibition range as that for nasal isolates. All isolates were sensitive to vancomycin with inhibition range from 1 mg/L to 2 mg/L. 14.3% isolates were inhibited at 1 mg/L while remaining 85.7% inhibited at 2 mg/L and MRSA and MSSA were equally distributed between the range (One way ANOVA, $P>0.05$). All isolates were resistant to tetracycline showing growth inhibition above breakpoint 1 mg/L. MIC ranged from 8 mg/L to 256 mg/L isolates were equally distributed in these range (One way ANOVA, $P>0.05$).

MICs of ciprofloxacin ranged from 0.25 mg/L to 128 mg/L. Out of 14 isolates, 03 (21.42%) were susceptible having MIC less than breakpoint concentration of 1 mg/L and 11 (78.57%) were resistant to ciprofloxacin with high MIC than breakpoint (One way ANOVA, $P<0.05$). Oxacillin MICs ranged from 0.5 mg/L to 512 mg/L for peri-rectal isolates of *S. aureus*. 21.4% isolates gave MIC at 0.5 mg/L which is less than breakpoint concentration of 2 mg/L and labeled as MSSA and 78.6% isolates showed resistance, MIC ranged from 64-512 mg/L, as they are above breakpoint concentration and considered as MRSA and showed great variation of MICs among MRSA and MSSA ($P<0.05$) (Table 4.7b).

Table 4.7b: MICs of peri-rectal MRSA (n=11) and MSSA (n=3) isolates

	MIC with Tetracycline	MIC with Ciprofloxacin	MIC with oxacillin	MIC with Vancomycin
Antibiotic Dilutions (mg/L)	N (%)	N (%)	N (%)	N (%)
0.25	----------	1 (7.1)	----------	----------
0.5	----------	2 (14.3)	3 (21.4)	----------
1	----------†	1 (7.1) †	----------	2 (14.3)
2	----------	----------	----------†	12 (85.7)
4	----------	----------	----------	----------
8	1 (7.1)	----------	----------	----------†
16	----------	1 (7.1)	----------	----------
32	2 (14.3)	----------	----------	----------
64	5 (35.7)	2 (14.3)	4 (28.6)	----------
128	4 (28.6)	7 (50)	----------	----------
256	2 (14.3)	----------	----------	----------
512	----------	----------	7 (50)	----------
ANOVA°	F=0.105 (p=0.751)	F=10.199 (p= 0.008)	F=6.728 (p=0.023)	F=0.571 (p=0.464)

† Breakpoint concentration, °One way ANOVA

4.4.1. MICs of VRE and VSE isolates

In case of nasal enterococcal isolates, 05 (8.9%) were confirmed as VRE while other 51 (91.1%) were VSE with MIC less than 8 mg/L (breakpoint concentration). all antibiotics have the same breakpoint concentration as that for *S. aureus* except ciprofloxacin which have breakpoint at 4 mg/L. MIC varied greatly but significantly for vancomycin among VRE (*E. faecalis* and *E. faecium)* and VSE (*E. faecalis* and *E. faecium*) (one way ANOVA, P<0.001). For tetracycline the MICs of isolates were ranged from 2 mg/L to 256 mg/L and all isolates were distributed almost equally in this range (one way ANOVA, P>0.05). All isolates were having MICs for tetracycline higher than breakpoint and considered as resistant. The MIC for ciprofloxacin ranged from 1 mg/L to 512 mg/L. Ciprofloxacin inhibited 7.2% isolates at concentration of <4 mg/L and considered susceptible, rest of 92.8% were inhibited at ≥4 mg/L. ANOVA statistic gave P>0.05 for ciprofloxacin indicating almost equal distribution of enterococci in MIC range. Oxacillin MICs were ranged from 4 mg/L to 512 mg/L and isolates were having equal distribution in MIC range (one way ANOVA, P> 0.05). Table 4.8a shows the MICs for nasal enterococci isolates.

Table 4.8a: MICs of nasal VRE (n=5) and VSE (n=51) isolates

Antibiotic Dilutions (mg/L)	MIC with Tetracycline N (%)	MIC with Ciprofloxacin N (%)	MIC with Oxacillin N (%)	MIC with Vancomycin N (%)
1	——— †	1 (1.8)	----------	28 (50)
2	4 (7.1)	3 (5.4)	——— †	22 (39.3)
4	7 (12.5)	2 (3.6) †	1 (1.8)	1 (1.8)
8	4 (7.1)	6 (10.7)	2 (3.6)	——— †
16	1 (1.8)	2 (3.6)	6 (10.7)	----------
32	2 (3.6)	5 (8.9)	5 (8.9)	----------
64	7 (12.5)	4 (7.1)	7 (12.5)	----------
128	19 (33.9)	12 (21.4)	11 (19.6)	----------
256	12 (21.4)	17 (30.4)	12 (21.4)	----------
512	----------	4 (7.1)	12 (21.4)	5 (8.9)
ANOVA°	F=0.447 (p=0.720)	F=0.296 (p= 0.828)	F=0.985 (p=0.407)	F=1212997.5 (p<0.001)

† Breakpoint concentration, °One way ANOVA

Total 103 isolates of enterococci were subjected to MICs determination. All antibiotics gave statistically significance value (one way ANOVA, $P<0.05$) representing unequal distribution of MIC among enterococcal groups. MICs of vancomycin ranged from 1 mg/L to 4 mg/L. Out of 103 isolates, 90.2% were inhibited at ≤4 mg/L and considered as VSE. Remaining 9.7% had MICs >8 mg/L and were regarded as VRE. MICs of tetracycline ranged from 1 mg/L to 256 mg/L and all isolates were resistant. MICs of ciprofloxacin and oxacillin ranged from 2 mg/L to 512 mg/L and 8 mg/L to 512 mg/L respectively. These concentration limits showed that the isolates were resistant to both of these antibiotics and there were equal distribution of enterococci among MICs range (Table 4.8b).

Table 4.8b: MICs of peri-rectal VRE (n=10) and VSE (n=93) isolates

	MIC with Tetracycline	**MIC with Ciprofloxacin**	**MIC with Oxacillin**	**MIC with Vancomycin**
Antibiotic Dilutions (mg/L)	**N (%)**	**N (%)**	**N (%)**	**N (%)**
1	4 (3.9) †	----------	----------	43 (41.7)
2	2 (1.9)	1 (1)	---------- †	43 (41.7)
4	19 (18.4)	7 (6.8) †	----------	7 (6.8)
8	2 (1.9)	16 (15.5)	5 (4.9)	---------- †
16	3 (2.9)	5 (4.9)	9 (8.7)	----------
32	4 (3.9)	14 (13.6)	17 (16.5)	----------
64	17 (16.5)	5 (4.9)	8 (7.8)	3 (2.9)
128	26 (25.2)	20 (19.4)	14 (13.6)	----------
256	26 (25.2)	23 (22.3)	21 (20.4)	----------
512	----------	12 (11.7)	29 (28.2)	7 (6.8)
ANOVA°	F=3.555 (p=0.017)	F=3.369 (p= 0.022)	F=2.683 (p=0.051)	F=106.428 (p<0.001)

† Breakpoint concentration, °One way ANOVA

4.5: Statistical analysis

In this study patient stayed an average 6.72 (SD ± 9.11) days in ICUs department. During this stay the frequency of MRSA and MSSA in 216 nasal samples was 17% and 15% respectively. Approximately 47% samples were coagulase negative and were put under heading coagulase-negative staphylococci (CoNS). These samples were not further processed for molecular identification. Twenty one percent samples gave no growth for staphylococci on selective culture media (Fig. 4.17a).

4.5.1: Frequency of *S. aureus* in nasal and peri-rectal samples

The frequency of MRSA were come out to be 5% in peri-rectal samples out of total 216 samples. MSSA and CoNS were 2% and 48% respectively. CoNS were the most frequent organism in peri-rectal samples. 45% samples no staphylococci were isolated (Fig. 4.17b). The chi sq. test give significant $P<0.001$ which demonstrate that all isolates were randomly distributed in nasal and peri-rectal samples.

Fig. 4.17a: Pie chart showing the proportion of staphylococci isolated from nasal samples

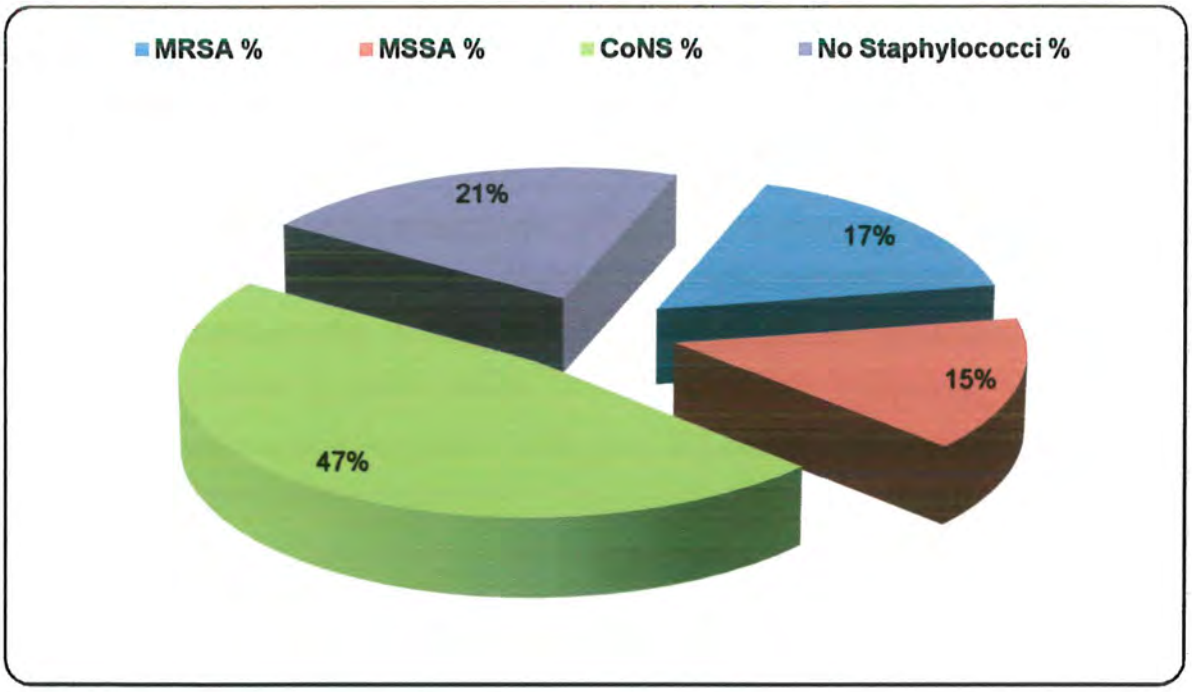


Fig. 4.17b: Pie chart showing the proportion of staphylococci isolated from peri-rectal samples

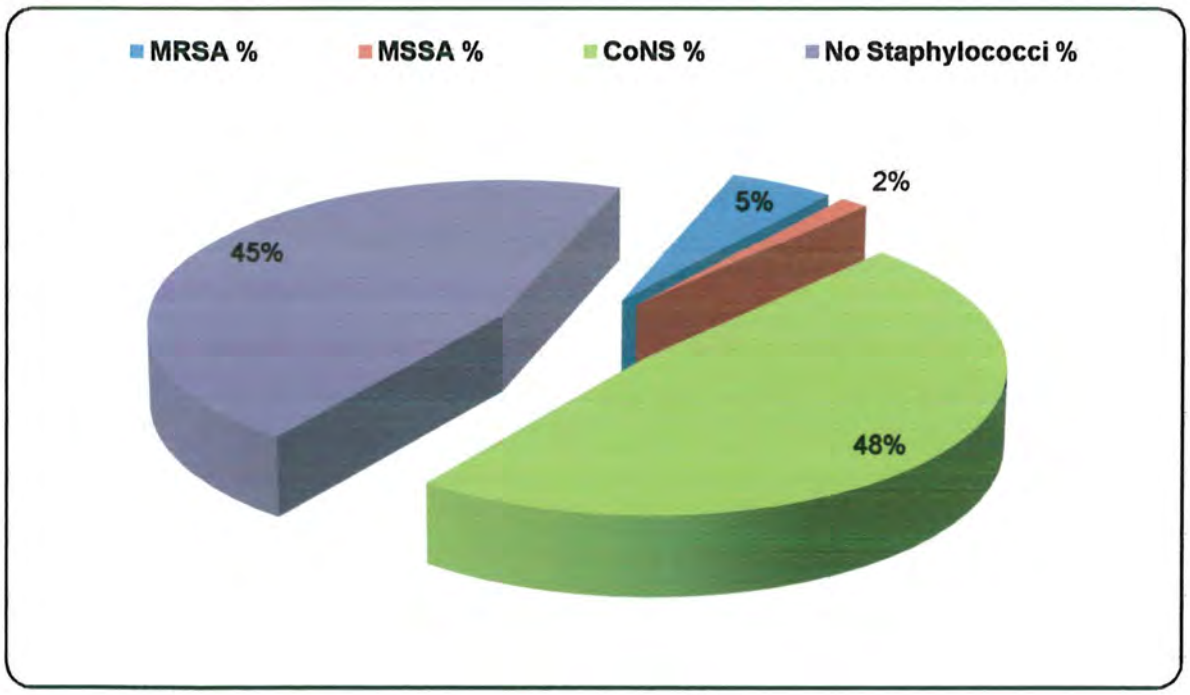


Fig. 4.18b: Pie chart showing the proportion of enterococci isolated from peri-rectal samples

