DETECTION OF *erm* GENE AMONG INDUCIBLE CLINDAMYCIN RESISTANT *STAPHYLOCOCCAL* ISOLATES IN CLINICAL SAMPLES

Dissertation submitted to

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

CHENNAI- 600032

In partial fulfillment of the requirement for the degree of

Doctor of Medicine in Microbiology (Branch IV)

M. D. (MICROBIOLOGY)

DEPARTMENT OF MICROBIOLOGY

TIRUNELVELI MEDICAL COLLEGE

TIRUNELVELI- 11

APRIL 2015

CERTIFICATE

This is to certify that the dissertation entitled, **''DETECTION OF *erm* GENE AMONG INDUCIBLE CLINDAMYCIN RESISTANT *STAPHYLOCOCCAL* ISOLATES IN CLINICAL SAMPLES ''** by **Dr. A.UMA MAHESWARI**, Post graduate in Microbiology (2012-2015), is a bonafide research work carried out under my direct supervision and guidance and is submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai, for M.D. Degree Examination in Microbiology, Branch IV, to be held in April 2015.

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NAME OF PRINCIPAL INVESTIGATOR: Dr. A. Umamaheswari, MBBS.,
DESIGNATION OF PRINCIPAL INVESTIGATOR: Post Graduate in MD Microbiology
DEPARTMENT & INSTITUTION: Department of Microbiology, Tirunelveli Medical College

Dear Dr. A. Umamaheswari, *The Tirunelveli Medical College Institutional Ethics Committee (TIREC) reviewed and discussed your application during the IEC meeting held on 14.05.14.*

THE FOLLOWING DOCUMENTS WERE REVIEWED AND APPROVED

1.	TIREC Application Form	☐
2.	Study Protocol	☐
3.	Department Research Committee Approval	☐
4.	Patient Information Document and Consent Form in English and Vernacular Language	☐
5.	Investigator's Brochure	☐
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THE PROTOCOL IS APPROVED IN ITS PRESENTED FORM ON THE FOLLOWING CONDITIONS

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

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CONTENTS

ABBREVIATIONS

MSSA	**-**	**Methicillin sensitive *Staphylococcus aureus***
MRSA	**-**	**Methicillin resistant *Staphylococcus aureus***
MSCONS	**-**	**Methicillin sensitive *Coagulase Negative Staphylococcus***
MRCONS	**-**	**Methicillin resistant *Coagulase Negative Staphylococcus***
CL	**-**	**Clindamycin**
ERY	**-**	**Erythromycin**
iMLSB	**-**	**inducible Macrolide Lincosamide Streptogramin B**
cMLSB	**-**	**constitutive Macrolide Lincosamide Streptogramin B**
MS phenotype	**-**	**Macrolide Lincosamide Streptogramin B phenotype**
PCR	**-**	**Polymerase chain reaction**

ABSTRACT

DETECTION OF *ERM* GENE AMONG INDUCIBLE CLINDAMYCIN RESISTANT *STAPHYLOCOCCAL* ISOLATES IN CLINICAL SAMPLES

BACKGROUUND OF THE STUDY:

Staphylococcus aureus infection is associated with hospital and infections which arise from the community . The emergence of resistance to most antimicrobial agents in *Staphylococci* indicates the need for new effective agents in the treatment of *Staphylococcal* infection. Among the alternatives available Clindamycin is considered to be safe, effective and less costly agent.In vitro routine tests for Clindamycin susceptibility may fail to detect inducible Clindamycin resistance due to *erm* genes, resulting in treatment failure, thus necessitating the need to detect such resistance by a simple D-test on routine basis.

AIM:

The present study is aimed to detect *erm* gene in inducible Clindamycin resistant *Staphylococcal* isolates and to study the relationship between Clindamycin and Methicillin resistance.

MATERIALS AND METHODS:

During the study period a total of 100 non duplicate clinical isolates of *Staphylococci* were collected from different clinical samples like aural swabs, wound swabs, pus and vaginal swabs from both in-patient and out-patient departments of Tirunelveli Medical College The *Staphylococcal* species were identified by standard biochemical techniques.and their antibiotic susceptibility tested by standard disk diffusion method on Muller Hinton agar [MHA] according to the standards of clinical and laboratory standards institute[CLSI].Detection of inducible Clindamycin resistence was performed by D-test on a Mueller Hinton agar plate with a lawn culture of the isolate which was adjusted to 0.5 Mcfarland's concentration . Discs of Clindamycin(2µg),Erythromycin(15µg) were kept at a distance of 15mm. The disc diffusion D test, showed

- **Inducible MLSB phenotype (iMLSB)**
- **Constitutive MLSB phenotype (cMLSB)**
- **MS phenotype**

The *Staphylococcal* isolates from MLSBi were further tested by Real-Time PCR for *erm*A/*erm*B/*erm*C genes.

RESULTS:

In this study among the 100 *Staphylococcal* isolates 80% were *Staphylococcus aureus* and rest 20% were *coagulase negative staphylococcus.* Out of strains isolated 80% were from hospitalized patients . The study group contained 55% males and 45% females and Male to female sex ratio was 1.2 2:1. Majority of the study group were found among the age group 21-30 yrs.

Majority of the *Staphylococcal aureus* 75% and CONS 100% were isolated from pus and majority of the MSSA 76.36% and 72% MRSA were also from pus. Inducible MLSB resistance was detected by Disc diffusion test (D-test).

In D-test the phenotypic distribution of iMLSB , cMLSB and MS were 35%, 12.5% and 52.5% respectively. In the phenotypic distribution of iMLSB *Staphylococcus .aureus* was 37.5% and *CONS* 25%,. MSSA 31.58% and MRSA 46.15%.

Majority of the iMLSB was susceptible to Ciprofloxacin ,Cotrimoxazole and Gentamycin. while cMLSB and MS phenotype were resistant to ciprofloxacin when compaired to iMLSB .There was high rate of resistance exhibited by cMLSB towards Cefoxitin when compaired to iMLSB. No resistance was observed to vancomycin.

Genetic analysis by real time PCR performed on iMLSB showed 21.42% *erm* A,7.14% *erm* B,14.3% *erm* C,50% *erm* A and *erm* C and 7.14% *erm* B and *erm* C.

CONCLUSION:

In this present study D-test is easy to perform and found inexpensive for practical purpose than PCR which is the gold standard .This test is used to detect an inducible Clindamycin resistance in staphylococci as a routine work in clinical microbiology laboratories. This test help us to provide confident laboratory reports and Clindamycin can be omitted in patients with infections caused by inducible Clindamycin resistance staphylococci, and therapeutic failures may be thus avoided.

Key words: *Staphylococcus aureus, Coagulase Negative Staphylococcus,* Inducible MLSB phenotype (iMLSB), *erm* A, *erm* B and *erm* C genes.

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1. INTRODUCTION

Staphylococcus aureus infection is associated with hospital and infections which arise from the community . *Staphylococci* mainly colonizes various skin and mucosal surfaces. Infections occur due to trauma or abrasion to these surfaces. Person to person spread of *Staphylococci* gets established as part of normal flora of the recipient or produces infection on introduction to sterile sites during surgery.

Staphylococci produces a wide spectrum of infections. Several toxins and enzymes are produced by *Staphylococci* that cause tissue invasion and survival of the organism resulting in life threatening infections.

Most of the *Staphylococci* causing serious nosocomial infections have acquired antimicrobial resistance. The rising level of resistance has been found in *Staphylococcus aureus* and *Coagulase Negative Staphylococcus (CONS*) to a wide range of antibiotics . This has lead to significant threat in the treatment efficacy of both *Staphyloccus aureus* and *Coagulase Negative Staphylococcus (CONS*). [1]

Healthy children are common carrier of *Staphylococci.* Individuals who are nasal carriers may develop clinical infection . The emergence

of Methicillin resistant *Staphylococcus aureus (MRSA*) has become a notorious hospital acquired infection . In early 1940s Penicillin was introduced and shortly resistance to Penicillin group of antibiotics began emerging .

Resistance arised due to the production of enzyme β-lactamase that hydrolyzed the ring present the β-lactam antibiotics. The genes that are responsible for this resistance are found on class II plasmid [2] Methicillin was introduced in Britain for clinical use and soon resistant strains developed against other β-lactamase-resistant Penicillins and to Methicillin. Today 75-95 percent Penicillin-resistant strains has been recognized with the maximum being reported among hospital isolates. In recent days there is a rise in resistance for Methicillin.

MRSA strain was first isolated in 1982, and intermediate resistant *MRSA* strain to Vancomycin (Mu50) was identified in 1997.[3] *MRSA* resulted in a wide variety of suppurative infections including abscesses, exudative dermatitis, severe pyoderma, fistulas and postoperative wound infections. Methicillin resistance has become an increasing problem as resistance to other antimicrobial agents occur in *MRSA* [4]

The utility of older antimicrobials like Trimethopriim Sulfamethoxazole and Clindamycin was encouraged due to the

emergence of Methicillin resistance .This raised an interest to use Macrolide-Lincosamide-Streptogramin B (MLSB) antibiotics to correct *Staphylococcal* infections.

It is not advisible to treat empirically *Staphylococcal* infection due to the rise of multi drug resistance among *MRSA.* Clindamycin has been an better option due to good tissue penetration of all tissues except central nervous system.[5] . Thus Clindamycin has become one of the attractive options to treat *MRSA i*nfections those acquired in the community (*CA-MRSA*) as well as in the hospital. [6]

The Macrolide-Lincosamide Streptogramin B (MLSB) antibiotics which was used for treating *Staphylococcal* infections also resulted in resistant strains. [7] Since 1968 Clindamycin resistance induced by Macrolides was noted among the *Staphylococci* that resulted in treatment failure with Clindamycin . The development of resistant mutants resulted in failure in the treatment with Clindamycin .This kind of resistance could not be tested by the routein disc diffusion method but can be detected by D-test.

Two different resistant mechanisms occur in Macrolides – Lincosamide Streptogramin B (MLSB) antibiotic resistance .The. *msrA* gene codes efflux mechanism which is responsible for the MS phenotype .The resistance to Erythromycin and either inducible or

constitutive resistance to Clindamycin which is due to methylation of 23 S rRNA is coded by *erm* gene.