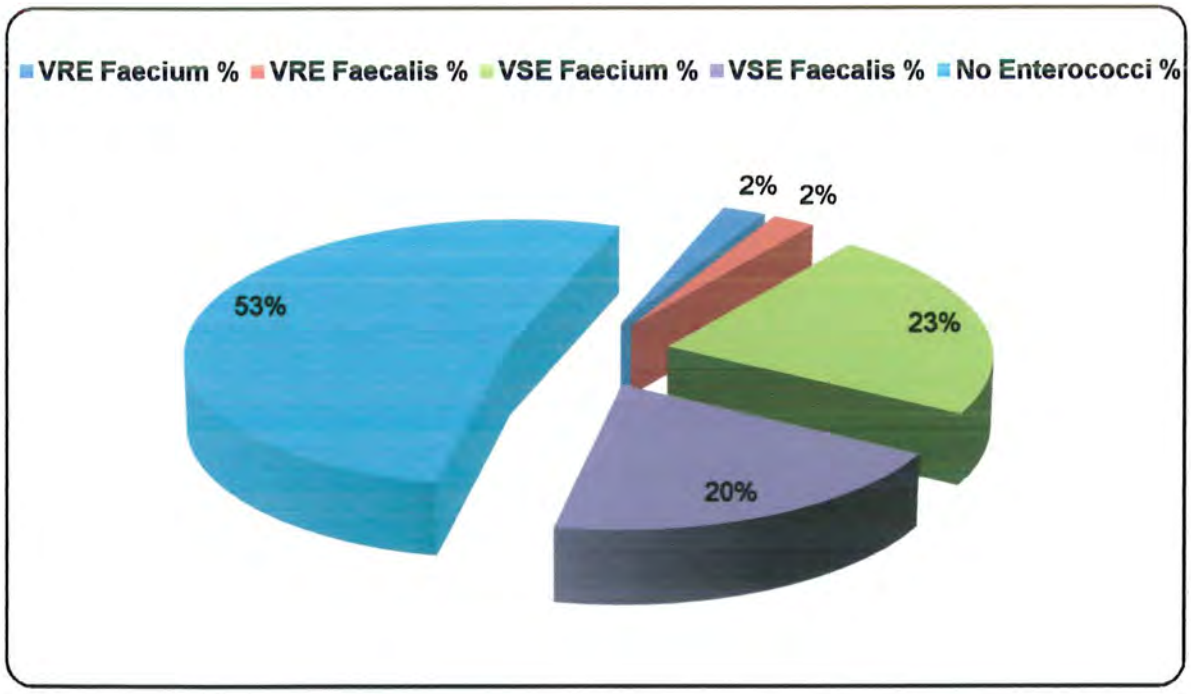
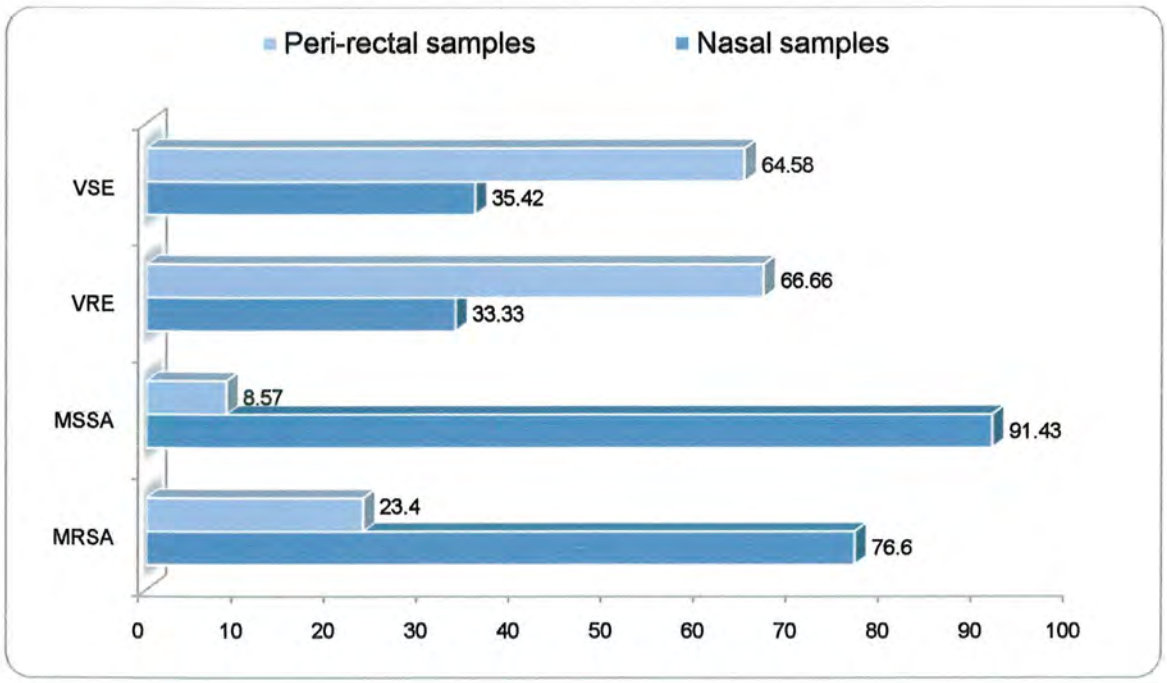


Fig. 4.19: Percentages of MRSA, MSSA, VRE and VSE in nasal and peri-rectal samples



4.5.3: Frequency of MRSA and MSSA among age groups

Out of 432 nasal and peri-rectal samples from 216 patients, there were 47 MRSA, 35 MSSA, 206 CoNS and 144 samples gave no growth for staphylococci. There were 69 (31.9%) patients with age group <1 year, 29 (13.4%) with 1to <6 years, 13 (6%) with 6 to <12yreas, 15 (6.9%) with 12 to <18 years, 32 (14.8%) with 18 to <35 years and 58 (26.9%) with >35 years age group.

The MRSA were randomly distributed in nasal and peri-rectal isolates within different age groups (P<0.001 and <0.05 respectively). High number 18 (31%) nasal MRSA were found in age group >35 years and 05 (8.6%) were in peri-rectal MRSA. In these isolates from age group >35, there were 03 patients who were colonized with MRSA both in nasal and peri-rectal area. The frequency was high in age group 12 to < 18 years that was 07 (46.7%) out of 15 samples. Similarly the nasal MSSA were high in the same group of patient i.e. 11(19%) and in peri-rectal isolates only 03 (1.4%) MSSA were isolated. CoNS were the most frequent isolate in nasal and peri-rectal samples in patients of <1 year of age. These were 43 (62.3%) isolates from nasal and 33 (47.8%) from peri-rectum. Total 46 nasal and 98 peri-rectal samples came with no result for staphylococci (Table 4.9).

4.5.4: Frequency of MRSA and MSSA among gender groups

The staphylococcal distribution was random among gender for nasal isolates (P<0.001) while almost equal for peri-rectal (P>0.05). There were 54 (25%) male patients, 51(23.6%) female patients, 56 (25.9%) male child patients and 55 (25.5%) female child patients. Male category was more colonized with MRSA that is 38.9% in nasal and 14.8% in peri-rectal staphylococci samples than females and paeds (Table 4.9).

4.5.6: Distribution of staphylococcal isolates among rural and urban patients

There was 102 (47.2%) patients from rural area and 114 (52.8%) from urban areas. So there were more patients from urban areas admitted in the ICU wards. But patients came from rural setting were colonized more with nasal MRSA than urban i.e. 24 (23.5%) and 12 (10.5%) respectively. Rural patients colonized with nasal MSSA were 11(10.8%) and urban were 21 (18.4%) which mean more urban were colonized with nasal MSSA. In case of peri-rectal isolates, urban patients were more colonized with MRSA and MSSA than rural patients. There were 06 (5.3%) MRSA in urban and 05 (4.9%) in rural and 02 (1.8%) MSSA were in urban and 01(1%) in rural. The overall isolation from nasal samples were random ($P<0.05$) and almost equal from peri-rectum ($P>0.05$).

The patients from lower class, middle class and higher class were 117, 97 and 02 in number respectively. Only two patients were from higher class. Nasal MRSA were equally isolated from both the lower and middle class. In 117 lower class patients 17 (14.5%) were MRSA, 18 (18.6%) and 01 (50%) isolate of MRSA were from middle class and higher class respectively. Whereas 07 (6%), 03 (3.1%) and 01(50%) were peri-rectal MRSA from lower, middle and higher class respectively. MSSA isolation was almost equal among nasal and peri-rectal isolates. The MSSA were distributed among lower and middle class only, none of the case from higher class. 14 (12%) and 18 (18.6%) were nasal MSSA, 02 (1.7%) and 01 (1%) peri-rectal MSSA from lower and middle class patients respectively. Fig. 4.21 and 4.22 shows the graphical presentation of prevalence according to residence and socioeconomic status of the patients. The chi square test gave p value 0.014 for nasal samples which is statistically significant whereas $P=0.106$ (statistically insignificant) in case of peri-rectal isolates, with equal distribution of isolates among the group.

Table 4.10: Association between clinical diagnosis and staphylococcal colonization

		Staphylococci from nasal cavity					Staphylococci from peri-rectum					
		MRSA	MSSA	CoNS	No staph	P value	MRSA	MSSA	CoNS	No staph	P value	Total
Diagnosis/ Reason for admission												
Aspiration Pneumonia	N (%)	2 (33.3)	2 (33.3)	2 (33.3)	0		1 (16.7)	0	2 (33.3)	3 (50)		6 (100)
Meningitis	N (%)	1 (9.1)	2 (18.2)	4 (36.4)	4 (36.4)		0	0	7 (63.6)	4 (36.4)		11 (100)
Pneumonia	N (%)	2 (2.8)	5 (6.9)	47 (65.3)	18 (25)	<0.001	1 (1.4)	1 (1.4)	33 (45.8)	37 (51.4)	0.178	72 (100)
Renal Failure	N (%)	3 (13.6)	4 (18.2)	10 (45.5)	5 (22.7)		1 (4.5)	0	10 (45.5)	11 (50)		22 (100)
Tetanus	N (%)	6 (54.5)	0	4 (36.4)	1 (9.1)		1 (9.1)	0	9 (81.8)	1 (9.1)		11 (100)
Tuberculosis	N (%)	0	3 (33.3)	3 (33.3)	3 (33.3)		0	1 (11.1)	2 (22.2)	6 (66.7)		9 (100)
Other	N (%)	22 (25.9)	16 (18.8)	32 (37.6)	15 (17.6)		7 (8.2)	1 (1.2)	41 (48.2)	36 (42.4)		85 (100)

MRSA: Methicillin-resistant *S. aureus*, MSSA: Methicillin-sensitive *S. aureus,* CoNS: Coagulase-negative staphylococci.

4.5.8: Frequency of enterococci (nasal and peri-rectal isolates) among age groups

Over all frequencies of enterococci are highlighted in Table 4.12b according to which total 15 VRE and 144 VSE were isolated from 432 nasal and peri-rectal (2 samples/patient) samples. These were distributed as 05 VRE and 51 VSE in nasal, whereas 10 VRE and 93 VSE from peri-rectal samples. 273 samples gave no result for enterococci (160 nasals and 113 peri-rectal).

Considering the age group first, there was only 05 VRE isolates from 216 nasal samples, 02 (0.9%) *E. faecium* and 03 (1.4%) were *E. faecalis*. These five isolates were distributed in the different age groups. Out of 51 nasal VSE, 24 (11.1%) were *E. faecium* and 27 (12.5%) were *E. faecalis*. Of these 24 *E. faecium*, 12 (50%) were isolated from age group <1 year. The remaining 12 isolates are distributed randomly among other age groups. In case of VSE (*E. faecalis*) majority of isolates were from age group >35 years and age group <1 year i.e. 14 (24.1%) and 8 (11.6%) respectively. The p value for nasal enterococci come to be <0.05 which is statistically significant (Table 4.11).

Peri-rectal samples gave 10 VRE isolates (2.3% each *E. faecium* and *E. faecalis*) which were distributed almost equally in all age group (Table 4.11). There were 50 peri-rectal VSE (*E. faecium*) isolates major proportion was from age group <1 year that is 14 (20.3%), 13 (22.4%) from >35 years and 10 (31.3%) from 18 to <35 years but the frequency was high in 18 to <35 years group as the 10 (31.3%) isolates were out of 32. Similarly the total 43 (19.9%) VSE (*E. faecalis*) were isolated predominantly form age group <1 year i.e. 17 (24.6%). The overall enterococci frequency was random in all age groups (P=0.722).

There were 11 cases from age group <1 which was having VSE (both *E. faecium* & *E. faecalis)* in both nasal and peri-rectal samples. Whereas one case positive for VRE in peri-rectal area was harboring VSE in nasal cavity and one case was positive in VRE colonization both in nasal and peri-rectal area. Other enterococci harboring predominant age group >35 showed colonization either in nasal or peri-rectal area but not in both.

4.5.9: Frequency of enterococci among genders

The patients in each gender group were almost equal as mentioned before. Gender group has the unequal distribution of enterococci (Table 4.11), only 05 isolates of nasal VRE were isolated. Among males, frequency was 1.9% for VRE (each for *E. faecalis* and *E. faecium*). In females, there was no isolation of VRE, whereas 01 (1.8%) nasal VRE was *E. faecium*, isolated from male child and 02 (3.6%) *E. faecalis* were isolated from female child. *E. faecium* sensitive to vancomycin, was more frequent in female child that was 09 (16.4%) and 08 (14.3%) in male child. Whereas vancomycin-sensitive *E. faecalis,* were distributed among the group as: males 12(22.2%), females 06 (11.8%), male child group 04 (7.1%) and female child group 05 (9.1%). Frequency of these isolates was high among males (Table 4.11).

In peri-rectal samples, out of 10 VRE, 05 were *E. faecium* from which 02 (3.7%) were isolated from males, 01 (1.8%) from male child and 02 (3.6%) from female child group making child group more predominant. Same is the case with other 05 VRE which were *E. faecalis*, 02 (3.9%) were isolated from females, 02 (3.6%) from male child and 01 (1.8%) from female child group. VSE especially *E. faecium* was equally divided, 16 (29.6%) and 09 (17.6%) from males and females while 09 (16.1%) and 16 (29.1%) from male child and female child group respectively. In contrast the frequency of *E. faecalis* specie of VSE was 15 (26.8%) and 11 (20%) in male and female child groups and 08 (14.8%) and 09 (17.6%) in males and females adults. The p value for peri-rectal isolates vs. gender was 0.492 (statistically insignificant) (Table 4.11).

Table 4.11: Frequency of enterococci among different age groups and gender

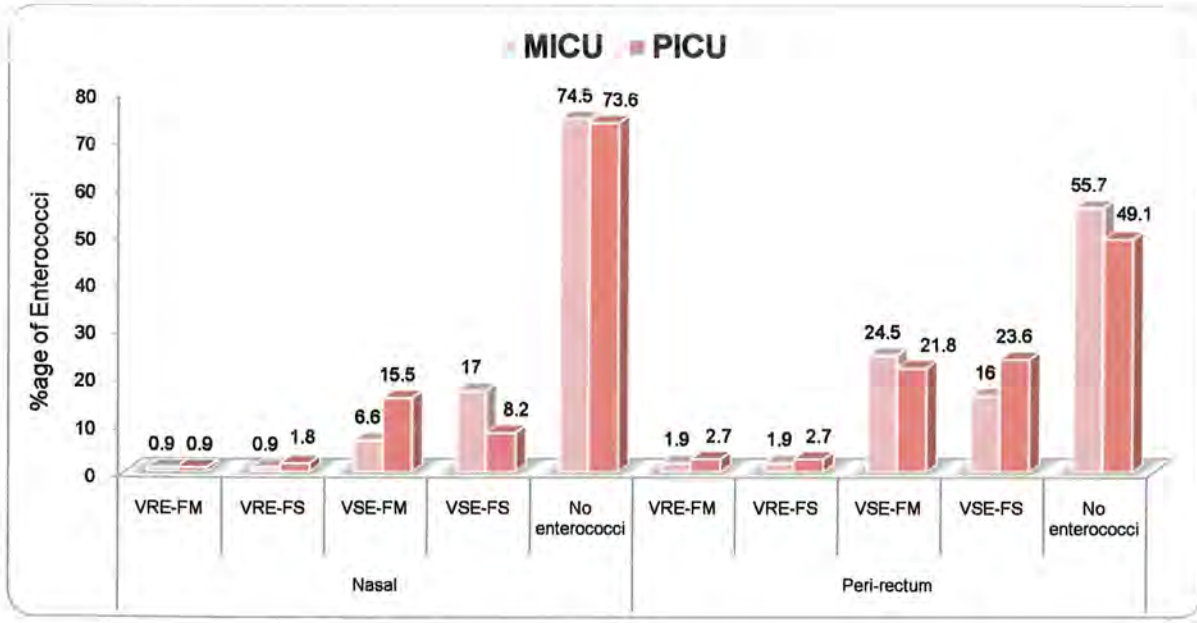
		Enterococci from nasal cavity						Enterococci from peri-rectum						
		VRE-FM	VRE-FS	VSE-FM	VSE-FS	No enterococci	P Value	VRE-FM	VRE-FS	VSE-FM	VSE-FS	No enterococci	P Value	Total
Age (In Years) Groups														
<1	N (%)	0	1 (1.4)	12 (17.4)	8 (11.6)	48 (69.6)		1 (1.4)	1 (1.4)	14 (20.3)	17 (24.6)	36 (52.2)		69 (100)
1 to <6	N (%)	0	1 (3.4)	5 (17.2)	0	23 (79.3)		2 (6.9)	1 (3.4)	7 (24.1)	7 (24.1)	12 (41.4)		29 (100)
6 to <12	N (%)	1 (7.7)	0	0	1 (7.7)	11 (84.6)	0.017	0	1 (7.7)	4 (30.8)	2 (15.4)	6 (46.2)	0.722	13 (100)
12 to <18	N (%)	1 (6.7)	0	2 (13.3)	2 (13.3)	10 (66.7)		1 (6.7)	1 (6.7)	2 (13.3)	2 (13.3)	9 (60)		15 (100)
18 to <35	N (%)	0	0	3 (9.4)	2 (6.3)	27 (84.4)		1 (3.1)	0	10 (31.3)	6 (18.8)	15 (46.9)		32 (100)
>35	N (%)	0	1 (1.7)	2 (3.4)	14 (24.1)	41 (70.7)		0	1 (1.7)	13 (22.4)	9 (15.5)	35 (60.3)		58 (100)
Total	N (%)	2 (0.9)	3 (1.4)	24 (11.1)	27 (12.5)	160 (74.1)		5 (2.3)	5 (2.3)	50 (23.1)	43 (19.9)	113 (52.3)		216 (100)
Gender														
Male	N (%)	1 (1.9)	1 (1.9)	4 (7.4)	12 (22.2)	36 (66.7)		2 (3.7)	0	16 (29.6)	8 (14.8)	28 (51.9)		54 (100)
Female	N (%)	0	0	3 (5.9)	6 (11.8)	42 (82.4)	0.182	0	2 (3.9)	9 (17.6)	9 (17.6)	31 (60.8)	0.492	51 (100)
Male Child	N (%)	1 (1.8)	0	8 (14.3)	4 (7.1)	43 (76.8)		1 (1.8)	2 (3.6)	9 (16.1)	15 (26.8)	29 (51.8)		56 (100)
Female Child	N (%)	0	2 (3.6)	9 (16.4)	5 (9.1)	39 (70.9)		2 (3.6)	1 (1.8)	16 (29.1)	11 (20)	25 (45.5)		55 (100)
Total	N (%)	2 (0.9)	3 (1.4)	24 (11.1)	27 (12.5)	160 (74.1)		5 (2.3)	5 (2.3)	50 (23.1)	43 (19.9)	113 (52.3)		216 (100)

4.5.10: Frequency of enterococci in wards

There were 02 VRE (01 (0.9%) each *E. faecalis* and *E. faecium*) isolated from nasal samples of MICU patients and 03 VRE (01 (0.9%) *E. faecium* and 02 (1.8%) *E. faecalis*) were from PICU patients. Similarly there was 25 nasal VSE isolated from MICU patients in which 07 (6.6%) were *E. faecium* and 18 (17%) were *E. faecalis* whereas in PICU, 26 nasal VSE were isolated, in these 17 (15.5%) were *E. faecium* and 09 (8.2%) were *E. faecalis*. Total 79 (74.5%) samples from MICU and 81 (73.6%) from PICU gave no enterococcal growth and p value for nasal isolates was P>0.05 (Fig. 4.24).

In peri-rectal samples, 04 VRE (02 (1.9%) each *E. faecalis* and *E. faecium*) were from MICU and 06 VRE (03 (2.7%) each *E. faecium* and *E. faecalis*) were from PICU. The VSE were more frequent in PICU that was 50 in number. Of these, 24 (21.8%) were *E. faecium* and 26 (23.6%) were *E. faecalis*. Whereas in MICU 43 VSE (26 (24.5%) *E. faecium* and 17 (16%) *E. faecalis)* were isolated from peri-rectal samples (P>0.05) (Fig. 4.24).

Fig. 4.24: Frequency of enterococci in wards

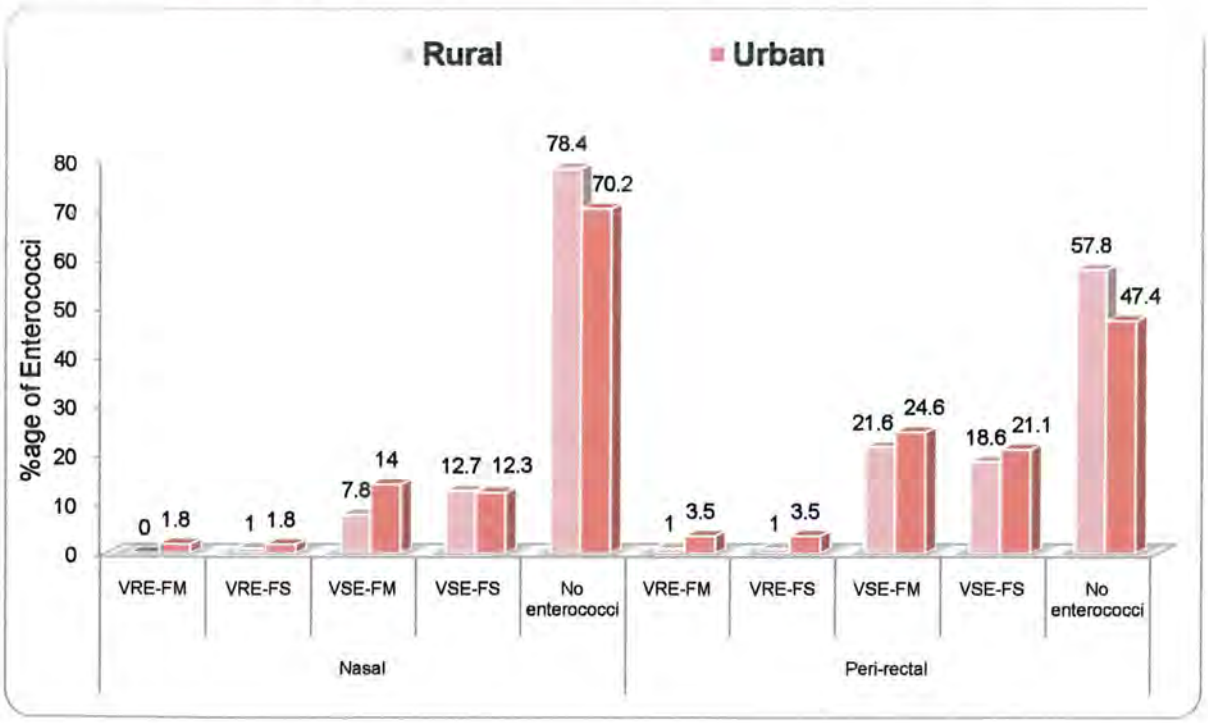


4.5.11: Frequency of enterococci among rural and urban patients

Urban patients have the major portion of nasal and peri-rectal VRE and VSE than rural patients. There were 04 nasal VRE from urban patients, 02 (1.8%) each *E. faecium* and *E. faecalis*. There were 20 nasal VSE in urban patients, of which 16 (14%) were *E. faecium* and 14 (12.3%) were *E. faecalis*. Patients from rural setting were nasal colonized with 01 (1%) VRE (*E. faecalis*) and 21 VSE (08 (7.8%) *E. faecium* and 13 (12.7%) *E. faecalis*). P value was statistically insignificant as it was >0.05).

From peri-rectal isolates, urban patients were colonized with 08 VRE (04 (3.5%) each *E. faecium* and *E. faecalis*) and 52 VSE (28 (24.6%) *E. faecium* and 24 (21.1%) *E. faecalis* while rural patients were colonized with 02 VRE (01 (1%) each *E. faecium* and *E. faecalis*) and 41 VSE (22 (21.6%) *E. faecium* and 19 (18.6) *E. faecalis*) with P value >0.05 for both nasal (P=0.357) and peri-rectal (P=0.346) isolates (Fig. 4.25).

Fig. 4.25: Frequency of enterococci among rural and urban patients

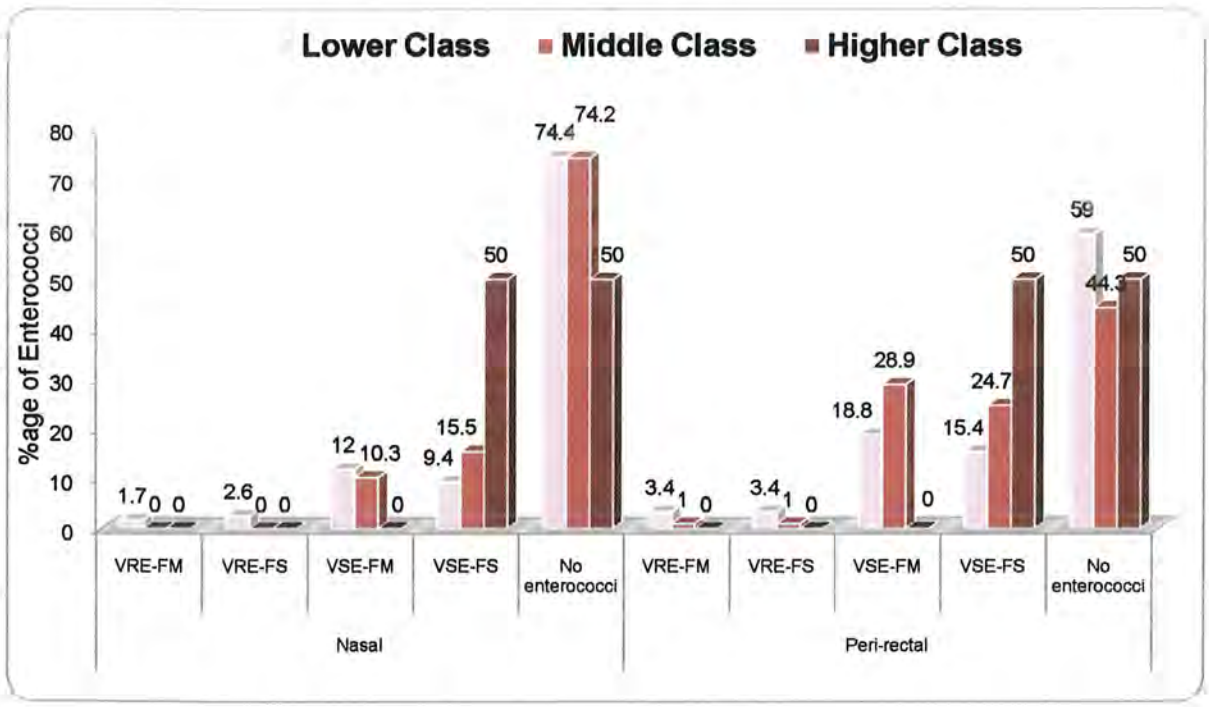


4.5.12: Enterococcal frequency among socio-economic classes

Socio-economic status of the patients showed unequal distribution of VRE and VSE isolates within the group (statistically insignificant). Patients from lower class group were colonized more with VRE isolates. 05 nasal (02 (1.7%) *E. faecium* and 03 (2.6%) *E. faecalis*) and 08 peri-rectal VRE (04 (3.4%) each *E. faecalis* and *E. faecium*) were from lower class. Both lower class and middle class were colonized with equal number (25 isolates each) of nasal VSE isolates. There were only two patients from higher class, only one was colonized with VSE (*E. faecalis*).