There are numerous reports of inducible resistance to Clindamycin (iMLSb). This kind of resistance cannot be detected by routine testing methods. When Clindamycin susceptible *Staphylococci* that are resistant to Erythromycin are isolated, the D-zone test for inducible resistance to Clindamycin has to be performed .[7] It is mandatory to check for inducible resistance before declaring Clindamycin sensitivity . A negative D-test confirms the Clindamycin sensitivity .This test helps us to use Clindamycin to treat infections produced by *Staphylococcus*.[4]

The erythromycin ribosomal methylase *(erm)* genes *responsible for Staphylococcal* strains show cross resistance to MLS antibiotics .There are three methylase genes in *Staphylococci*, namely *ermA, ermB* and *ermC* The expression of *ermA* is inducible by erythromycin, but *ermB* and *ermC* may be either inducible or constitutive.[8]

In this present study the antimicrobial susceptibility patterns for Erythromycin, Clindamycin and Cefoxitin was evaluated by D test and Cefoxitn disc diffusion test .The *erm* gene responsible for inducible Clindamycin resistance was detected by PCR . It is essential to know the accurate antibiotic susceptibility pattern of any pathogen for making

appropriate therapeutic decision. .Hence it is necessary to identify the mechanisms that confer resistance to MLS antibiotics with regard to Clindamycin for *Staphylococcal* infections.

It is easy to perform the 'D'Test along with routine susceptibility testing . This helps us to know about the local data regarding inducible resistance to clindamycin and guides in treatment of Staphylococcal infections ,thereby avoiding therapeutic failures.[9]

Thus the purpose of this study was to detect the incidence of inducible resistance to Clindamycin among *Staphylococcal* strains from clinical samples of our geographical area.

AIMS & OBJECTIVES

2. AIMS AND OBJECTIVES

- To know the antibiotic susceptibility pattern of isolated *Staphylococcal* strains from clinical samples.
- To known the Methicillin susceptibility pattern among clinical samples of *Staphylococcus aureus* and *CONS* by Cefoxitin disc diffusion test.
- To know the incidence of inducible resistance to Clindamycin among Methicillin resistant *Staphylococci* and Methicillin sensitive *Staphylococcal* isolates by Double disk approximation test in both *Staphylococcus aureus* and *CONS* isolates.
- To detect *erm* gene in inducible Clindamycin resistant *Staphylococcal* isolates by Real Time PCR.

REVIEW OF LITERATURE

3. REVIEW OF LITERATURE

The name *Staphylococcus* was first named by Ogston (1883) for cocci occurring in groups which caused infection and prqduction of pus in soft tissues. He also differentiated pathogenic cocci into one in groups called "*Staphylococcus*" and the other which appeared in chains named "*Streptococcus*."

The genus *Staphylococcus* produced a group of species which were wide different from *Micrococcus. Staphylococcus* had only three species until 1970 .[10] To date, there are 32 species and eight sub-species most of which preferentially colonise the human body.

3.1 *STAPHYLOCOCCAL* SPECIES:

Staphylococci are Gram positive bacteria which divides in planes to form grape-like clusters. There are two important species . Due to the rise in antibiotic resistance *Staphylococcus aureus* has become a major pathogen of increasing importance

3.2 GENERAL CHARACTERS:

Staphylococci are non-spore forming ,non motile anaerobes, which grow by aerobic ,anaerobic methods of respiration. Most strains have a relative complex nutritional requirement, so that they require an

organic source of nitrogen, supplied by essential amino acids, e.g. arginine, valine. Members of *Staphylococci* genus are catalase-positive and oxidase-negative, and are tolerant to high concentration of salt.[11]

3.3 STAPHYLOCOCCUS AUREUS:

Despite their phylogenic similarities *Staphylococcus aureus* is distinct from the *CoNS* (e.g. *Staphylococcus epidermidis*) and more virulent. The named *aureus*, refers to the colonies that are golden colour when grown in solid media, while *CoNS* form pale ,translucent and white colonies. Pathogenic *Staphylococci* clot blood due to coagulase production .[11]

3.4 STAPHYLOCOCCUS EPIDERMIDIS:

Staphylococcus epidermidis are gram-positive cocci nonmotile and coagulase negative that are most commonly found in the human skin. It is non-pathogenic in healthy people but there is risk for developing an infection in people with compromised immunity.[29] .Various strains of *Staphylococcus epidermidis* form biofilms which impairs wound healing. [12]

3.5 HABITAT:

Staphylococci are the major bacteria inhabiting the skin,and mucous membranes. The important areas of colonization of Staphylococci in the human body are the face, axilla and groin. The

axilla contains high humidity, higher pH than the general skin surface and with rich eccrine and apocrine glands that augment the growth of *Staphylococci*.

The nose provides the dominant ecological site for *Staphylococcus aureus* and a major habitat for *Staphylococcus epidermidis*. The populations of *Staphylococci* reach very high densities in these habitat (c. 104–106 cfu/cm2). *Staphylococcus epidermidis* and *Staphylococcus hominis* are the major species of the axillae and groins [13]

Staphylococcus aureus is a member of commensal microflora and readily colonises the anterior nares. Nasal carriage acts as an endogenous reservoir for clinical infections in the colonised individuals or as a source of cross-colonisation for community spread.[11]

3.6 CARRIER:

Staphylococcus aureus is localized to the nares by attaching to a region of moist epithelium devoid of cilia as its adherence is poor to ciliated epithelium. The elimination of nasal carriage by topical antibiotics leads to loss of carriage in both axilla and perineum which appears to be a temporary resident.

There are three *Staphylococcus aureus* carriage patterns described in adult population, with 20 %- being persistent *Staphylococcus aureus* carriers, 60%-intermittent carriers, and 20 %- persistent non-carriers.

Staphylococcus aureus carriage are found to be higher in those individuals who are infected with human immunodeficiency virus , insulin-dependent diabetes, in intravenous drug abusers, in hemodialysis patients and patients with continuous ambulatory peritoneal dialysis . [13]

3.7 MORPHOLOGY:

The genome of *Staphylococcus aureus* is 2.8Mb in size and its cell wall of is about 20 nm .It is the outer coat present and beneath this is the cytoplasmic membrane that covers the cytoplasm.[11]

3.8 CELL WALL :

Peptidoglycan is the basic component that makes up the major portion of the cell wall . It helps in the development of the compact several layers that withstands the high internal osmotic pressure of *Staphylococci* . The next major component are the teichoic acids contributes to about one third of cell wall.

Peptidoglycan together with teichoic acid thus forms the major portion of the mass of cell wall and the rest is contributed by surface proteins, peptidoglycan hydrolases and exoproteins . Two major types of teichoic acids are present . They are that present in the cell wall and other one associated with cell membrane. They are composed of N-acetylamino sugar and sometimes D-alanine [11].

Most *Staphylococcal* species posses teicholic acids which are found sometimes slightly exposed to the exterior and this forms region for the attachment of antibodies and bacteriophages [13] Teichoic acids produce a negative charge to the *Staphylococcal* cell surface and play an important role in the localization of metal ions and the activities of autolytic enzymes . [11]

Some of the cell surface components act as virulence determinants and are involved in attaching the bacteria to surfaces . About 90% clinical strains of *Staphylococcus aureus* possess capsular polysaccharides . Capsule production decreases phagocytosis *in vitro*, and enhances virulence of *Staphylococcus aureus* in a mouse bacteraemia model therefore acts as a form of biofilm.[64]

3.9 BIOFILM:

Biofilm is a mode of survival during unfavorable conditions for various microbes. The bacterial cells form aggregates in this mode of living thus become resistant to antimicrobial agents, harsh environmental conditions and to immune cells. They form channels which serve to diffuse waste products away and nutrients into the biofilm .[12]

The two steps in formation of biofilms in *Staphylococcus epidermidis* are first, bacteria adhere to a surface by teichoic acids, surface material unspecific factors such as hydrophobicity, and several proteins.

This is followed by the actual accumulation of biofilm . Biofilm associated protein (*bap),* accumulation associated protein (*aap)* autolysin E (*altE),* and intercellular adhesion (*ica*). are the major genes involved biofilm formation .

3.10 STAPHYLOCOCCAL INFECTIONS

Staphylococci causes skin infection which may be localised folliculitis, deeper furuncles and still deeper infections like carbuncles and impetigo. Bacteremia leads to spread of infection to internal organs. [1] Burns patients are more susceptible to staphylococcal infection[14]. The major concern for people with catheters, heart valves or other implants is that various strains of *Staphylococcus epidermidis* that forms biofilms on various metals surgical implants and materials other than surgical implants.[12]

3. 11 METHICILLIN SENSITIVE AND RESISTANT STRAINS:

In humans, both *MRSA* and *MSSA* strains of *Staphylococci* are found as normal commensals on the skin mainly the arm pits and the nose . Most hospital associated MRSA develop in people who harbor the organism in the nares, but community-associated *MRSA* occurs in those who have colonizing sites other than the nares. Clinical cases occur in patients who are not colonized.

Colonization with *Staphylococcal aureus* occurs after birth at any time and carriage may be transient or may be persistent. MRSA are transmitted, often via the hands by direct contact of colonized or infected people. The carrier state persists as long as person is infectious or when the lesions remain active. *MRSA* can be transmitted through fomites , food contaminated by carriers and in aerosols. They can also be transmitted during delivery from the mother to her baby. [12.]