In peri-rectal isolates, frequency of VSE was high in middle class. There were 52 VSE, of which 28 (28.8%) were *E. faecium* and 24 (24.7%) were *E. faecalis* from middle class patient group. The p value was >0.05 for both nasal (P=0.379) and peri-rectal isolates (P=0.26) from different socio-economic group of patients (Fig. 4.26).

Fig. 4.26: Enterococci vs. Socioeconomic status



4.5.13: Association between clinical diagnosis and enterococcal colonization

4.5.13: Association between clinical diagnosis and enterococcal colonization

Patients presented with pneumonia and with different other diseases were more colonized with VRE and VSE. In nasal samples, there were 02 (2.8%) VRE (*E. faecalis*), 20 VSE (18.1% *E. faecium* and 9.7% *E. faecalis*) from pneumonia patients. Similarly 01 VRE (1.2%*E. faecium*) and 17 VSE (07 (8.2%) *E. faecium* and 10 (11.8%) *E. faecalis*) were isolated from patients admitted with different disease conditions (Table 4.12).

In peri-rectal samples, "Other disease" group was more colonized with enterococci i.e. 02 VRE (2.4% *E. faecalis*) and 43 VSE (31.8% *E. faecium* and 18.8% *E. faecalis*), whereas the second most frequent group was of patients suffering with pneumonia which were having 03 VRE (1.4% *E. faecium* and 2.8% *E. faecalis*) and 31 VSE (22.2% *E. faecium* and 20.8% *E. faecalis*). The p value was < 0.05 in case of nasal isolates and $P=0.117$ for peri-rectal isolates (Table 4.12).

Table 4.12: Association between clinical diagnosis and enterococcal colonization

		Enterococci from nasal cavity						Enterococci from peri-rectum						
		VRE-FM	VRE-FS	VSE-FM	VSE-FS	No enterococci	P value	VRE-FM	VRE-FS	VSE-FM	VSE-FS	No enterococci	P value	Total
Clinical Diagnosis														
A. Pneumonia	N (%)	0	0	0	3 (50)	3 (50)		0	0	0	2 (33.3)	4 (66.7)		6 (100)
Meningitis	N (%)	0	0	1 (9.1)	0	10 (90.9)		2 (18.2)	0	1 (9.1)	3 (27.3)	5 (45.5)		11 (100)
Pneumonia	N (%)	0	2 (2.8)	13 (18.1)	7 (9.7)	50 (69.4)		1 (1.4)	2 (2.8)	16 (22.2)	15 (20.8)	38 (52.8)		72 (100)
Renal Failure	N (%)	1 (4.5)	0	1 (4.5)	2 (9.1)	18 (81.8)	0.015	1 (4.5)	1 (4.5)	4 (18.2)	4 (18.2)	12 (54.5)	0.117	22 (100)
Tetanus	N (%)	0	1 (9.1)	0	5 (45.5)	5 (45.5)		0	0	0	3 (27.3)	8 (72.7)		11 (100)
TB	N (%)	0	0	2 (22.2)	0	7 (77.8)		1 (11.1)	0	2 (22.2)	0	6 (66.7)		9 (100)
Others	N (%)	1 (1.2)	0	7 (8.2)	10 (11.8)	67 (78.8)		0	2 (2.4)	27 (31.8)	16 (18.8)	40 (47.1)		85 (100)
Total	N (%)	2 (0.9)	3 (1.4)	24 (11.1)	27 (12.5)	160 (74.1)		5 (2.3)	5 (2.3)	50 (23.1)	43 (19.9)	113 (52.3)		216(100)

VRE-FS: VRE-*E. faecalis*, VRE-FM: VRE- *E. faecium*, VSE-FS: VSE- *E. faecalis*, VSE-FM: VSE- *E. faecium*, TB: Tuberculosis

4.5.14: Association of invasive devices with MRSA and VRE isolates

Table 4.13 presents the association among invasive devices like urine catheter, suction catheter, ventilator etc., with multi drug resistant isolates of MRSA and VRE. As there was no blood samples or urine samples were collected from these patients so it was difficult to associate the invasive devices with the colonization of MRSA and VRE. However patients on ventilators were more colonized with MRSA i.e. 20 (55.6%) patients ($P < 0.001$) similarly the patients with intubation were also more colonized with MRSA i.e. 23 (63.9%) patients ($P<0.05$).

Table 4.13: Association of invasive devices with isolation of resistant isolates

		MRSA in nasal cavity			MRSA in peri-rectum			VRE in nasal cavity			VRE in peri-rectum		
		Negative	Positive		Negative	Positive		Negative	Positive		Negative	Positive	
Invasive Devices		N (%)	N (%)	P value	N (%)	N (%)	P value	N (%)	N (%)	P value	N (%)	N (%)	P value
Central Catheter	No	172 (95.6)	29 (80.6)	0.001	191 (93.2)	10 (90.9)	0.774	196 (92.9)	5 (100)	0.537	191 (92.7)	10 (100)	0.537
	Yes	8 (4.4)	7 (19.4)		14 (6.8)	1 (9.1)		15 (7.1)	0		15 (7.3)	0	
Intubation	No	108 (60)	13 (36.1)	0.008	116 (56.6)	5 (45.5)	0.469	118 (55.9)	3 (60)	0.856	114 (55.3)	7 (70)	0.362
	Yes	72 (40)	23 (63.9)		89 (43.4)	6 (54.5)		93 (44.1)	2 (40)		92 (44.7)	3 (30)	
I/V Cannula	No	13 (7.2)	3 (8.3)	0.816	15 (7.3)	1 (9.1)	0.827	15 (7.1)	1 (20)	0.277	15 (7.3)	1 (10)	0.749
	Yes	167 (92.8)	33 (91.7)		190 (92.7)	10 (90.9)		196 (92.9)	4 (80)		191 (92.7)	9 (90)	
Renal Dialysis	No	170 (94.4)	33 (91.7)	0.522	193 (94.1)	10 (90.9)	0.660	199 (94.3)	4 (80)	0.184	195 (94.7)	8 (80)	0.57
	Yes	10 (5.6)	3 (8.3)		12 (5.9)	1 (9.1)		12 (5.7)	1 (20)		11 (5.3)	2 (20)	
Suction catheter	No	173 (96.1)	29 (80.6)	0.001	194 (94.6)	8 (72.7)	0.004	199 (94.3)	3 (60)	0.002	194 (94.2)	8 (80)	0.075
	Yes	7 (3.9)	7 (19.4)		11 (5.4)	3 (27.3)		12 (5.7)	2 (40)		12 (5.8)	2 (20)	
Urine Catheter	No	118 (65.6)	9 (25)	0.000	125 (61)	2 (18.2)	0.005	124 (58.8)	3 (60)	0.956	118 (57.3)	9 (90)	0.040
	Yes	62 (34.4)	27 (75)		80 (39)	9 (81.8)		87 (41.2)	2 (40)		88 (42.7)	1 (10)	
Ventilator	No	151 (83.9)	16 (44.4)	0.000	162 (79)	5 (45.5)	0.010	163 (77.3)	4 (80)	0.885	157 (76.2)	10 (100)	0.079
	Yes	29 (16.1)	20 (55.6)		43 (21)	6 (54.50)		48 (22.7)	1 (20)		49 (23.8)	0	

I/V: Intravenous, N: Number

4.5.15: Single and multiple antibiotic treatment

Only four groups of antibiotics which were frequently used in treatment were focused. These were aminoglycosides, most common in use was amikacin, beta-lactams including amoxicillin/clavulanic acid, ampicillin, ampicillin/cloxacillin and imipenem. 3rd generation cephalosporins most frequently used was ceftriaxone. Other was ceftazidime, cefotaxime and cefoperazone. Fluoroquinolones were ciprofloxacin and levofloxacin. The multiple antibiotics used for the treatment of patients were divided into 10 categories on the basis of combinations (Table 4.14)

Table 4.14: Antibiotic combinations used in treatment of patients

	ANTIBIOTIC COMBINATION
Category 1	BL+FQ
Category 2	A+BL+3GC+FQ
Category 3	A+3GC
Category 4	A+BL+3GC
Category 5	A+BL
Category 6	A+3GC+FQ
Category 7	A+FQ
Category 8	BL+3GC
Category 9	BL+3GC+FQ
Category 10	3GC+FQ

A: Aminoglycosides, BL: Beta Lactams, 3GC: 3rd Generation Cephalosporins, FQ: Fluoroquinolone

Out of 216 patients, 80 (37.04%) patients were treated with single antibiotics and 110 (50.92%) with multiple antibiotics and 26 (12.04%) were not given any antibiotic treatment (Table 4.15). Of these 80, 10 (12.5%) were colonized with MRSA and out of 110, 18(19.6%) patients were colonized with MRSA. In case of single antibiotic treatment, 03 (30%) cases each were found associated with patients using beta lactams, 3rd generation cephalosporins, and fluoroquinolones whereas 05 (27.8%) patients were using combination therapy of 3rd generation cephalosporins + fluoroquinolones. On chi square test only significant association was found in antibiotics and nasal MRSA colonization with p values <0.05 for both single and multiple antibiotic therapy (Table 4.15 & 4.16).

The patients in group <1 year and 1 to <6 years of age were treated more frequently than other age groups with aminoglycosides, beta lactams and 3rd generation cephalosporins and combination antibiotics. 26 (12%) patients from age group <1 year and 13 (6%) patients from age 1 to <6 years were taking single antibiotic treatment and 38 (17.6%) and 14 (6.5%) of the same age group were using antibiotic combinations respectively. Other group was age group >35 years with 16 (7.4%) using single antibiotic regimen and 33 (15.3%) were on multiple antibiotic regimen. The two group >35 was also more colonized with MRSA and VRE.

Table 4.15: Staphylococcal colonized patients on single antibiotic treatment regime

		Patient's Categories MRSA Positive	MRSA Negative	Total	P value
Antibiotics					
Aminoglycosides	N (%)	1 (10)	6 (8.6)	7 (8.8	
Beta Lactams	N (%)	3 (30)	6 (8.6)	9 (11.3)	
Cephalosporins 3rd generation	N (%)	3 (30)	52 (74.3)	55 (68.8)	0.022
Fluoroquinolones	N (%)	3 (30)	6 (8.6)	9 (11.3)	
Total	N	10	70	80	

N: Number

Table 4.16: Staphylococcal colonized patients on multiple antibiotic treatments regime

		Patient's Categories MRSA Positive	MRSA Negative	Total	P value
Antibiotic combination					
cat-1	N (%)	1 (5.6)	6 (6.5)	7 (6.4)	
cat-2	N (%)	1 (5.6)	0	1 (0.9)	
cat-3	N (%)	2 (11.1)	14 (15.2)	16 (14.5)	
cat-4	N (%)	0	4 (4.3)	4 (3.6)	
cat-5	N (%)	3 (16.7)	4 (4.3)	7 (6.4)	
cat-6	N (%)	1 (5.6)	2 (2.2)	3 (2.7)	0.024
cat-7	N (%)	0	1 (1.1)	1 (0.9)	
cat-8	N (%)	4 (22.2)	51 (55.4)	55 (50)	
cat-9	N (%)	1 (5.6)	2 (2.2)	3 (2.7)	
cat-10	N (%)	5 (27.8)	8 (8.7)	13 (11.8)	
Total	N	18	92	110	

Cat: Category, N: Number

4.5.16: Association of history of vancomycin intake with VRE colonization

The history of vancomycin intake was monitored to see its effect with the isolation of vancomycin resistant enterococci. Results showed that peri-rectal VRE isolates are statistically correlated with the patient using vancomycin (Table 4.17).

On chi square test, peri-rectal VRE isolates were found to be having some association with vancomycin usage (P= 0.027). The total 21 (9.7%) patients were receiving vancomycin (Table 4.19). Of 07 peri-rectal VRE colonized patients, 03 (30%) were using vancomycin (P<0.05). while with other antibiotics, only 07 VRE positive case were using 3rd generation cephalosporins (P >0.05).

Table 4.17: Association of history of vancomycin intake with VRE-positive patients

		VRE in nasal cavity		VRE in peri-rectum		
		Positive	Negative	Positive	Negative	Total
History of vancomycin intake						
Yes	N (%)	1 (20)	20 (9.5)	3 (30)	18 (8.7)	21
No	N	4	191	7	188	195
	%	80	90.5	70	91.3	90.3
	Total	5	211	10	206	216
P value		0.433		0.027		

DISCUSSION

DISCUSSION

The purpose of this study was to isolate, screen and identify MRSA and VRE to determine their frequency, co-colonization and co-existence in nasal and peri-rectal samples of patients admitted in MICU and PICU. In the mean time antibiotic susceptibility was also determine for these organisms. Both these organism are critical as they can be the cause of severe nosocomial infections so colonization of these are alarming to already ill patient. There is also a potential threat of transfer of vancomycin resistance gene from enterococci to *S. aureus* when both of these organisms co-exist or shear the same niche. This can result in emergence of vancomycin resistant *S. aureus* (VRSA) which now-a-days is becoming more prevalent resistant organism and infection caused by this organism is difficult to treat. Song et al, 2004 have also been reported the emergence of heterogeneous vancomycin resistant *S. aureus* strains from India and its neighboring countries.

Furthermore, the extensive and irrational use of antibiotics making these organisms more and more resistant which needed to be evaluated for proper treatment of critical ill patients. In enterococci, horizontal transfer of mobile genetic elements is the major cause of acquiring antibiotic resistance and spread. This transmission is mediated primarily by conjugative plasmids of the pheromone-responsive and broad host range incompatibility group 18 type. These plasmids were recently shown to promote genome plasticity in antibiotic resistant *E. faecalis*, and their involvement has been implicated in *E. faecium* as well. Furthermore, incompatibility group 18 plasmids played an important mediator role in transfer of vancomycin resistant gene from enterococci to MRSA (Palmer et al., 2010).

The areas which were focused for this study were MICUs and PICUs of tertiary care hospitals as in these areas nosocomial infection are more common. The reasons for this are critical illness with which patients come into ICUs, low immunity, usage of invasive devices, poor hygiene, multiple antibiotic treatment, medical and paramedical staff, bedding and clothing etc., these are the risk factors for colonization and cross contamination of these resistant bugs (Mona et al. 2005; Richard et al. 1999a, 1999b).

In this study, total number of MRSA and VRE isolated from both nasal samples and peri-rectal samples of 216 patients were 47 and 15, while 35 and 144 were MSSA and VSE respectively.

5.1: Frequency of MRSA and MSSA in nasal and peri-rectal samples

The nasal colonization of *S. aureus* was 68 out of 216 (31.5%). In these 16.7% were MRSA and 14.8% were MSSA (Fig. 4.17a). Various studies on MRSA and MSSA reported different rate of nasal and peri-rectal colonization. In Korea 36.1% nasal MRSA colonization was reported in hospitalized patients (Eun et al., (2006). Chen et al., (2010) reported overall 42% and 32% prevalence of *S. aureus* and MRSA nasal carriage among the patients respectively. Present study shows lower carriage rate of *S. aureus* and MRSA than these studies but higher carriage rate than Acton et al., (2009) reported results that was 20% for *S. aureus* and 9% for MRSA intestinal carriage rate in healthy individuals and patients.

From peri-rectum samples frequency was 7% (14 out of 216) with 5% MRSA and 2% MSSA (Fig. 4.17b). In comparison with peri-rectal isolates, nasal cavity was more colonized with MRSA and MSSA. Comparison of nasal MRSA with peri-rectal MRSA (16.7% vs. 5%; $P < .001$) resulted that 05 patients were having MRSA both in nasal and peri-rectal samples and rest of 31 and 06 cases were purely nasal and peri-rectal MRSA isolates respectively. This finding is somehow related to previous study of Zhang et al., (2007) which reported after sensitive PCR assay, that nasal swabs alone missing 24% of positive patients and addition of rectal swabs sampling significantly enhanced sensitivity.

Male patients were more colonized with MRSA than female and child group both in nasal and peri-rectal samples and found to be 21 (58.3%) out of 36 nasal MRSA, and 08 (72.7%) out of 11 peri-rectal MRSA (Table 4.9). A study conducted by Akpaka et al., (2006) reported that there was no significant difference in the gender distribution of the *S. aureus* isolates in terms of their susceptibility to methicillin. There were 49% (817/1668) male patients and 51% (851/1668) females patients which gave MSSA isolates whereas MRSA isolates were 49.6% (121/244) in males and 50.4% (123/244) in females. In a similar type of study, Osmon et al., (2004) reported no difference in the prevalence of MSSA and MRSA isolates from the

studied cases. Currie et al., (2008) reported nasal and rectal swabs sensitivity for detection of MRSA colonization as 68% and 62%, respectively which is much high than the present study. The difference in frequencies and colonization in our study may be due to geographical difference or difference in hygienic conditions of the patients.

The age group >35 years was the predominant group in nasal and peri-rectal colonization of MRSA and MSSA (Table 4.9). There were 18 (50%) out of 36 nasal MRSA isolates and 11 (34%) out of 32 nasal MSSA isolates from this group. Whereas peri-rectal colonization was 5 (45%) out of 11 MRSA and 1 (33.3%) out of 3 MSSA. In a similar study, Panhotra et al., (2005) screened 600 patients for nasal colonization and isolated 122 (20.2%) *S. aureus*. Of these 07 (1.1%) were MRSA and 155 (19.1%) were MSSA. Nasal colonization was high in young and adult patents and significant high colonization was seen among females like this study nasal colonization was high among adults in present study. In another study equal distribution of MSSA and MRSA was reported among different age groups with the exception of newborns (Hafeez et al. 2004).

5.2: Frequency of VRE and VSE in nasal and peri-rectal samples

Facklam et al., (1999) reported that the normally inhabit of enterococci in human is gastrointestinal tract however, they are also found in the mouth and vagina and on the skin of healthy individuals. In present study enterococci were 25.9% (56 out of 216) in nasal and 57.7% (103 out of 216) from peri-rectum swabs (Fig. 4.18a and 4.18b). The VRE isolates were 2.3% from nasal and 4.6% from peri-rectal samples and frequency of VSE isolates was 23.6% from nasal and 43.1% from peri-rectal samples. In a recent study by Navidinia et al., (2009) it is reported that 16.9% VRE which isolated from stool samples of hospitalized children. This carriage rate is much high then present study. Chadwick and Oppenheim (1997) reported carriage rate of glycopeptide-resistant enterococci from different body sites. They concluded that the glycopeptide-resistant enterococci were isolated most frequently from stool samples (95%) whereas from other site including mouth, nose, throat, rectum and perineum recovery was poor (25%). Present study also high in peri-rectal carriage rate of enterococci but nasal carriage rate is also comparatively high. On comparison of nasal-VRE and peri-rectal-VRE (2.3% vs. 4.6%; $P < .001$), it was

found that out of 15, four patients were positive for both nasal and peri-rectal-VRE isolates. So there were 56 patients (25.9%) harbouring enterococci in nasal cavity other than VRE although the main areas of colonization and isolation for enterococci are stool, rectum and peri-rectum area (Weinstein et al., 1996b). In this present study the frequency of VRE is also low in peri-rectal samples as well as in nasal samples but there is high frequency of nasal VRE and VSE isolates which may be due to poor hygiene of the patients.

Unlike MRSA & MSSA colonization among different age groups, VRE and VSE carriage rate in nasal and peri-rectal samples were high in age group <1 year patients (Table 4.11). Out of 51 nasal VSE colonized patients, 20 (39.2%) patients were under 1 year of age and 16 (31.4%) patients with age more than 35 years. Whereas out of 93 peri-rectal VSE, 31 (33.3%) were from age group <1 year and 22 (23.7) were from age group >35 years. So these two groups were more colonized with VSE both in nasal and peri-rectum area than rest of the groups. The VRE in these two groups were 3 (20%) in group <1 year and 2 (13%) in >35 year, out of total 15 isolates. An annual report on VRE from Louisiana office of public health, infectious disease epidemiology section (2006) reported that adults over the age of 65 years are of high risk of infection with VRE and high incidence rate than other age groups. According to this adult might be more likely to be admitted to hospitals than younger peoples so chances of VRE colonization and infection are more in them. The present study VRE and VSE colonization is more in children. This trend might be due to geological difference or more likely due to different hygienic conditions.

5.3: Co-colonization and co-existence of MRSA and VRE

In present study, on comparison of isolates of nasal MRSA and nasal VRE (16.7% vs. 2.3%; $P > 0.05$) no co-existence was found in any patient. Whereas in case of nasal MRSA and peri-rectal VRE (16.7% vs. 6.5%; $P > 0.05$), only 01 (10%) out of 10 VRE colonized patient was colonized with nasal MRSA. This showed very low frequency of co-colonization/co-existence i.e. 01 (0.5%) case out of 216 patients. As compared with studies of Furuno et al., (2005) they reported co-colonization rate as 2.7% while on further analysis of peri-rectal samples of 57 patients revealed 40.4% peri-rectal MRSA/VRE co-colonization. As compared with this, in present study the

co-colonization in much less. A study was carried out by Karchmer, et al., (2002) in a tertiary care facility where they founded that 2.5% of all patients were co-colonized with both MRSA and VRE. Franchi, et al, also observed that 28.6% colonized patients with VRE isolates were also colonized with MRSA.

In china, Wang et al., (2009) isolated predominant MRSA clone at Beijing Chaoyang Hospital from 2006 to 2007 that had the type III SCCmec element. All MRSA isolates were resistant to different antimicrobial agents and emergence of coexistence of MRSA and VRE in the same patient was found. From seven patients they isolated MRSA and VRE (E. faecium) simultaneously during their inpatient stay.

Similarly there were 02 (0.92%) patient having co-existence of peri-rectal MRSA/peri-rectal VRE. Out of these 02, one patient was also having nasal VRE-positive sample. There was also no co-existence of nasal MRSA and nasal VRE. Five cases of nasal MRSA-positive were also positive for peri-rectal MRSA similarly 04 peri-rectal VRE-positive were also positive in nasal VRE samples. Ray et al., (2003) studied the prevalence of gastrointestinal *S. aureus* colonization among thirty seven patients colonized with VRE and reported that 20 (54.1%) of these patients were also colonized with MRSA confirming their co-existence. The difference in co-colonization and co-existence in this present study with these studies might be due to geographical difference, difference in hygiene of the patients or use of antibiotics.

5.4: Frequency of MRSA and VRE in MICU and PICU

Frequency of nasal MRSA in MICU was 29.2% (31 out of 106) and 9.4% (10 out of 106) in peri-rectum (Fig 4.19). Of these 10 patients sampled from peri-rectum, 05 were colonized with nasal MRSA as well. A study conducted in India by Shagufta Naseer and Jayaraj (2010) reported 44.6% nasal MRSA colonization in MICU. In present study the isolation of nasal MRSA were much lower in MICU than this previous report.

There were 19 (17.9%) and 2 (1.9%) nasal and peri-rectal MSSA from MICU and two nasal MSSA positive were peri-rectal MRSA-positive as well. Qu et al., (2010) reported a total of 209 (20%) *S. aureus* isolates from 1044 nasal swabs of Chinese

military volunteers in which all were methicillin susceptible. Roghmann et al., (2007) reported that 24% patients carried *S. aureus* on their perineal skin, in which 16% were MSSA and 10% were MRSA and most of perineal carriers were also colonized in the anterior nares. All the patients in this cohort study were adults with spinal cord dysfunction. In present study the nasal isolation of MSSA is similar to the previous reported rate whereas in case of peri-rectal isolation, there is low isolation rate than the previous reported studies.

In PICU, among 110 patients there were 4.5% (5 out of 110) nasal and 0.9% (1 out of 110) peri-rectal MRSA (Fig. 4.20). There was one case of peri-rectal MRSA-positive was also colonized with MRSA in nasal cavity. MSSA were 13 (11.8%) and 1 (0.9%) were nasal and peri-rectal samples. El-Sayed et al., (2005) reported 25% colonization of MRSA in PICU and Merrer et al., (2004) mentioning 23% prevalence in nasal samples of PICU. This present study is much low in frequency of MRSA in PICU and MICU then previous studies.

As far as enterococci were concern, there were total 15 (6.9%) isolates of VRE from both nasal and peri-rectal samples. In MICU there were 2 (1.9%) nasal and 4 (3.8%) peri-rectal VRE colonize patients (Fig. 4.24). One peri-rectal VRE positive patients were also nasal VRE positive and one peri-rectal VSE positive patient was nasal VSE positive. VSE were 25 (23.6%) nasal and 43 (40.5%) peri-rectal isolates which were isolated from MICU and 16 patients were positive for VSE both in nasal and peri-rectal samples. Bonten et al., (2004) reported that 14% (43 out of 301) patients VRE-positive on admission in MICU. Rectal swabs were taken for VRE isolation in these patients. Weinstein et al., (1996b) reported that rectal and peri-rectal swabs equally sensitive for detection of VRE.

In PICU, there were 3 (2.7%) nasal and 6 (5.5%) peri-rectal VRE (Fig. 4.24). Three peri-rectal VRE-positive patients were nasal VRE-positive and one patient with peri-rectal VRE-positive was colonized with nasal VSE. A very low frequency of VRE was reported in PICU of Ain Shams University Hospitals that was 4% and further a lower rate of 1.5% VRE colonization were found after prolong admission (Sheren et al., 2005). A similar study by Glenn et al., (2004) reported that 18% patients of PICU were colonized with VRE on admission and 25% patients which were susceptible, became colonized later on.