There is an increasing incidence of *MRSA* among the normal persons and those hospitalized .This increasing trend has led to increase in the invasive infections .[15] The resistance to antimicrobial agents among nosocomial pathogens is an increasingly global problem worldwide. [16]

The genetic material present on the movable portion of *Staphylococcal* chromosome *which is the mecA* gene is responsible for the production of resistance to Penicillin group of antimicrobials.[17]

Household Methicillin resistant strains are maintained due to the colonization of pets which is often transient.[18] Clustered outbreaks of community-associated *MRSA* infection are isolated within prisons, and among athletes who share equipment in Native American communities. [19]

The sensitivity of Trimethoprim - sulphamethoxazole and Tetracycline, among the Vancomycin intermediate and resistant strains

of *Staphylococcal* isolates led to the rise in the efficiency of older antimicrobials which can be used to treat infections due to *MRSA*.[20]

3.12.MACROLIDE LINCOSAMIDE AND STREPTOGRAMIN B

The re is a increased rise in the resistance to antimicrobials among microorganism that necessitates the need to know the susceptibility of the organism towards antimicrobials and to select the appropriate antibiotics for the treatment of infection.[21]

This has led to an interest to use of Macrolide Lincosamide–Streptogramin B (MLSB) antibiotics in the treatment of Methicillin resistant *Staphylococcus aureus (MRSA*) infections .[22]

Antibiotic collectively named MLSB are a group of Macrolides (e.g. Erythromycin, Azithromycin, Spiramycin) Lincosamides (e.g.Clindamycin ,Lincomycin),and Streptogramin B (e.g., Quinupristin) which are different in their chemical nature, but are found to have similar mechanism of action on inhibition effects on bacterial protein synthesis .[23]

Erythromycin (*ERY*) a Macrolide and Clindamycin (*CLI*) a Lincosamide belongs to different classes of antibiotics of the MLSB family with similar mechanism of action and resistance. They inhibit by binding to the 50s ribosomal subunit .[23]

The tremendous use of MLSB antibiotics led to the emergence of acquiring resistance to MLSB antibiotics by increased number of *Staphylococcal* strains. The target site modification by *erm* genes has been the mechanism for such resistance . Phenotypically this has been seen one in the i MLSB phenotype the other found to be c MLSB phenotype[24]

In the presence of Macrolides, but not Lincosamides the inducible phenotype is expressed . This results in failure in treatment with the use of Clindamycin in iMLSB phenotype and this lead to the selection of cMLSB especially in deep seated infections leading to bacterial burden.[25]

It is necessary to assess the frequency or prevalence of iMLSB because during therapy these strains have a great level of potential in the production of *erm* gene that lead to develop constitutive resistance to Clindamycin .[26]

The resistance occurs in *Staphylococcal* isolates which are susceptible to Clindamycin among resistant strains to Erythromycin The expression of *erm* gene which occurs as a product of methylase of ribosome which is produced in small amount . These strains are usually produced in resistant strains to Erythromycin as this induced the methylase production but they can also be produced in the absence of

inducer which occurs in mutations of the promoter region of *erm* gene.[27]

3.13 CLINDAMYCIN:

Clindamycin belongs to the antibiotic group Lincosamide . It inhibits protein synthesis .It is available in Capsules of strength of 150 mg, 300 mg . Tropical forms are available as creams .For vaginal use , Vaginal Suppository , foam and gel are available. Parental intravenous injections are also available

There are many types of Lincosamides that have been prepared but only Clindamycin and Lincomycin are the antimicrobials used to treat infections .Clindamycin is produced chemically while Lincosamide is produced from *Streptomyces lincolnensis*.[28]

Clindamycin is one of the alternative drug used in Penicillin allergy patients to treat deeply situated infections. It inhibits Panton-Valentine Leukocidin which is a toxin.[14]Clindamycin can be used in *Staphylococcal* isolates resistance to Methicillin. Furthermore it gets deposited in deeply situated abscesses and same dose can be used in kidney infections.. This drug has excellent oral absorption making it as an great option for follow-up treatment after parental route of administration during hospitalisation[29]

The following reasons makes Clindamycin as a drug of choice.It is available in oral as well as parental formulations. , the drug is remarkably present in skin and finally it is used in MRSA infections which are rapidly emerged in recent years , is frequently susceptible to Clindamycin .[30] So this has been a better option for the treatment of these resistant strains causing infections. [31]

Staphylococcus aureus isolates with Clindamycin resistance is mostly accompanied by Macrolide resistance. This is due to the *erm* genes, which mediates Clindamycin resistance that occurs due to the modified site which is shared by Macrolides, Lincosamides and group B Streptogramin (MLSB) antibiotic groups.[31]

The cause for the unsuccessful therapy with lincosamide in erythromycin-resistant strains with the *msr*(A) gene is that the therapeutic effect of lincosamides is maintained which favors the switch from the inducible to the constitutive type of *erm* expression . [32]

3.14 MACROLIDES:

Macrolides are group of bacteriostatic antibiotics that act by binding reversibly to 50S ribosomal subunits of the susceptible organism. The mechanism of acquired resistance to Macrolides,Lincosamide and Streptogramin B (MLSB) antibiotics in

Staphylococcus aureus is due to the target site modification.This confers cross-resistance to the MLS antibiotics. [33]

The pathogenic *Staphylococcus aureus*, and the potentially pathogenic *Coagulase-Negative Staphylococci (CoNS)* are carriers of genes for resistance to macrolides[34]

Nowadays resistance to Macrolides is increasing worldwide. Inducible resistance has been found to be more than 50% in *MSSA* and constitutive phenotype resistance more than 80% in *MRSA* . It has been 10.8% in *MSSA* and 82% in *MRSA* in Sout Africa[35]

3.15 STREPTOGRAMIN - B

The treatment of infections caused by multidrug resistant and Gram-positive pathogens can be done using Quinupristin Dalfopristin (Synercid,30:70 ratio) which is the first parenteral Streptogramin licensed for clinical use in the United States and Europe .Quinupristin and Dalfopristin enter bacterial cells by diffusion .It results in an irreversible inhibition of bacterial protein synthesis by binding to different sites on the 50S ribosomal subunit, [23]

3.16 **MECHANISM OF RESISTANCE TO MLSB ANTIBIOTICS:**

Lincosamides and Macrolides binds to the same or closely related binding sites in the bacterial ribosome .Resistance to Macrolide, Lincosamide, and Streptogramin B antibiotics (MLS phenotype) occurs

through methylase enzyme that alters the antimicrobial drug binding site by removing the methyl group from an adenine residue in the 23S rRNA component of the 50S subunit of the ribosome .This alters the efficacy of the drug .[36] The most effective inducer of inducible MLSB resistance is the low levels of Erythromycin [37]

The Macrolide- Lincosamide –StreptograminB (MLSB) family of antibiotics which are structurally unrelated but serves as drug of choice in resistant Gram positive organisms, including both staphylococci and streptococci The MLS antibiotics are related microbiologically by their similar modes of action as they inhibit protein synthesis by binding to the 23S r RNA.

Clindamycin has excellent tissue penetration except in CNS because it accumulates in abscesses.It has good oral absorption and no dosage requirement in renal diseases .Thus is the preferred agent in this group .

In penicillin allergic patient it is also a useful alternative for penicillin [38].

Acquiring resistance to MLSB antibiotics occurred due to the widespread use of MLSB antibiotics in Staphylococcal strains[39] . This acquired resistance can be either constitutive where methylase is always produced or inducible type.[40]

The modification of the ribosomal target is encoded by erythromycin ribosome methylase *(erm)* gene which is a multiallele plasmid borne and

leads to the production of the methylase enzymes. The A2058 residue located in the conserved domain V of the 23S rRNA which is a component of the 50S ribosomal subunit, is methylated by the methylase enzyme .This lead to cross resistance and the formation of the phenotype of the resistance pattern, called as the MLSB – resistant phenotype[41].

Methylase is always produced in case of constitutive resistance, whereas in inducible resistance , methylase is produced only in presence of inducer like Erythromycin [42]

Macrolide resistance may be conferred by efflux mechanism encoded by *msr(A*) (MS-phenotype) or as ribosomal target modification that affects activities of Macrolides ,type B Streptogramins and Clindamycin (MLSB-resistance) [43]

In *Staphy-lococcus species* Clindamycin resistance can be either constitutive or inducible. The target site modification mediated by the *erm* genes,is expressed either constitutively(constitutive MLSB phenotype) or inducibly (inducible MLSB phenotype). It is difficult to detect in the routine laboratory the strains with Inducible resistance to Clindamycin [44]

In the presence of low levels of inducers, such as Erythromycin the expression of the MLSB phenotype can be either constitutive or inducible . The erm genes are encoding enzymes that confer inducible or constitutive

resistance. There are three expression of MLSB phenotypes which can be described as constitutive (MLS Bc), inducible (MLS Bi) or MS phenotype[33]

In MSB resistance due to the expression of *mrs (A)* gene . does not produce resistance in clindamycin which remains active . In this resistance an active efflux occurs , due to energy-dependent pump,. The modifi cation of the drug-binding site on the bacterial ribosome, encoded by *erm* genes (*erm* (A) or *erm* (C) in staphylococci results in resistance to Macrolides,Lincosamides and type B Streptogramin (MLSB resistance[64]

When not placed adjacent to each other they appear as Erythromycin resistant and Clindamycin sensitive in vitro but they show therapeutic failure in-vivo therapy with Clindamycin [45]

Constitutive resistance can be easily but it is difficult to detect inducible resistance is by routine antimicrobial susceptibility tests. In order to detect inducible clindamycin resistance (ICR) in isolates of staphylococci,according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI), it is necessary to subject to the D-zone test.[46]

3.17.D-zone test:

A lawn culture adjusted to 0.5Mcfarland's concentration of the isolate was made on a Mueller Hinton agar plate. Along with routine

antibiotic susceptibility testing the discs of Clindamycin (CL (2μg) and Erythromycin ER (15μg) were placed at a distance of 15mm (edge to edge) as per the CLSI recommendations. Based on the D test, the disc diffusion test showed four phenotypes.

The Inducible MLSB phenotype (iMLSB): D shape in the Clindamycin zone is known as inducible resistance to clindamycin .

The Constitutive MLSB phenotype (cMLSB): strains appearing resistant to both drugs.

The MS phenotype: strains of erythromycin resistantce and Clindamycin susceptible .

There is a zone of flattening near to the erythromycin disk (D – test positive.) indicate iMLSB . sometimeswithin the zone of inhibition around clindamycin growth may occur indicates resistance to Clindamycin.