VSE colonization in PICU was 26 (23.7%) in nasal and 50 (45.4%) in peri-rectal samples. Fourteen patients were colonized with VSE both in nasal and peri-rectal samples. Trabulsi et al., (1998) surveyed rectal colonization of VRE in paediatric patients with no active VRE infection and reported 42 patients (56%) out of 93 were colonized with enterococci and all were VSE. In present study the isolation of peri-rectal VSE from PICU is also near to this previous study but, isolation of nasal VSE are exceptional case. As enterococci are potential pathogen especially *E. faecalis*, its presence and colonization in nasal cavity is of great health hazard. Definitely this finding is indicating poor hygiene and poor cleanliness either of the ward or medical, paramedical personals and attendants around patient. This area needed more extended study to evaluate the reason of such large nasal colonization in paeds.

5.5: Identification of isolates with PCR

In this present study, the identification of isolates was confirmed by PCR analysis by using specie specific gene primers and antibiotic resistance gene primers to detect resistant gene. The primers used were already designed and studied (Table 3.1 and 3.3). These primers included, were specific for identification of *Staphylococcus* (Staph 16S), *S. aureus* (*nucA* and *coag.*), for identification of enterococcal specie (*ddl E. faecium and ddl E. faecalis*), antibiotic resistant genes, methicillin resistant gene (*mecA),* vancomycin resistant gene (*vanA* and *vanB*).

Identification of total 68 isolates of nasal *S. aureus,* which were coagulase and catalase positive, were confirmed with PCR by amplification of *nucA* gene. The positive isolates for *nuc* gene were compared with antibiotic susceptibility pattern by disc diffusion using cefoxitin, methicillin and oxacillin discs. It was found that all 32 (47.1%) susceptible and 36 (52.9%) resistant isolates to these three antibiotics were positive with *nucA* primer (Table 4.3). Whereas comparison with *mecA* primer result showed that 36 (100%) were positive and amplify *mecA* gene and identified as MRSA. Staph 16S and *coag* primer also showed positive result for all these 68 isolates.

A similar study with *nucA* primer was done by Kuzma et al., (2003) and reported that a single DNA band of 270 bp was detected in 29 *S. aureus* isolates isolated from milk samples and the reference strain and the primers were not able to amplify and detect DNA from bacteria other than *S. aureus*. Many other studies emphasize

on multiplex PCR for simultaneous detection *S. aureus* and MRSA by using two or more genes specific primers in PCR reaction mixture. Such a study was conducted in India by Rallapalli et al., (2008) reporting that MRSA isolates can be detected within three hours using multiplex PCR with *mecA* and *coag* gene specific primers and MRSA isolates gave two amplicon at 533 bp for *mecA* and 810 bp for *coag* specific products. Multiplex PCR is a successful tool for rapid identification and characterization of MRSA by targeting different genes. In a study by Oliveira and Lencastre (2002), they reported the development, validation, and application of a multiplex PCR strategy for quick identification and characterization of several MRSA clones. They suggested that this technique might be the efficient molecular typing tools for the characterization of MRSA clonal types and for the rapid identification of structural variants of the *mec* element (Oliveira and Lencastre 2002). Louie et al., (2002) described a multiplex PCR-based assay for the direct detection of methicillin-resistant staphylococci from blood culture bottles. They designed multiplex PCR assay to detect the *nuc*, *mecA*, and bacterial 16S rRNA genes with 99.2% and 100% sensitivity and specificity, respectively.

Perez-Roth et al., (2001) similarly described a multiplex PCR assay for the detection of clinically relevant antibiotic resistance genes present in *S. aureus* isolates and for the simultaneous identification of such isolates at the species level. They targeted *mecA*, *ileS*-2 (encoding high-level mupirocin resistance), and *femB* (encoding a factor essential for methicillin resistance) genes.

In the present study, different combinations of primers were tested for multiplex PCR. One was with *nucA* and *mecA* primers for MRSA and MSSA, other was *coagulase* and staph 16S primer for *S. aureus* and Staph 16S, *nucA* and *mecA* primers for detection of *S. aureus* and MRSA simultaneously. It was observed that *nucA* and *mecA* successfully identify all isolates when multiplex PCR was done using these primers.

Similarly the specie identification of enterococci (*E. faecalis and E. faecium*) and VRE, *ddl E. faecium* and *ddl E. faecalis* primers were used successfully. There are several different genes mediating vancomycin resistance, including *vanA, vanB, vanC, vanD,* and *vanE*. Of these, *vanA* and *vanB* were focused. The reason for this was that these were more common and importance due to their transmissible

ability. And only two species of enterococci were focused because *vanA* and *vanB* genes are found most frequently in *E. faecalis* and *E. faecium*. And another reason was that these enterococcal species detected in human bowel most commonly as resident flora.

In this study, PCR analysis successfully identified 26 (46.4%) and 30 (53.6%) isolates as *E. faecium* and *E. faecalis* in nasal cavity respectively in which 05 (8.9%) isolates were VRE (02 *E. faecium* and 03 *E. faecalis*) and were positive for *vanA* gene (Table 4.4). No *vanB*-positive strain was found in nasal cavity. Whereas in peri-rectal isolates, there were 55 (53.4%) *E. faecium* and 48 (46.6%) *E. faecalis*, in which 10 (9.7%) isolates were VRE (05 each *E. faecalis* and *E. faecium*). In a study, Dutka-Malen et al., (1995) used both these *ddl* primers and *vanA* and *vanB* primers to identify *E. faecalis* and *E. faecium* as well as vancomycin resistant. They successfully identified 23 enterococcal isolates out of 26, and in them 13 were *E. faecalis* and 07 were *E. faecium.*

Colony PCR was performed on enterococci to identify vancomycin-resistance as well as specie identification directly from an overnight culture. Colony PCR is a method in which bacterial colonies are directly screened by PCR. Test is successful in the sense that the laborious DNA extraction process is by past. In the present study colony PCR was successful in identifying two enterococcal reference isolates of *E. faecalis* and *E. faecium* along with *vanA* gene.

In a similar type of study by Tsuchizaki et al., (2000), colony PCR was performed as multiplex PCR for identification of antibiotic resistance gene in MRSA and enterococci. The study identify two important factors in colony PCR, one was bacterial colony size and second was DNA polymerase with high performance. The study resulted good amplification of the target gene regions of *mec*A and aac(6')/aph(2") that are responsible for antibiotic resistance to methicillin and arbekacin in MRSA and enterococci respectively.

In a recent study, Yi et al., (2010) developed a method based on colony PCR for rapid screening of class IIa bacteriocin-producing lactic acid bacteria. The principle was simply to detect the presence of bacteriocin gene by using specific degenerate primers to amplify a 3 kb fragment from bacteriocin structural gene to histidine

kinase gene regions. Colony PCR is a novel method for identification and detection of different target genes in bacteria.

5.6: Antibiotic susceptibility testing

All the identified isolates of MRSA and VRE were subjected to antibiotic susceptibility study by disc diffusion and MICs. Both nasal and peri-rectal-MRSA isolates were 80 to 100% resistant to ciprofloxacin, erythromycin, gentamicin, levofloxacin and tetracycline where as all were susceptible to linezolid, quinupristin/dalfopristin, teicoplanin and vancomycin and moderately-resistant to imipenem. Of these amoxicillin/clavulanic acid was 97% resistant in nasal while 54% in peri-rectal isolates (Table 4.5). This high resistance is alarming as MRSA is a potential pathogen and can cause serious illness so its colonization and such resistance is a serious matter. In contrast, the nasal MSSA isolates were 93.8 % susceptible to ciprofloxacin, 78.1% for erythromycin and 65.6% for tetracycline while MSSA isolates from peri-rectal area were 66.7% susceptible to amoxicillin/clavulanic acid, ciprofloxacin, imipenem and erythromycin. All MSSA isolates of peri-rectal area were resistant to tetracycline. All nasal and peri-rectal MSSA isolates were susceptible to linezolid, teicoplanin, quinupristin/dalfopristin and vancomycin. A study on prevalence and antimicrobial resistance pattern of MRSA in Assam, India, reported that all MRSA isolates were significantly more resistant to antibiotics than MSSA isolates. Many of the isolates (37.5%) were resistant to all antibiotics (Saikia et al., 2009).

All isolates VRE of nasal and peri-rectal areas showed resistance to gentamicin, erythromycin, methicillin, oxacillin and vancomycin. Only linezolid and quinupristin/dalfopristin were susceptible against all VRE, while other antibiotics tetracycline, levofloxacin, ciprofloxacin, ampicillin and amoxicillin/clavulanic acid showed 40-60% resistance (Table 4.6). All nasal and peri-rectal VSE showed resistant to methicillin and oxacillin and other antibiotics susceptibility varied greatly but all were sensitive to linezolid, quinupristin/dalfopristin and vancomycin. Caballero-Granado et al., (1998) reported high-level resistance to gentamicin. Out of 93 patients with bacteremia 31(33%) were because of enterococci having high-level resistance to gentamicin. In present study both VRE and VSE isolates were resistant to gentamicin.

In a previous study by Aleyasin et al., (2007) at Tehran Baghyatallah Hospital reported antibacterial resistance among isolated enterococci. The VRE isolates were resistant to ampicillin (75%), ciprofloxacin (41.6%), chloramphenicol (33.3%), erythromycin (50%), gentamicin (41.6%) and tetracycline (58%). The reported MICs of these VRE isolates with vancomycin were ranged between 32-512 µg/ml.

MICs were measured against four antibiotics ciprofloxacin, oxacillin, tetracycline and vancomycin (Table 4.7a & 4.7b). Results showed that there was no MRSA and MSSA from nasal and peri-rectal area were found vancomycin resistant, so no vancomycin-resistant *S. aureus* (VRSA) and vancomycin-intermediate *S. aureus* (VISA) were isolated in this present study. There were 16 (23.5%) nasal *S. aureus* (both MSSA and MRSA) were susceptible to tetracycline and 25 (36.8%) were susceptible to ciprofloxacin. 32 (47.1%) isolates were sensitive for oxacillin all were MSSA remaining were MRSA having MIC greater than 2 mg/L. All nasal *S. aureus* isolates were susceptible to vancomycin with MIC 1-2 mg/L and all 14 peri-rectal- *S. aureus* (both MRSA and MSSA) were resistant to tetracycline whereas 3 out of 14 were susceptible to ciprofloxacin and oxacillin as these were having lower MICs than 1 mg/L and 2 mg/L respectively. These three isolates most probably were peri-rectal MSSA. All peri-rectal *S. aureus* were sensitive to vancomycin ranged from 1 to 2 mg/L.

All nasal enterococci (both VRE and VSE) were resistant to tetracycline and oxacillin (Table 4.8a & 4.8b). In both the case the MIC was higher than the breakpoint concentration. The 3 isolate were susceptible to ciprofloxacin. Fifty one (91.1%) out of 56 isolates that were most probably the VSE, were sensitive with vancomycin inhibiting in range 1 to 4 mg/L. The remaining five isolates were resistant with MIC of 512 mg/L. Similarly in case of peri-rectal enterococci all 103 isolates were resistant to tetracycline and oxacillin similar to nasal isolates. While in ciprofloxacin only 1 (1%) sample was sensitive. In case of vancomycin 93 (90.2%) were sensitive and were VSE while remaining 10 isolates were inhibited at 64 mg/L and 512 mg/L, and were know to be VRE.

5.7: Antibiotic therapy and colonization of MRSA & VRE

This present study evaluate that 13% patients who were either on single antibiotic therapy or multiple antibiotic treatment, were colonized with MRSA ($P < 0.05$) and 10 (4.6%) were colonized with VRE ($P > 0.05$). 15.3% of patients with age group >35 years were using single and combination antibiotics and found to be more colonized by MRSA and VRE. A study conducted by Srinivasan et al., (2006) studied the risk factors and associated problems in management of MRSA and reported that 44% of the patients that had consumed antibiotics (either as outpatients or as inpatients) for more than 2 to 3 weeks, developed infection by MRSA.

The patients on vancomycin treatment were found to be 21(9.7%) (Table 4.17). Of these, 4 (19%) were found to be VRE colonized (1 nasal and 3 peri-rectum). Similarly 7 (out of 15) VRE colonized patients were using 3rd generation cephalosporins and 3 (out of 15) were on combination therapy of beta lactams and 3rd generation cephalosporins. There is not much strong data that we can conclude association of VRE with vancomycin and other antibiotics usage. However a study conducted by Carmeli et al., (2002) reported that patients who were on prolonged treated with 3rd generation cephalosporins, metronidazole and quinolones were significantly at higher risk for VRE colonization and treatment with vancomycin is not a risk factor for VRE colonization and infection. Another study highlighting the same aspect reported that intravenous treatment with vancomycin does not significantly increase VRE in the stool so if given over a short period, it will not increase the risk of VRE infection (Nahum et al., 2003).

CONCLUSIONS

CONCLUSIONS

The present study ends up with the following conclusions:

- Frequency of MRSA was more in MICU than PICU and the patients above 35 years of age were of risk of acquiring more MRSA than other age groups. While VRE was more prevalent in PICU than MICU.
- Patients in PICU with age less than 1 year were more colonized with nasal VSE strains i.e. 50% VSE (*E. faecium*) and 29.6% VSE (*E. faecalis*).
- Only one (0.5%) patient was co-colonized with nasal MRSA/peri-rectal VRE, so the prevalence of MRSA/VRE was found to be so low. Similarly one (0.5%) patient was having co-existence of peri-rectal MRSA/peri-rectal VRE. There was also no coexistence of nasal MRSA and nasal VRE. Both these were isolates from MICU. Three patients were having MRSA both in nasal and peri-rectal samples. Similarly 03 were having VRE at both the sites.
- All MRSA and MSSA were successfully identified by simple and multiplex PCR with *nucA*, *mecA*, *coag.*, *coagulase*, staph 16S and 16SrRNA primers.
- VRE and VSE were identified for specie and vancomycin resistance successfully by PCR with *ddl E. faecalis*, *ddl E. faecium*, *vanA* and *vanB* primers.
- Majority of MRSA and VRE isolates were also resistant to other treated antibiotics which indicate the continuous irrational use of antimicrobials.
- Both nasal and peri-rectal-MRSA isolates were 80 to 100% resistant to ciprofloxacin, erythromycin, gentamicin, levofloxacin and tetracycline where as all were susceptible to linezolid, quinupristin/dalfopristin, teicoplanin and vancomycin and were moderately-resistant to imipenem. Of these amoxicillin/clavulanic acid was 97% resistant in nasal while 54% in peri-rectal isolates.
- All the MRSA isolates from MICU and PICU were susceptible to linezolid, quinupristin/dalfopristin, teicoplanin and vancomycin.
- All isolates of nasal and peri-rectal VRE showed resistance to gentamicin, erythromycin, methicillin, oxacillin and vancomycin. Tetracycline, levofloxacin, ciprofloxacin, ampicillin and amoxicillin/clavulanic acid showed 40-60 percent resistance for both types of isolates.

- Nasal and peri-rectal VRE isolates were sensitive to linezolid and quinupristin/dalfopristin only.
- Both nasal and peri-rectal isolates of MRSA have MICs rang for oxacillin from 32-512 mg/L and 0.125-1 mg/L for MSSA isolates and for vancomycin both MRSA and MSSA ranged from 1-2 mg/L for all. For ciprofloxacin and tetracycline both strains (nasal/peri-rectal-MRSA and nasal/peri-rectal MSSA) ranged from 0.125-128 mg/L and 0.25-256 mg/L respectively.
- The MIC range of vancomycin for nasal and peri-rectal VRE isolates ranged from 64-512 mg/L whereas the isolates which were in range between 1-4 mg/L were VSE. Both VSE and VRE were resistant to oxacillin with MIC ranged from 4-512 mg/L. MIC for Ciprofloxacin ranged from 1-512 mg/L and tetracycline ranged from 1-256 mg/L for both the strains.
- There were 13% patients who were either on single antibiotic therapy or multiple antibiotic treatment, were colonized with MRSA and 10 (4.6%) were colonized with VRE. 15.3 percent of patients from age group >35 years were on single and combination antibiotics and found to be more colonized by MRSA and VRE.

FUTURE PROSPECTS & RECOMMENDATIONS

FUTURE PROSPECTS AND RECOMMENDATIONS

The present study ends up with satisfactory conclusions but there are still many aspects which need more research including:

- Molecular typing of isolated MRSA and VRE with different molecular techniques like PFGE to check for their clonality.
- Sequencing of the resistant *mec*A and *van*A gene to check the genetic variation among the isolates from other prevalent worldwide.
- Study of virulent factors to judge the virulence of the organisms.
- Synergistic effect of antibiotic combinations or combinations with neon-particles on MRSA and VRE for assessing better treatment regimen in case of infections with these organisms.

Keeping in view the current scenario of antibiotic usage and increasing resistance against commonly available antibiotics, it is the need of time for the development of new antibiotics and other alternates to combat infections caused by resistant bugs. Biomedical research is in progress for better curable agents but resistant organisms like MRSA and VRE requires careful monitoring and surveillance programs to minimize the hazardous outcomes. Multiple antimicrobial therapy, irrational usage and improper doses of these drugs opens gateway for generating resistance.

There are many other aspects for working on these resistant organisms but the most recommended in my point of view is the synergistic study of antibiotics with neon-particles especially ZnO on MRSA and VRE. There is possibility of conjugating of ZnO or other neon-particles with available antibiotics which are now resistant to MRSA and VRE and check for enhancement in their activity. Another aspect is to target the virulent factor either gene or protein which is responsible for virulence as in MRSA or *S. aureus* coagulase gene is the one which enhances virulence of these organisms. By targeting this factor and inhibiting this can decrease the virulence of MRSA and in future that method can be used as a remedy for MRSA infections.

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APPENDIX

Appendix A: Colony morphology of staphylococci and enterococci on SBA, MSA and BAA media

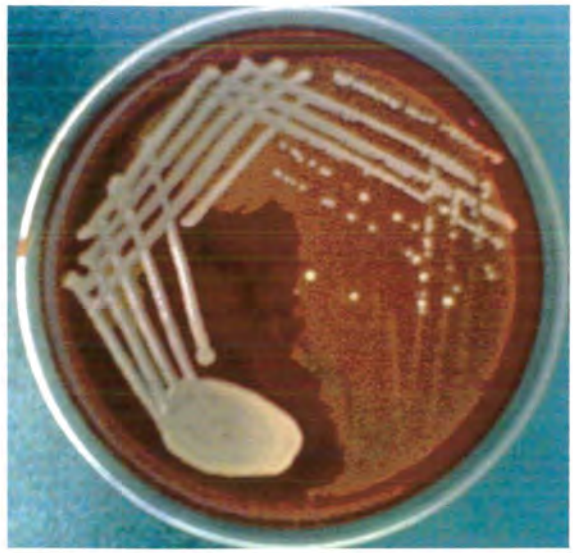

Fig. A-1a

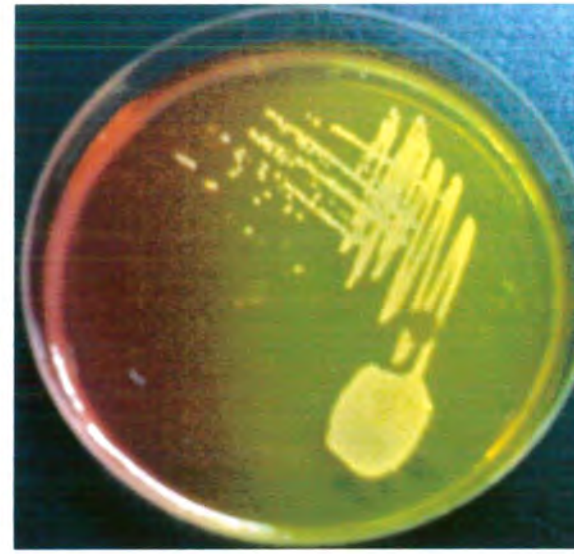

Fig. A-1b

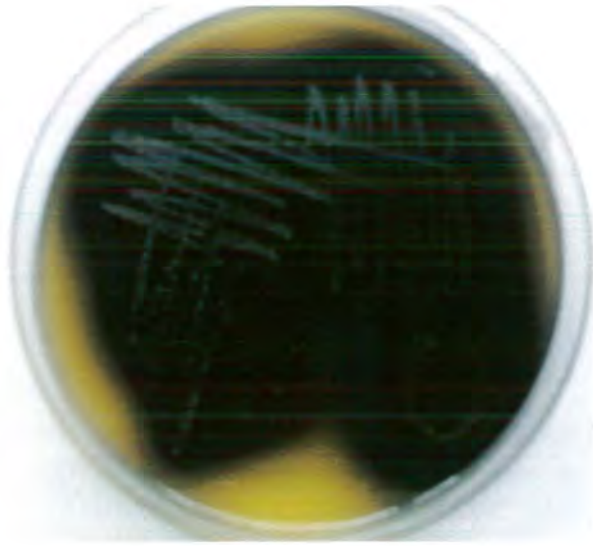

Fig. A-1c

Fig. A1a: Colony morphology of staphylococci on Sheep Blood Ager showing β-haemolysis after overnight growth.

Fig. A1b: Colony morphology of staphylococci on Mannitol Salt Agar showing yellow zone colonies.

Fig. A1c: Colony morphology of enterococci on Bile Aesculin Ager showing pin-point colonies showing black zone.

Appendix B: Agarose gel analysis of the 270 bp *nucA* amplicons from various *S. aureus*

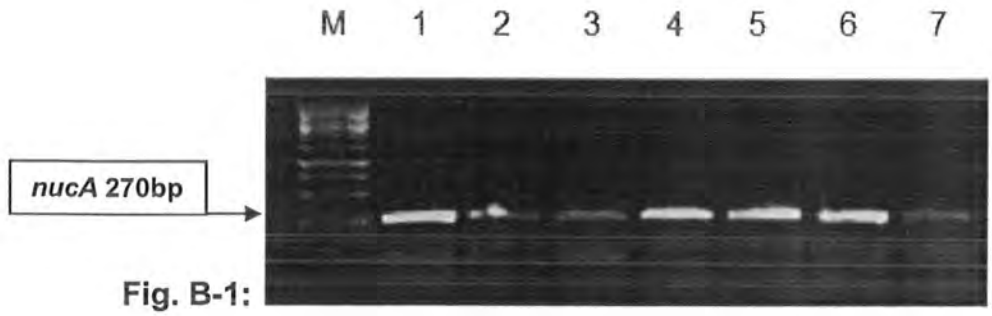

Fig. B-1:

Fig. B-2:

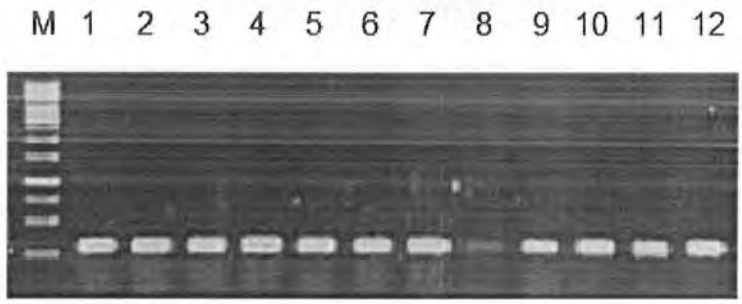

Fig. B-3:

Fig. B-1: M: 1 kb (GeneRuler), L1: SH1000, L2: N1S, L3: N3S, L4: N5S, L5: N12S, L6: P26S, L7: P27S, L8: N38S, L9: N50S.

Fig. B-2: M: 1 kb (GeneRuler), L1: SH1000, L2: N51S, L3: N52S, L4: N57S, L5: N60S, L6: N61S, L7: N65S, L8: N67S, L9: N70S, L10: N71S, L11:P71S.

Fig. B-3: M: 1 kb (GeneRuler), L1: SH1000, L2: N73S, L3: N74S, L4: N75S, L5: N83S, L6: N84S, L7: N85S, L8: N86S, L9: N87S, L10: N88S, L11: N89S, L12: P89S.

M: DNA Marker, **L:** Lane

Appendix B (Continue)

Fig. B-4:

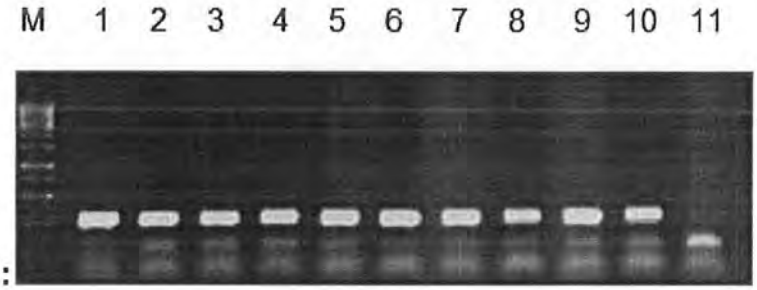

Fig. B-5:

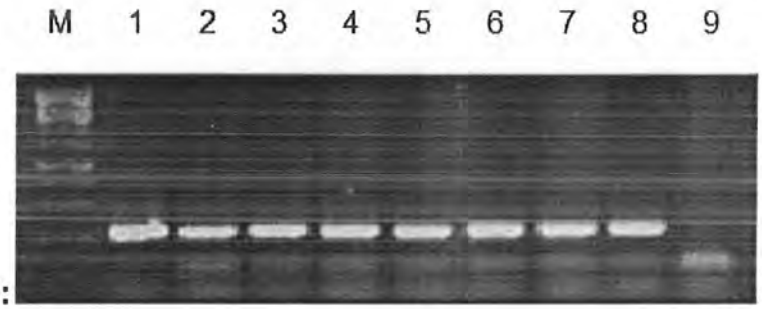

Fig. B-6:

Fig. B-4: M: 1 kb DNA Marker (GeneRuler), L1: SH1000, L2: P90S, L3: N91S, L4: P90S, L5: N93S, L6: P99S, L7: N101S, L8: P101S.

Fig. B-5: M: 1 kb DNA Marker (GeneRuler), L1: SH1000, L2: N102S, L3: N105S, L4: N109S, L5: P109S, L6: N110S, L7: N113S, L8: N119S, L9: N130S, L10: N133S, L11: NC.

Fig. B-6: M: 1 kb DNA Marker (GeneRuler), L1: SH1000, L2: N144S, L3: N157S, L4: N158S, L5: N159S, L6: N160S, L7: N162S, L8: N164S, L9: NC.