3.18 INTERPRETATION OF D-TEST:

When an isolate demonstrates inducible clindamycin resistance D-shaped zone is seen around the clindamycin disk .Clindamycin is reported as resistant in the report .There is highly predictive of the organism producing a positive D-test result in cases of diabetes, cystic fibrosis, immunodeficiency and postsurgical status. The relative frequency of inducible resistance is tested periodically which helps in tailoring empiric therapy for suspected *S aureus* infections[63]

D-test helps in the phenotypic determination which is confermed by PCR to detect erm A,ermB,ermC genes in clinical *Staphylococcal* isolates from the USA[62] *ermA* and *ermC* were found to be responsible for the iMLS and MS-phenotype showed strains that are not capable to hybridize with the probes of erm gene .[61]

The *erm* genes *erm*(F)], *erm*(A) and *erm*(C) are responsible for the iMLSB resistance in staphylococci, which occurs as either cMLSB or inducible[48]

The classes of the *erm* genes *erm*(A), *erm*(B), *erm*(C) are responsible for for iMLSB . Erythromycin induce methylase that produce Clindamycin to express its resistance.[49]

In MS resistance the mechanism, is that Streptogramin B 14- and 15-membered Macrolides to which partial cross-resistance occurs and which is conferred by gene *msrA* .This is due to the active efflux of antibiotics[50]

There is variation in the the amount of *Staphylococci* showing *in vitro* iMLSB among hospitals graphic region, age group, bacterial species and Methicillinsusceptibility[51]

The developing patterns of drug resistance should be kept a close watch by the clinical microbiologists and infectious diseases experts which helps them to guide therapy effectively. [60]

As Clindamycin resistance vary by methicillin susceptibility and geographic region and the high frequency of *MRSA* isolates with *in vitro* resistance to Clindamycin necessitates the determination of inducible Clindamycin resistnce in individual settings. This is of main concern as treatment failures with Clindamycin may occur with these Methicillin sensitive and resistant strains. [52]

Inappropriate therapy of *Staphylococcus aureus* which were reported as susceptible to Clindamycin without performing D-test . While a negative D-test result will confirms susceptibility for Clindamycin and helps to provide a good option for treatment.[53]

Prevalence of the two phenotypes necessitate us to test each isolate of *Staphylococcus* for inducible Clindamycin resistance. If MS resistance is uncommon, in the locality ,most of the labs will not do

D-test but they report as Clindamycin resistant for every Erythromycin resistant isolates .[54]

Multidrug resistant. Methicillin resistant strains (*MRSA)* remain in high priority for hospital epidemiologist, since these strains are virulent much more difficult and expensive to treat as they are virulent .[55]

The major problems faced while controlling *MRSA* in the tertiary care hospital are the environment .persistence of *MRSA* carriage increasing number of patients at risk of acquisition ,inadequate isolation

facilities for isolation of the organism ,problems in identifying the source of outbreak, inadequate antibiotic and admission policy .[59]

There is a high risk of colonization of Methicillin resistant *Staphylococci* in HIV- infected patients Increasing use of Clindamycin, among *MRSA* clones will complicate the treatment of these infections present in the community [56]

Resently there are many automated or semi-automated machines to detect antibiotic susceptibility testing (AST) . The BDXpert System, the BD Phoenix™ A and BD EpiCenter™ System are some of the automated AST systems which help us to provide test results as per CLSI guidelines.[57] Vitek (bioMerieux) – V1, and MicroScan MICroSTREP Plus(Dade Behring) – MS are few automated systems used for the interpretation of AST results.[58]

MATERIALS AND METHODS

4. MATERIALS AND METHODS

This study was conducted during the period from April 2013 to May 2014 at the Department of Microbiology, Tirunelveli Medical College , Tirunelveli.

4.1 INCLUSION CRITERIAS:

During the study period a total of 100 non duplicate clinical isolates of *Staphylococci* were collected from different clinical samples like aural swabs, wound swabs ,pus and vaginal swabs from both in-patient and out-patient departments of Tirunelveli Medical College . The *Staphylococcal* species were identified by standard biochemical techniques.

4.2 EXCLUSION CRITERIAS:

- Patients who were already on treatment with clindamycin .
- Samples from patients producing positive culture but with no signs of infection .

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

4.3 Ethical clearance:

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

4.4 Informed consent:

Informed consent obtained from all persons involved in this study.

4.5 Proforma:

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

4.6 Sample storage:

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

4.7 Primary isolation and identification of *Staphylococci* :

4.7.1 Samples:

During the study period pus collected from abscess, wound swabs from discharges in skin infections, ear swabs and vaginal swabs were collected from those patients attending outpatient& inpatient department

in Tirunelveli Medical College . Samples which were received was processed within two hours of receipt as per standard procedures.

4.7.2 Microscopy:

Gram positive stained cells appearing spherical on light microscopy . These cocci appear in clusters as division occurs in two are more planes *Staphylococcus aureus* mainly produced irregular clusters of cells and *Coagulase Negative Staphylococcus* mainly produced aggregates of tetrads and pairs.

4.7.3 MORPHOLOGY:

Staphylococci produced classical colonies on a variety of commercial agar media. In gram stain samples showing Gram positive cocci in clusters ,tetrads and pairs were processed . Samples were streaked into both nutrient and blood agar, incubated at 37^0c for 18 to 24 hours. Presence of large (2-4mm diameter), circular, smooth, golden yellow or cream coloured, opaque, easily emulsifiable colonies on nutrient agar were noted. On blood agar β-hemolytic colonies and no hemolysis on some plates were observed with similar morphology. Mannitol salt agar was used as selective media . Pigment present on colonies were of different shades depending on the particular species and strain.

4.7.4 Biochemical reactions:

Catalase test:

It was done by slide test or tube test.

Slide test-

A single colony taken from nutrient agar plate was placed over the clean glass slide, to this one drop of 3% H_2O_2(hydrogen peroxide) was added, effervescence was observed. When effervescence appeared it was a positive test.

Tube test-

1ml of 3% H_2O_2 was taken in a small test tube, small amount of bacterial growth was introduced with the help of glass rod or plastic applicator stick , effervescence was observed.

Control : Positive and negative strains were tested simultaneously .

COAGULASE TEST:

Tube coagulase test detects Free coagulase and slide coagulase test detects bound coagulase . Slide coagulase test was used to screen strains of *Staphylococcus aureus* and tube coagulase was used to confirm it.

SLIDE COAGULASE TEST:

Principle:

Other name for bound coagulase is clumping factor. fibrin clot that deposits on the cell wall is formed by cross-links of α and β chain of fibrinogen in plasma.So individual cocci stick to each other and form clumps.

Procedure:

Staphylococci dense suspensions from culture was made on both ends of a glass slide and labeled as "test" and "control". The control serves to rule out false positivity due to autoagglutination. The test sample was treated with a drop of citrated plasma .Clumping of cocci in 5-10 seconds was considered as positive. Some strains did not produce bound coagulase, and were identified by tube coagulase test.

TUBE COAGULASE TEST

Principle:

The free coagulase of *Staphyloccus aureus* reacts with coagulase reacting factor (CRF) in plasma to produce a complex, (ie) thrombin. This thrombin converts fibrinogen to fibrin clotting of plasma occurs.

Procedure:

Three test tubes labeled as “test”, “negative control” and “positive control” were taken and filled with 0.5 ml of 1 in 10 diluted rabbit plasma. 0.1 ml of overnight broth culture of test bacteria was added to the tube labeled test.andt to the tube labeled positive control, 0.1 ml of overnight broth culture of known *Staphyloccus aureus* was added and to the tube labeled, negative control 0.1 ml of sterile broth was added. The tubes were incubated at 37^{o}C for four hours. Gelling of the plasma, indicates positive result. The test which were negative, were kept at room temperature for overnight incubation and observed the next day.

4.8 Antibiogram:

All identified *Staphylococcal* strains were then tested for Antibiotic susceptibility on Mueller-Hinton agar plates by the Kirby-Bauer disc diffusion method. Antibiotic discs used are Ampicillin (10 µg), Amoxyclavulanic acid (20/10 µg), Ciprofloxacin (5 µg),) Doxycycline (30 µg), Erythromycin (15µg), Gentamycin (30 µg), Cefoxitin (30µg) Clindamycin (2 µg), Co-trimoxazole(23-75/1-25mg) and Vancomycin (30µg).

Control: *Staphylococcus* ATCC 25923 used as the control strain .

Methicillin resistance was detected by using Cefoxitin disc.

4.9 D-test:

4.9.1 PRINCIPLE:

Macrolides resistance can occur by two different mechanisms:

1) Efflux due to Macrolide Streptogramin resistance (*msrA* gene)

2) Ribosomal alteration due to erythromycin ribosome methylase (*erm gene*).

MLSB -resistant phenotype is the most frequent mechanism of resistance. Genes encoding these methylases have been designated erm (erythromycin ribosome methylation). Expression of resistance to MLSB in staphylococci are either constitutive (MLSBc) or inducible (MLSBi). If it is is constitutive, the strains are resistant to all MLSB antibiotics and if it is inducible, the strains are resistant to 14- and 15-membered macrolides.

In MLSBi strains, Macrolides will produce methylase, which allow Clindamycin resistance to develop and this resistance was identified with the D test

4.9.2 D –Test PROCEDURE:

The Erythromycin resistant isolates were subjected to D zone test for inducible Clindamycin resistance as per the CLSI guidelines. All antibiotic discs were procured from Himedia India, Private Ltd

D-zone test: - on a Mueller Hinton agar plate a lawn culture of the isolate which was adjusted to 0.5 Mcfarland's concentration was made and discs of Clindamycin(2µg),Erythromycin(15µg) were kept at a distance of 15mm.

The disc diffusion D test, showed

- **Inducible MLSB phenotype (iMLSB)**
- **Constitutive MLSB phenotype (cMLSB)**
- **MS phenotype**

The Inducible MLSB phenotype (iMLSB):

Clindamycin Inducible resistance was manifested by blunting of the Clindamycin zone neart the Erythromycin disc, giving a D shape.

The Constitutive MLSB phenotype (cMLSB):

Isolates which were resistant to both.

The MS phenotype:

Strains which were resistant to Erythromycin but susceptible to Clindamycin.

4.10 Real-Time PCR :

The *Staphylococcal* isolates from MLSBi were further tested gene by Real-Time PCR from Helini Biomolecules for

ermA/ermB/ermC genes. and procedure followed according to the manufacturer's instructions.

4.10.1 Safety precautions:

A Biosafety cabinet Level-2 was used to perform all procedures with due precautions by wearing a suitable lab coat, disposable gloves, and protective goggles. The assay wastes were discarded as per our local safety regulations.

4.10.2 Material Required:

- Vortex mixer.
- Water bath.
- Centrifuge with rotor for 1.5ml reaction tubes.
- 1.5ml/2ml centrifuge tubes.
- Disposable powder-free gloves.
- Micro pipettes and tips.
- 0.2 ml PCR tubes/8 well strips /96 well plate according to real time PCR machine and model.
- Thermo cycler (Biorad CFX 96)
- Computer for data storage
- 70% Ethanol

- Isoproponal

4.10.3 DNA extraction:

Each silica based spin column recovered up to 20µg of DNA and yielded purified DNA of more than 30 kb in size. Isolated DNA was used directly for PCR reaction.

4.10.4 Components of extraction:

- Phosphate buffered saline
- Lysozyme
- Digestion buffer
- Binding buffer
- Proteinase K
- Internal control template
- Isopropanol
- 70% ethanol
- Elution buffer

4.10.5 Storage and stability:

- The kit was stored at 25˚C.
- Proteinase K and Lysozyme was stored at -20˚C.

4.10.6 Sample preparation:

Four to five colonies of *Staphylococcal* isolates of MLSBi grown on NAP slope was inoculated into one ml of PBS in a 1.5ml sterile microcentrifuge tube. The tube was then centrifuged for five minutes at 6000 rpm at room temrerature. The supernatant was discarded thoroughly and the bacterial pellet was ready to use for DNA purification.

4.10.7 Principle of extraction:

The Cells are lysed by Proteinase K which immediately inactivates all nucleases .Cellular nucleic acids can bind glass fibres in the Pure Fast spin column. This bound nucleic acid is further purified by rapid "wash and spin" steps which removes all contaminations . Finally with the help of elution buffer the nucleic acids are eluded from the fibre spin column . The above step eliminates the use of organic solvent extractions and DNA precipitation, of many samples simultaneously.

4.10.8 Extraction procedure:

- All the steps were performed at room temperature.
- The bacterial pellet was mixed in 200µl of buffered saline and vortexed 30 seconds to dislodge the pellet.

- 180µl of Digestive buffer and 20µl of Lysozyme was added and gently vortexed for 10 seconds. It was incubated at 37^0 C for 15 min.
- Binding buffer of 200µl , 20µl of Proteinase K, and 5µl of internal control template was added to the suspension and pulse vortexed.
- This was mixed immediately by inverting several times and incubated at 56°C for 15 minutes in a water bath.
- 300µl of Isopropanol was added and vortexed for 15 seconds.
- Entire sample was pipette into a PureFast spin column.
- This was centrifuged for one minute at 12,000 rpm for 1min. The column placed back into the same collection tube after discarding the flow through.
- To the spin column add 500µl of 70% ethanol .
- This was centrifuged for 1min at 12,000 rpm.
- To the spin column 500µl 70% ethanol added .
- At 12,000 rpm this was centrifuged for 1min and discard the flow through and at 13,000 rpm centrifuged for two minute so as to remove the residual ethanol.

- A fresh 1.5ml microcentrifuge tube was used to transfer the spin column
- 75µl of the Elution buffer (pre-warmed to 56˚C) was added to the centre of the spin column membrane. Care was taken not to touch the membrane with pipette tip.
- It was then incubated for two minutes at room temperature and further centrifuged I min.
- Discard the column and purified DNA stored at -20^{0}C.

4.10.9 PCR amplification

Real time PCR ready-to-use kit used to detect *erm* A/B/C gene bearing bacterium using polymerase chain reaction (PCR).It contains reagents and enzymes for the specific amplification of *erm* A/B/C gene to directly detect the amplicon in fluorescence channels cycling green.There is an Internal control to amplify and identify the inhibition. External positive controls (Positive Template) are supplied ,which can be used as both qualitative and quantitative to determine the amount of bacterial load.

4.10.10 Specificity:

The *erm* A/B/C primer and probe are designed so as to in vitro quantify *erm* A/B/C genes in bacterium. The target sequences are

highly conserved .It act as a genetic marker. A comprehensive bioinformatics analysis is used to find the primers and probe sequences in this kit that have 100% homology with the reference sequences .

4.10.11 Kit components:

- Probe PCR Master mix
- *erm* A/IC PP mix
- *erm* B Mix containing Primer Probe
- *erm* C Mix of Primer Probe
- Internal control template
- Water, Nuclease Free
- erm A/B/C Positive Template

4.10.12 *erm* A/B/C primer & probe mix

The *erm* A/B/C primer & probe mix consists of Taq Man probe which is florescent labeled with FAM, forward primer and reverse primer.

erm A primer-

5'- TCA GGA AAA GGA CAT TTT ACC -3'

erm B Primer- -

5'- GGT AAA GGG CAT TTA ACG AC -3'

erm C Primer--

5’- CTTGTTGATCACGATAATTTCC -3’

erm A Probe - -

5’- GAG CTT TGG GTT TAC TAT TAA TGG -3’

erm B Probe- -

5’- CTT ACC CGC CAT ACC ACA -3’

erm C Probe—

5’- CATAAGTACGGATATAATACGCA -3’

4.10.13 Internal Control primer / probe Mix

The limited concentrations of the internal control primer /probe mix in PCR allows multiplexing with the target primers. Detection of the pathogen target gene is not affected by the amplification of the Internal control template .with the HEX channel the Internal control is detected and gives a CT value of 26±3.

The reason for including the internal control is to make sure that PCR inhibitors are not present in the extracted sample DNA and the performance of PCR mix ingredients are good. When no amplification was observed in internal control, it indicates that PCR inhibitors are present in the sample and efficiency of the nucleic acid purification is not optimum. It helps the

false negative results to be ruled out .5µl of the internal control template is added to sample/lysis buffer complex.

4.10.14 Positive and Negative contol

5 µl of Positive control added in the place of sample DNA and 5µl of Nuclease free water as negative control used in the place of DNA sample.

4.10.15 PCR amplification kit storage

The kit was stored at -20˚C.

Table.1 : *erm* detection mix for samples

S.No	Components	*ermA*	*ermB*	*ermC*
1.	Probe PCR Master Mix	10µl	10µl	10µl
2.	*erm*A/Internal Control Primer Probe Mix	10µl	-	-
3.	*erm*B Primer Probe Mix	-	10µl	-
4.	*erm* C Primer Probe Mix	-	-	10µl
5.	Purified DNA sample	5µl	5µl	5µl
6.	Total reaction volume	25µl	25µl	25µl

4.10.16 The *erm* reaction mix

The *erm*A reaction mix for the samples consisted of probe PCR master mix 10µl, *erm*A/IC primer probe mix 10µl, purified DNA sample 5µl and a total volume of 25µl.(Table.1)

The *erm*B reaction mix for the sample consisted of probe PCR master mix 10µl, *erm* B Primer Probe Mix 10µl, purified DNA sample 5µl and a total volume of 25µl.(Table.1)

The *erm*C reaction mix for the sample consisted of probe PCR master mix 10µl, *erm* C Primer Probe Mix 10µl, purified DNA sample 5µl and a total volume of 25µl.(Table1.)

For positive control mix, 5µl of positive control template was added instead of sample DNA and for negative control mix, 5µl of nuclease free water was added instead of sample DNA. (Table.1)

Initially negative control, followed by samples and finally positive control was added to prevent cross contamination. After adding all the ingredients, they were centrifuged and placed in the thermo cycler and the pcr reaction was made to run.

4.10.17 Basic steps in amplification

- Initial denaturation - First, the temperature is raised to 95˚C for five minutes for Taq enzyme activation.
- Denaturation- The temperature elevated to 95˚C for 20 seconds, separates template DNA strand to two complementary strands.
- Annealing- temperature decreased to 55˚C for 20 seconds, binds the primer to the DNA template complementarily. the temperature raised to 72˚C for 20 seconds leads to extension. DNA polymerase extends the primers ., two single template of DNA strands and the

newly synthesized complementary DNA strands combine together forming two new double stranded DNA copies after extension

- The copy of DNA may function as one template for further amplification. The products will be doubled in each cycle and for 40 cycles

- The final PCR products is 2n copies of template DNA. Data collection was made at the end of extension and the computer produces the cross threshold (Ct) value by calculating the fluorescence emitted at the end of each cycle.

Table 2: Amplification profile for erm gene

	Step	**Time**	**Temp**
	Taq enzyme activation	5min	95^0 C
40cycles	Denaturation	20sec	95^0 C
	Annealing/ Data collection	20sec	55^0 C
	Extension	20sec	72^0 C

*erm*A/B/C=FAM Channel

Internal control=JOE Channel

Ct value

- When Ct value was less than 38, it was considered as positive for *erm* gene.
- The test was repeated with Ct values between 37- 40.
- Negative result if no amplification occured.

4.10.18 Interpretation of results:

Negative control

In the negative control reactions growth curves which cross the threshold line and exhibiting fluorescence are not found.

Control-Positive

The Positive control reactions showing positive results before 40 cycles.

Test sample/specimen-Positive

A test sample/specimen is presumptive positive when all controls met the needed requirements.

Test sample/specimen-Negative

A test sample/specimen is presumptive negative when all controls met the needed requirements Sample amplification plot should cross threshold point before 38 cycles. (Ct value±38)

Internal control-Interpretation:

When used according to protocol CT value is expected within 24 to31. However this varies with extraction efficiency, the quatity of elute added to the PCR reaction and the individual machine settings. CT values of 31±3 are within the normal range.

Table 3: Interpretation of results

Test Sample	**Negative control**	**Internal control**	**Positive control**	**Interpretation**
Positive	Negative	Positive	Positive	**Positive**
Negative	Negative	Positive	Positive	**Negative**
Negative	Negative	Negative	Negative	**Repeat**
Positive	Positive	Positive	Positive	**Repeat**

RESULTS

Fig.1 BAP Plate - *Staphyloccus aureus* colonies

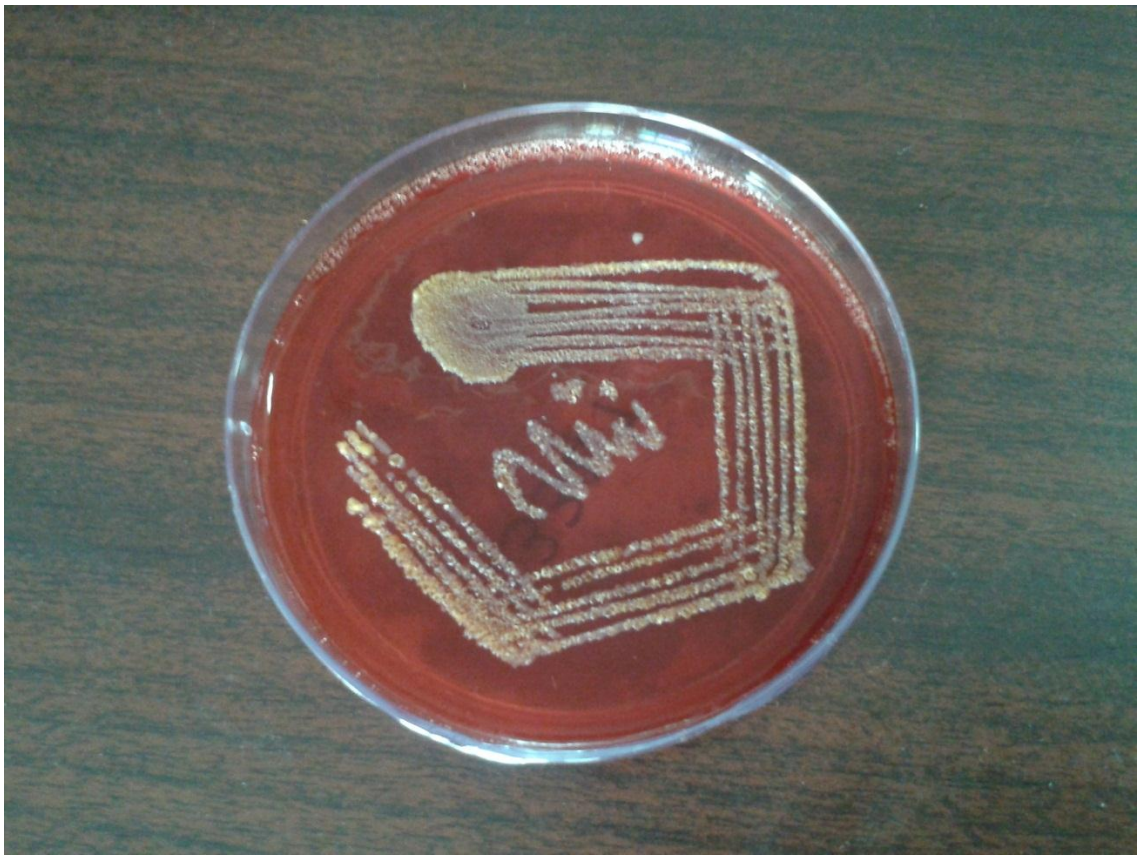


Fig. 2 NAP Plate - *Coagulase Negative Staphylococcus (CONS*).