NC: Negative Control (Sterilized Distilled Water)

Appendix B (Continue)

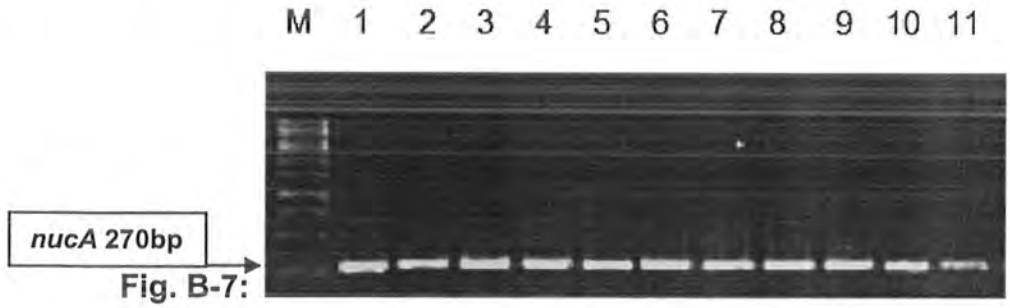

Fig. B-7:

Fig. B-8:

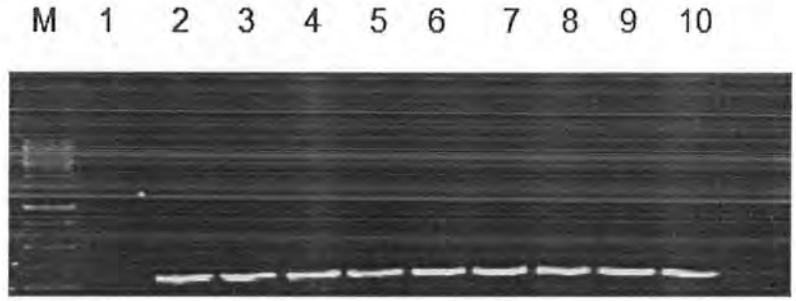

Fig. B-9:

Fig B-7: M: 1 kb DNA Marker (GeneRuler), L1: SH1000, L2: N169S, L3: N170S, L4: N177S, L5: N178S, L6: N184S, L7: P186S, L8: N187S, L9: N189S, L10: N190S, L11: N191S.

Fig B-8: M: 1 kb DNA Marker (GeneRuler), L1: SH1000, L2: N192S, L3: N203S, L4: N208S, L5: N209S.

Fig B-9: M: 1 kb DNA Marker (GeneRuler), L1: NC, L2: SH1000, L3: N210S, L4: N211S, L5: N212S, L6: N213S, L7: N214S, L8: P214S, L9: N215S, L10: N216S.

NC: Negative Control (Sterilized Distilled Water)

Appendix B (Continue)

Fig. B-10: M: 1 kb DNA Marker (GeneRuler), L1: MRSA252, L2: P72S (CoNS), L3: P83S (CoNS), L4: N89S, L5: N101S, L6: P110S (CoNS), L7: P133S (CoNS), L8: P136S (CoNS), L9: N177S, L10: N178S.

Appendix C: Agarose gel analysis of the 533 bp *mecA* amplicons from various *S. aureus* isolates

Fig. C-1:

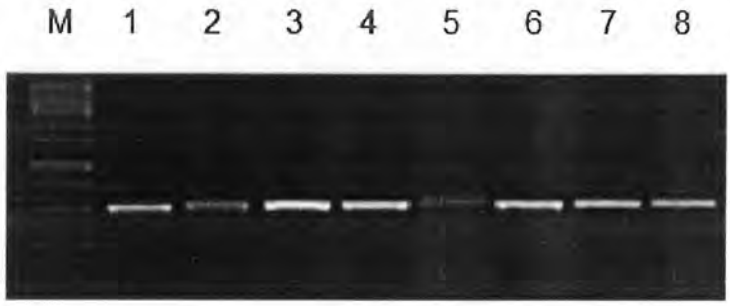

Fig. C-2:

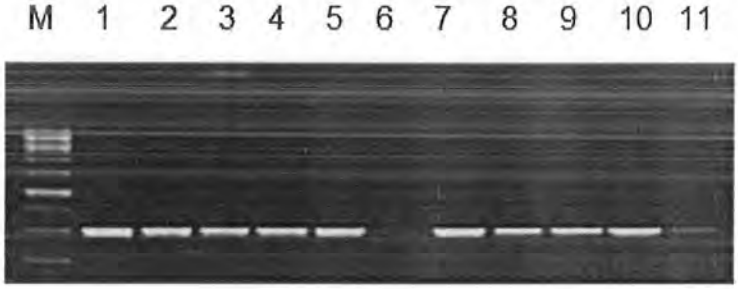

Fig. C-3:

Fig. C-1: M: 1 kb DNA Marker (GeneRuler), L1: MRSA252, L2: Negative Control (SD Water), L3: N74S, L4: N75S, L5: N83S, L6: N84S, L7: N85S, L8: N86S, L9: N87S, L10: P89S, L11: P90S, L12: N91S, L13: P91S, L14: N93S, L15: P99S.

Fig. C-2: M: 1 kb DNA Marker (GeneRuler), L1: MRSA252, L2: N101S, L3: N102S, L4: N105S, L5: N109S, L6: P109S, L7: N110S, L8: N113S.

Fig. C-3: M: 1 kb DNA Marker (GeneRuler), L1: N158S, L2: N164S, L3: N168S, L4: N169S, L5: N170S, L6: N171S, L7: N177S, L8: N178S, L9: N209S, L10: N213S, L11: P214S.

Appendix D: Detection of *E. faecalis* by *ddl E. faecalis* PCR

Fig. D-1:

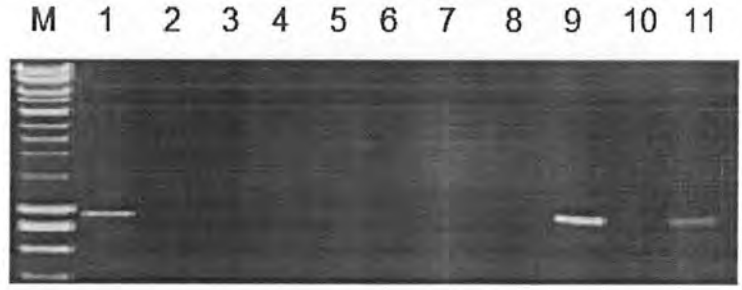

Fig. D-2:

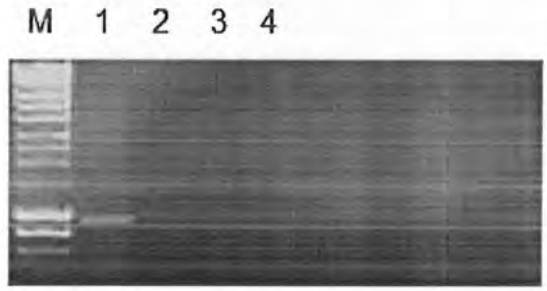

Fig. D-3:

Fig. D-1: M: 1 kb DNA Marker (Hyper ladder 1), L1: OG1RF Liv59, L2: P36E, L3: N36E, L4: P37E, L5: N37E: L6: P38E, L7: P40E, L8: N40E, L9: P42E, L10:P43E, L11: OG1RF Liv59

Fig. D-2: M: 1 kb DNA Marker (Hyper ladder 1), L1: OG1RF Liv59, L2: P5E, L3: P6E, L4: P11E, L5: P12E: L6: P15E, L7: P18E, L8: P21E, L9: N22E, L10:P45E, L11: N32E.

Fig. D-3: M: 1 kb DNA Marker (Hyper ladder 1), L1: OG1RF Liv59, L2: N47E, L3: P48E, L4: P69E.

Appendix E: Detection of *E. faecium* by *ddl E. faecium* PCR

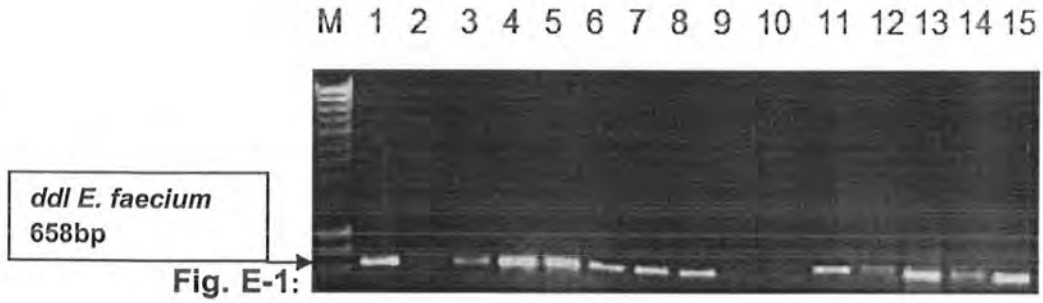

Fig. E-1:

Fig. E-2:

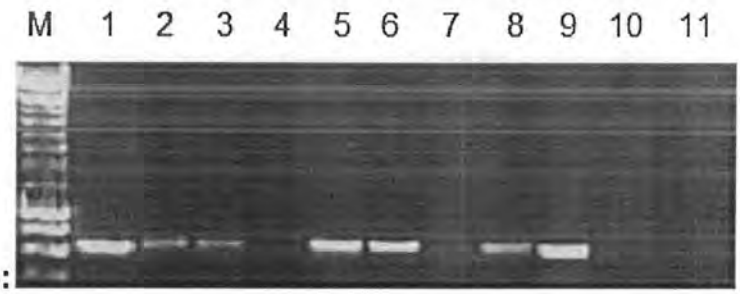

Fig. E-3:

Fig. E-1: M: 1 kb DNA Marker (Hyper ladder 1), L1: TX00016 Liv66, L2: P5E, L3: P6E, L4: P11E, L5: P12E: L6: P15E, L7: P18E, L8: P21E, L9: N22E, L10:P22E, L11: P25E, L12: P26E, L13: N26E, L14: P27E, L15: N27E.

Fig. E-2: M: 1 kb DNA Marker (Hyper ladder 1), L1: TX00016 Liv66, L2: P29E, L3: P31E, L4: N32E, L5: N32E: L6: P36E, L7: N36E, L8: N37E, L9: P37E, L10: P38E, L11: N40E.

Fig. E-3: M: 1 kb DNA Marker (Hyper ladder 1), L1: TX00016 Liv66, L2: P40E, L3: P42E, L4: P43E, L5: P45E: L6: N47E, L7: P48E, L8: N54E, L9: P69E.

Appendix F: Detection of VRE by *vanA* gene PCR

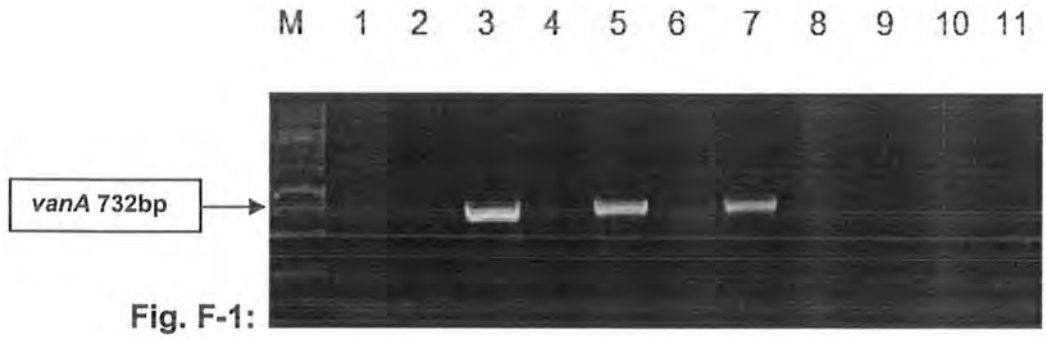

Fig. F-1:

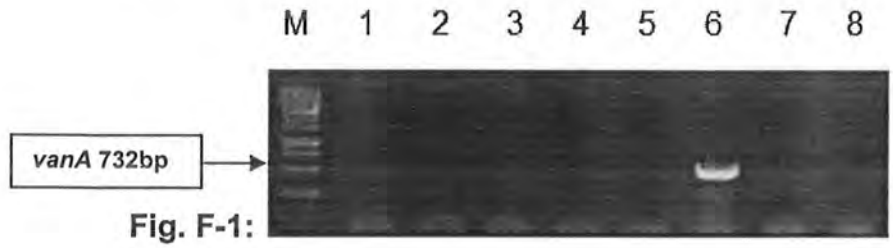

Fig. F-1:

Fig F-1: M: 1 kb DNA Marker (GeneRuler), L1: TX00016 LIV66, L2: BLANK, L3: P130E, L4: N141E, L5: P143E, L6: N148E, L7: P149E, L8: P153E, L9: P163E, L10: P167E, L11: P168E.

Fig F-2: M: 1 kb DNA Marker (GeneRuler), L1: P5E, L2: P6E, L3: P11E, L4: N12E, L5: P15E, L6: P18E, L7: P21E, L8: N22E.

Appendix G: Patient data form

Ref #

PATIENT DATA FORM

PERSONAL INFORMATION **DATE:**

Patient Name		Age Group	
Gender		Marital Status	
Admission No.			
Date of Admission		Time of Admission	

SOCIOECONOMIC STATUS ☐ Rural ☐ Urban

☐ Low Class ☐ Middle Class ☐ High Class

REASON FOR ADMISSION

FAMILY HISTORY

HISTORY OF VANCOMYCIN INTAKE ☐ Yes ☐ No.

If yes

Dosage (mg)	Daily Dose	Duration	When

INVASIVE PROCEDURES:

☐ Central venous catheterization ☐ Intubations ☐ Urine catheterization

☐ Renal dialysis ☐ Gastroscopy ☐ Colonoscopy.

☐ Ventilator ☐ Suction Catheter ☐ I/V Cannula

CURRENT MEDICATION

Name of Medication	Dosage (mg)	Daily Dose	Duration