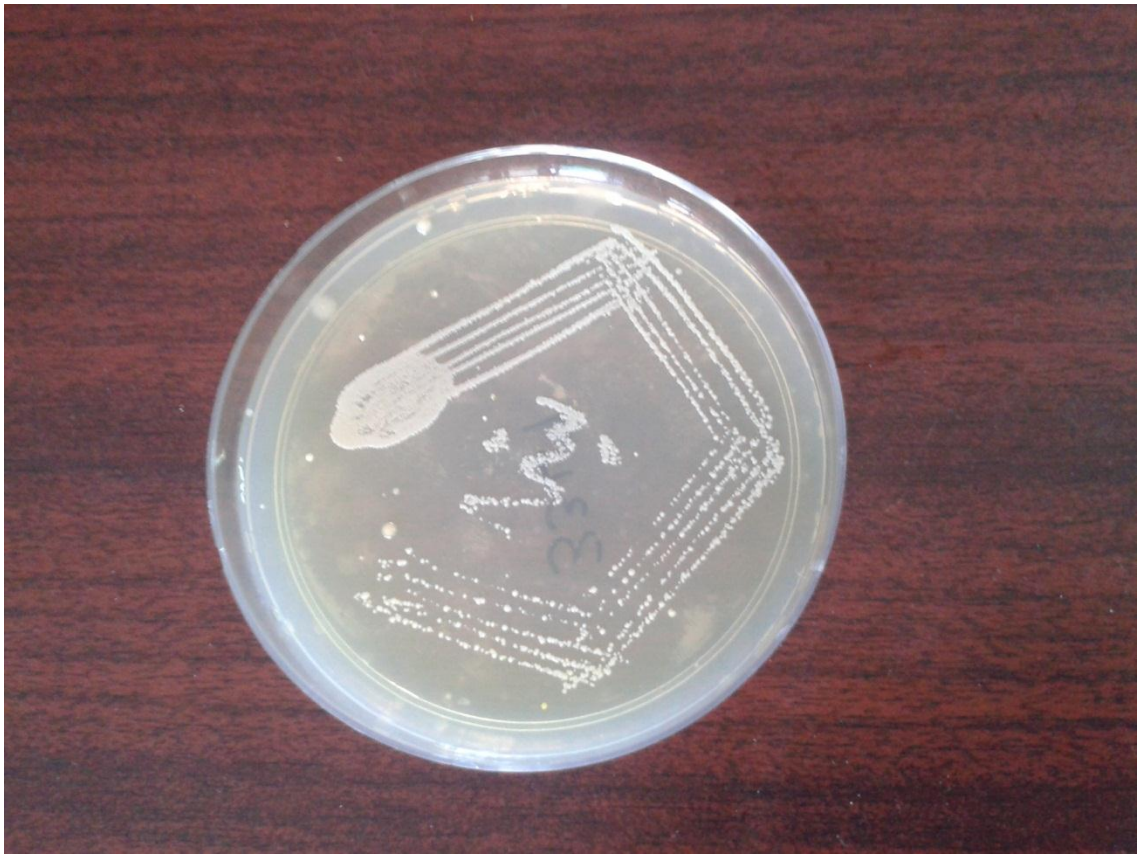


Fig.3 TUBE COAGULASE TEST

Fig. 4 ANTIBIOGRAM

Fig.5 Erythromycin sensitive pattern

Fig.6 Erythromycin resistance MLSB penotypes

Fig.6.1 Inducible MLSB phenotype (iMLSB)

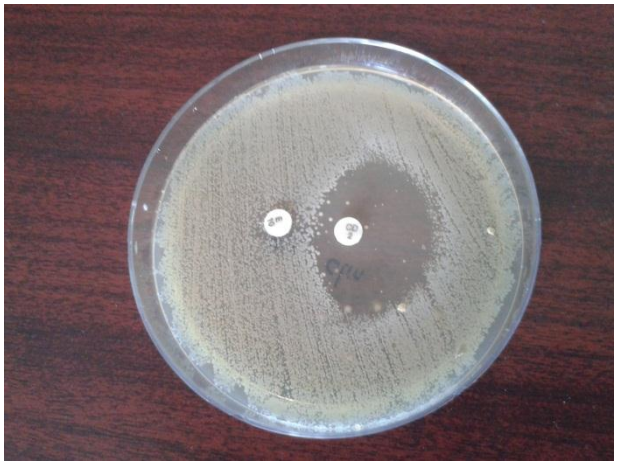


Fig. 6.2 Constitutive MLSB phenotype (cMLSB)

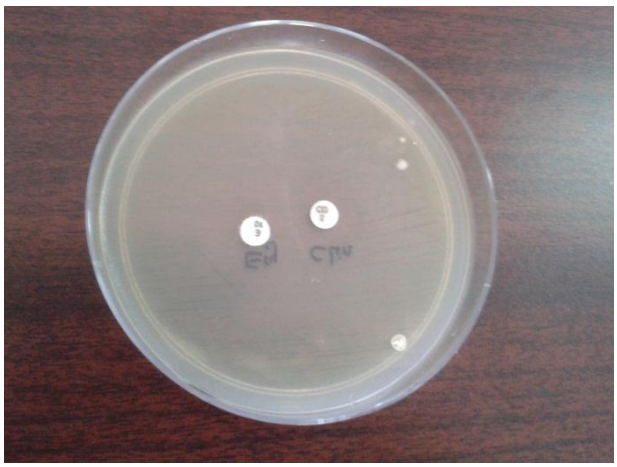


Fig. 6.3 MS phenotype

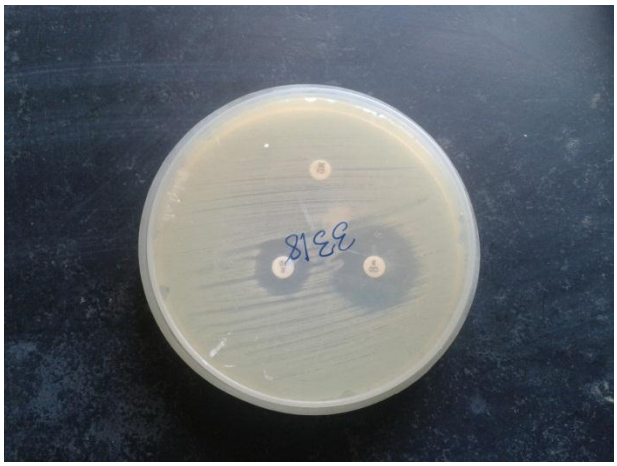


Fig. 7 MRSA

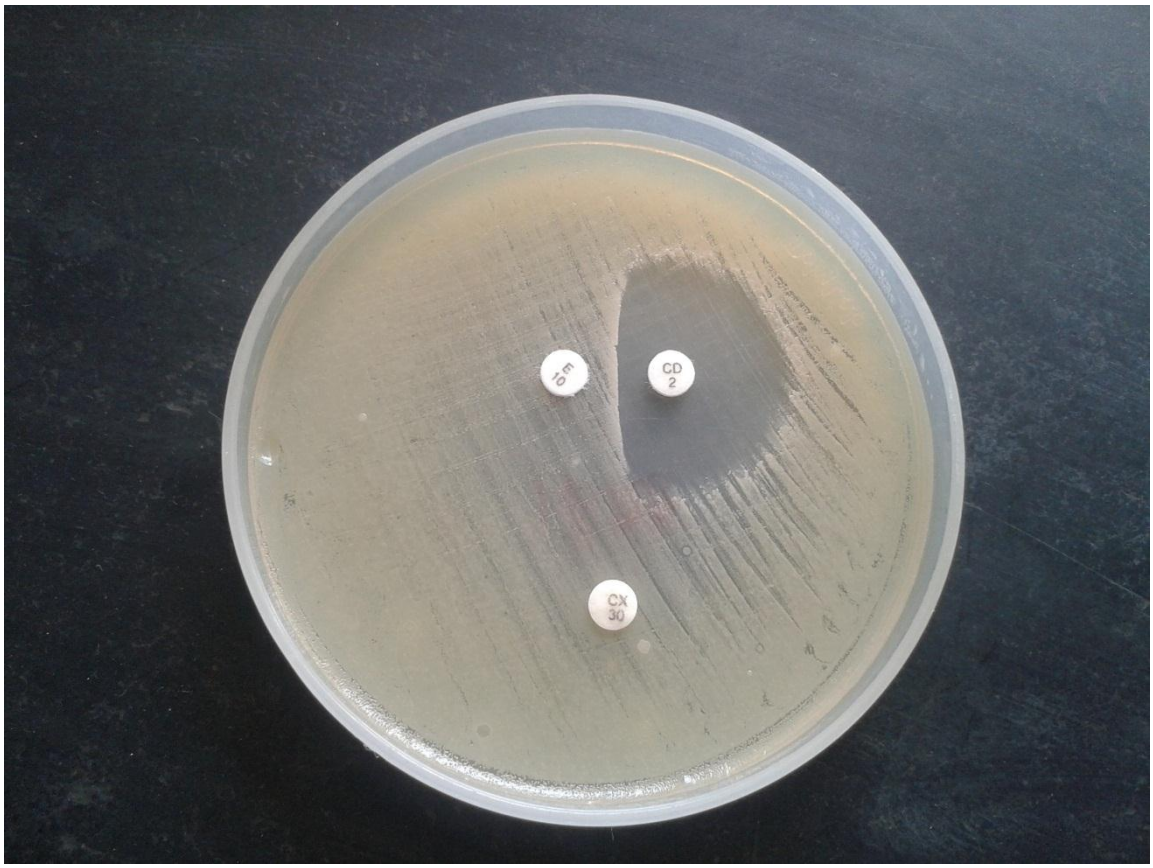


Fig. 8 D- Test

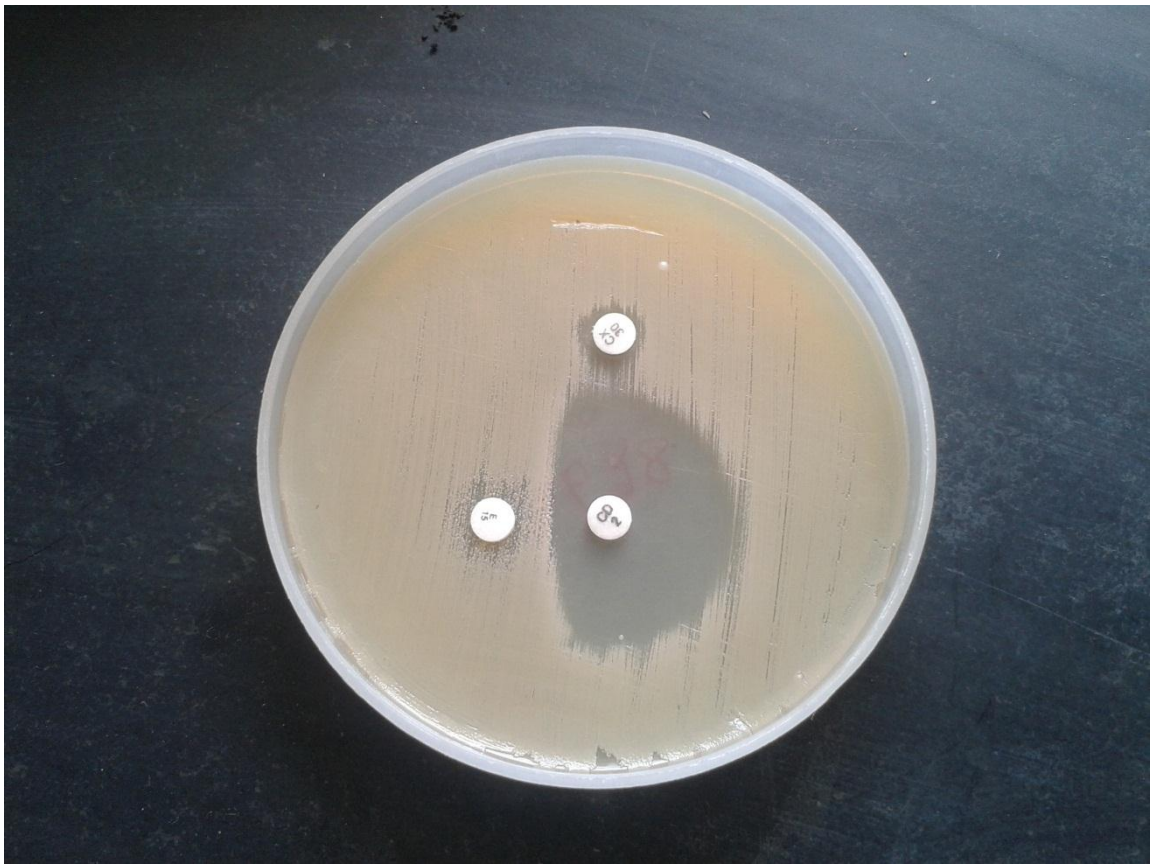


Fig. 9 PCR - DNA extraction kit

Fig. 10 PCR amplification kit

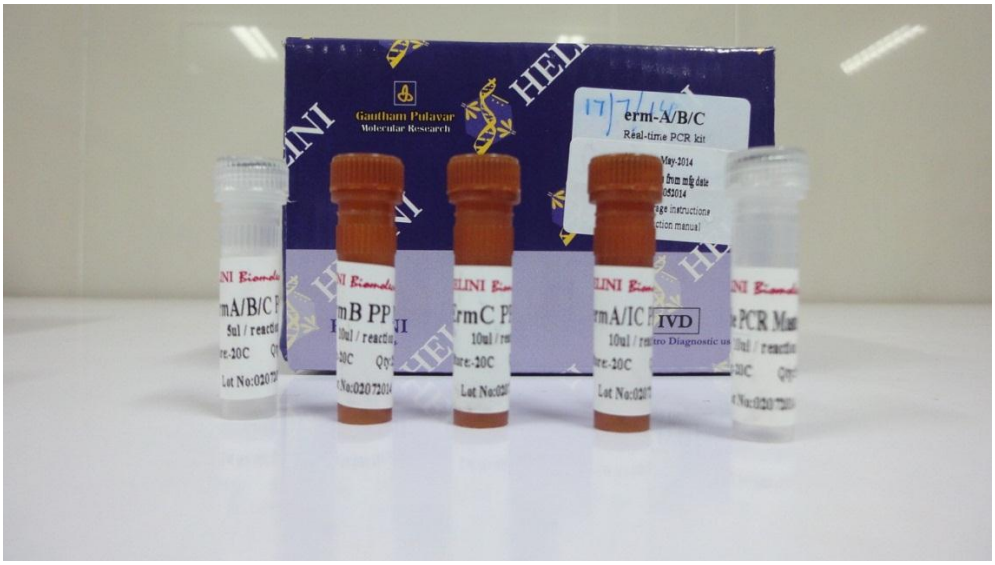
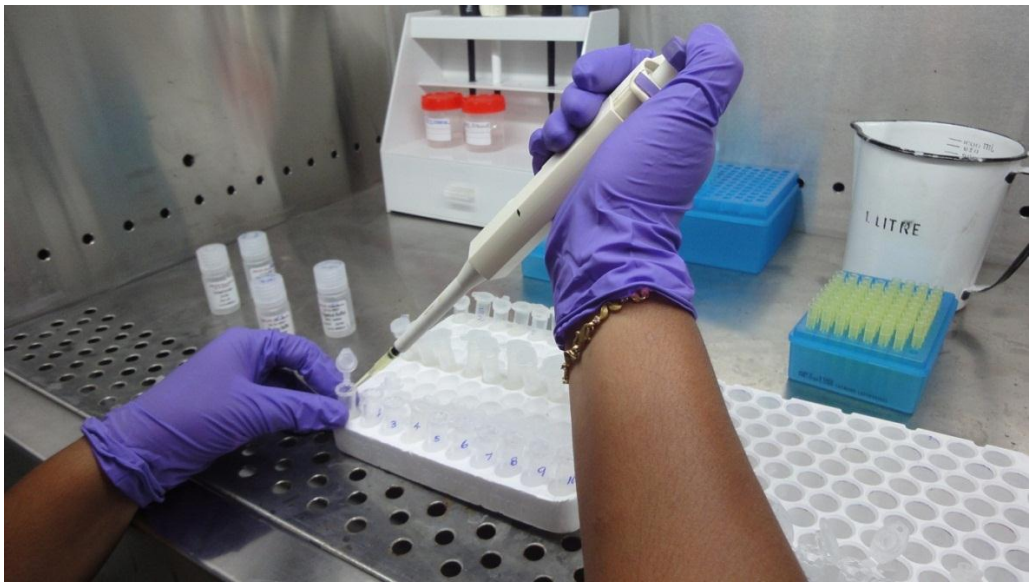
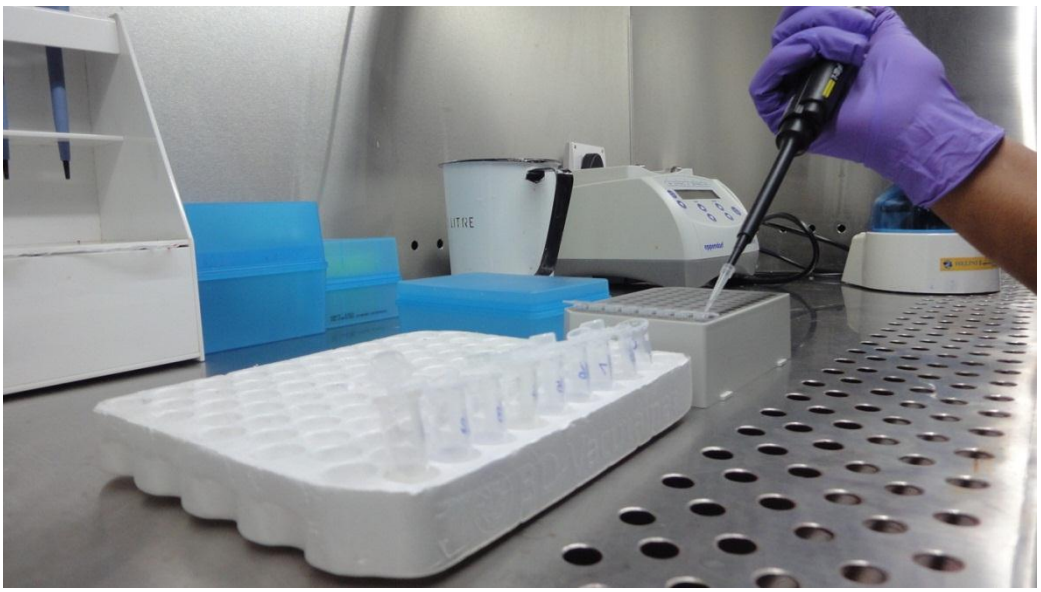


Fig. 11 DNA extraction





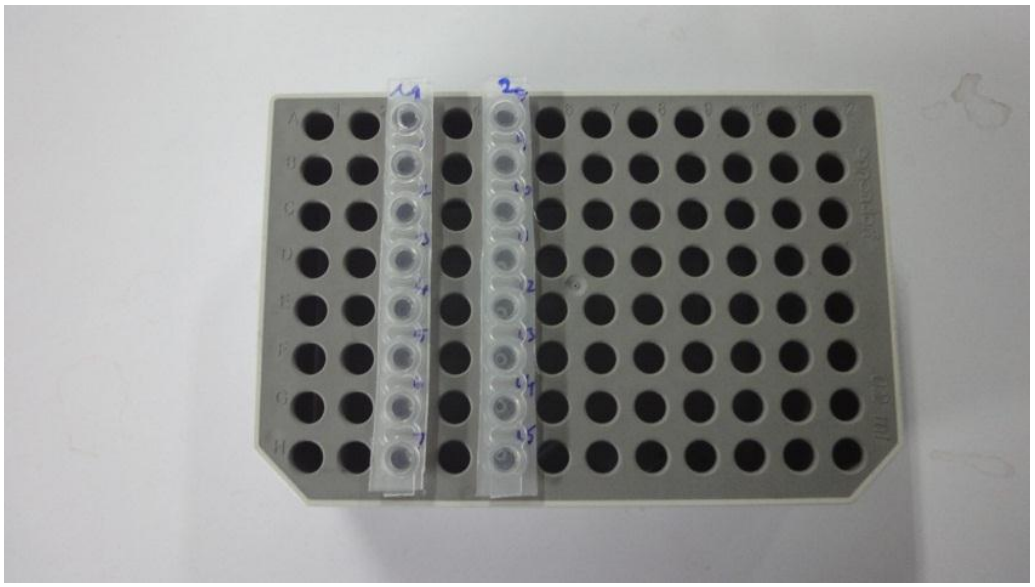


Fig. 12 Centrifuge

Fig. 13 Vortex mixer

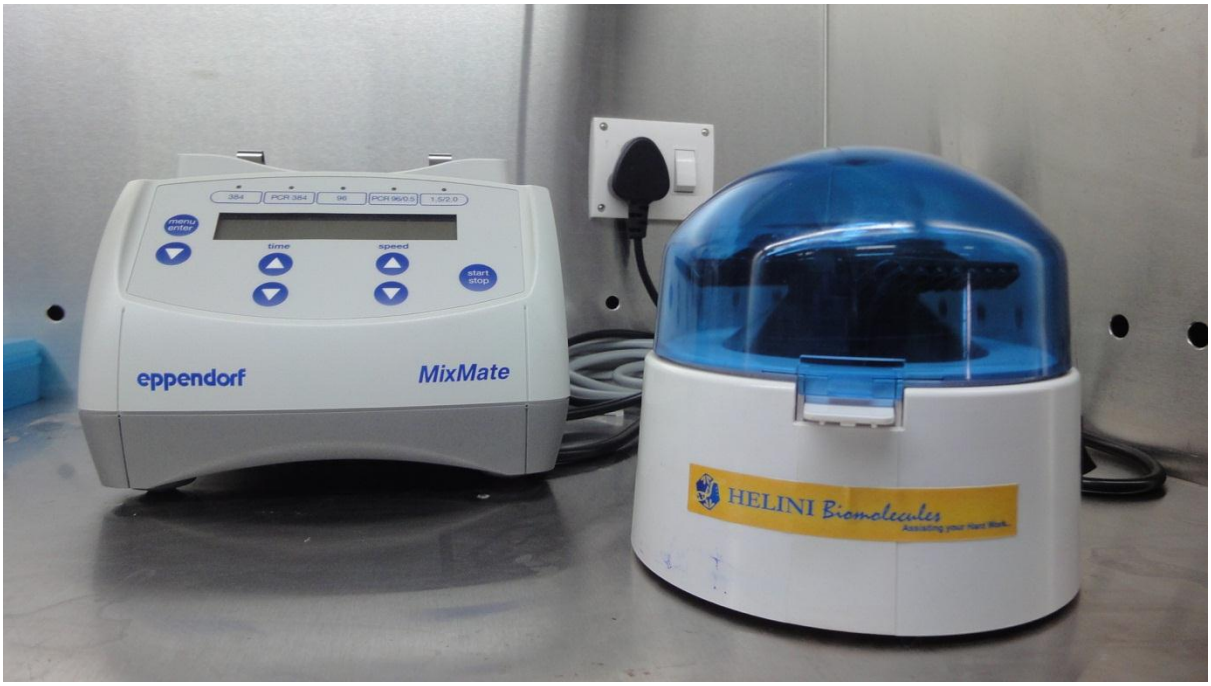


Fig. 14 Thermo cycler (Biorad CFX 96)

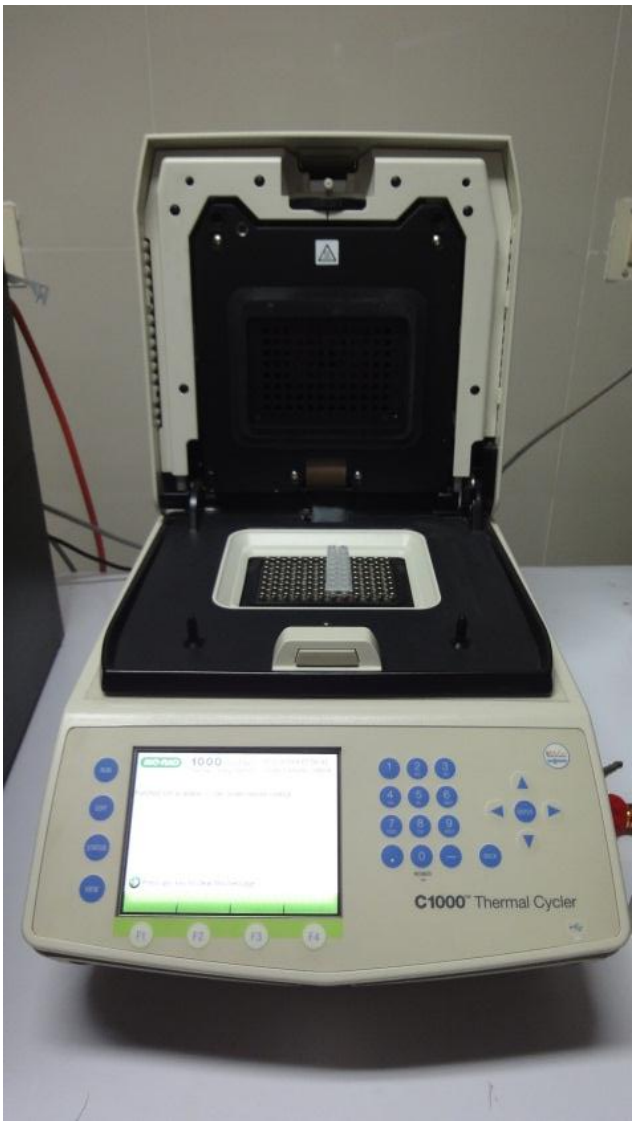


Fig.15 PCR Graph 1

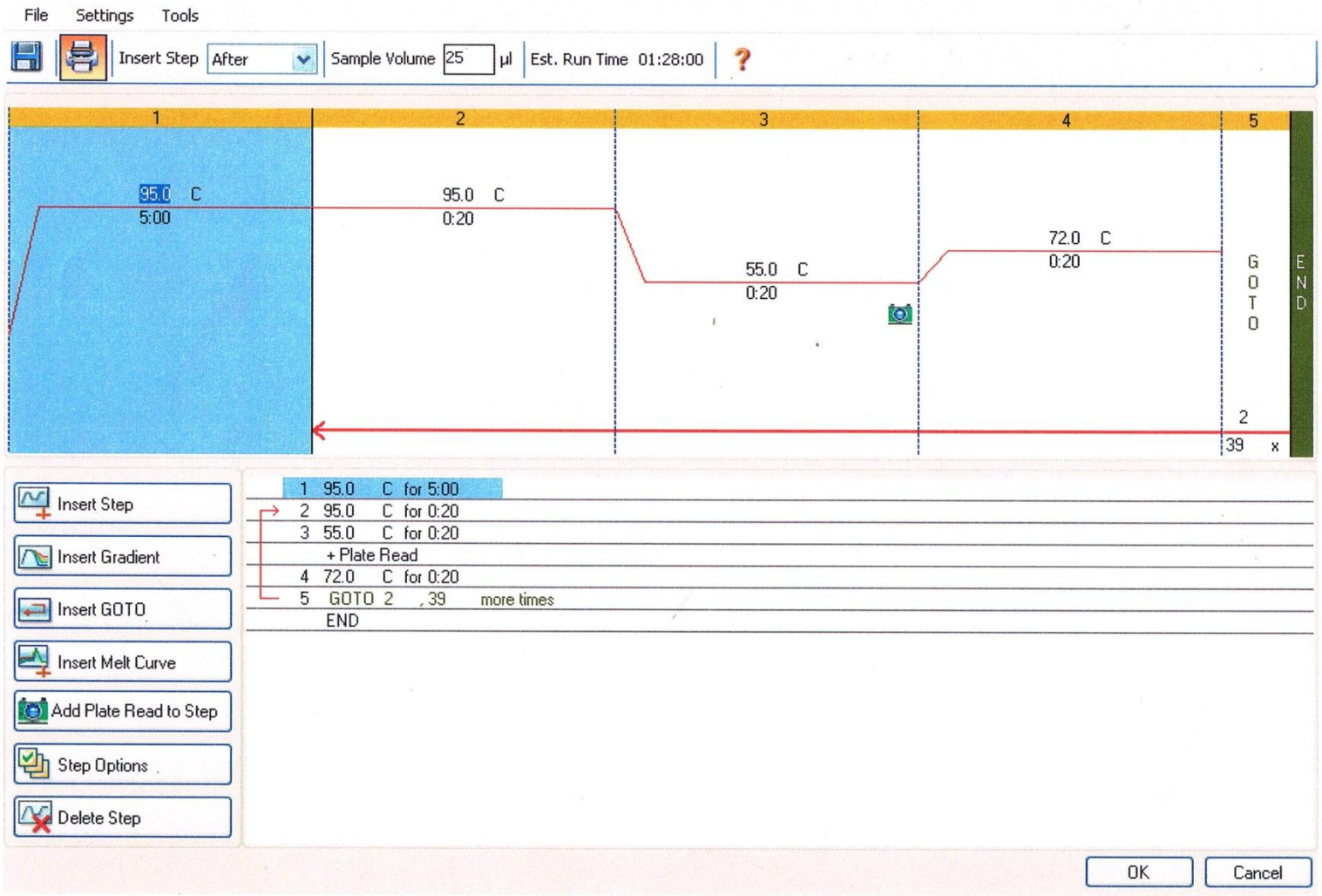


Fig.16 PCR Graph 2

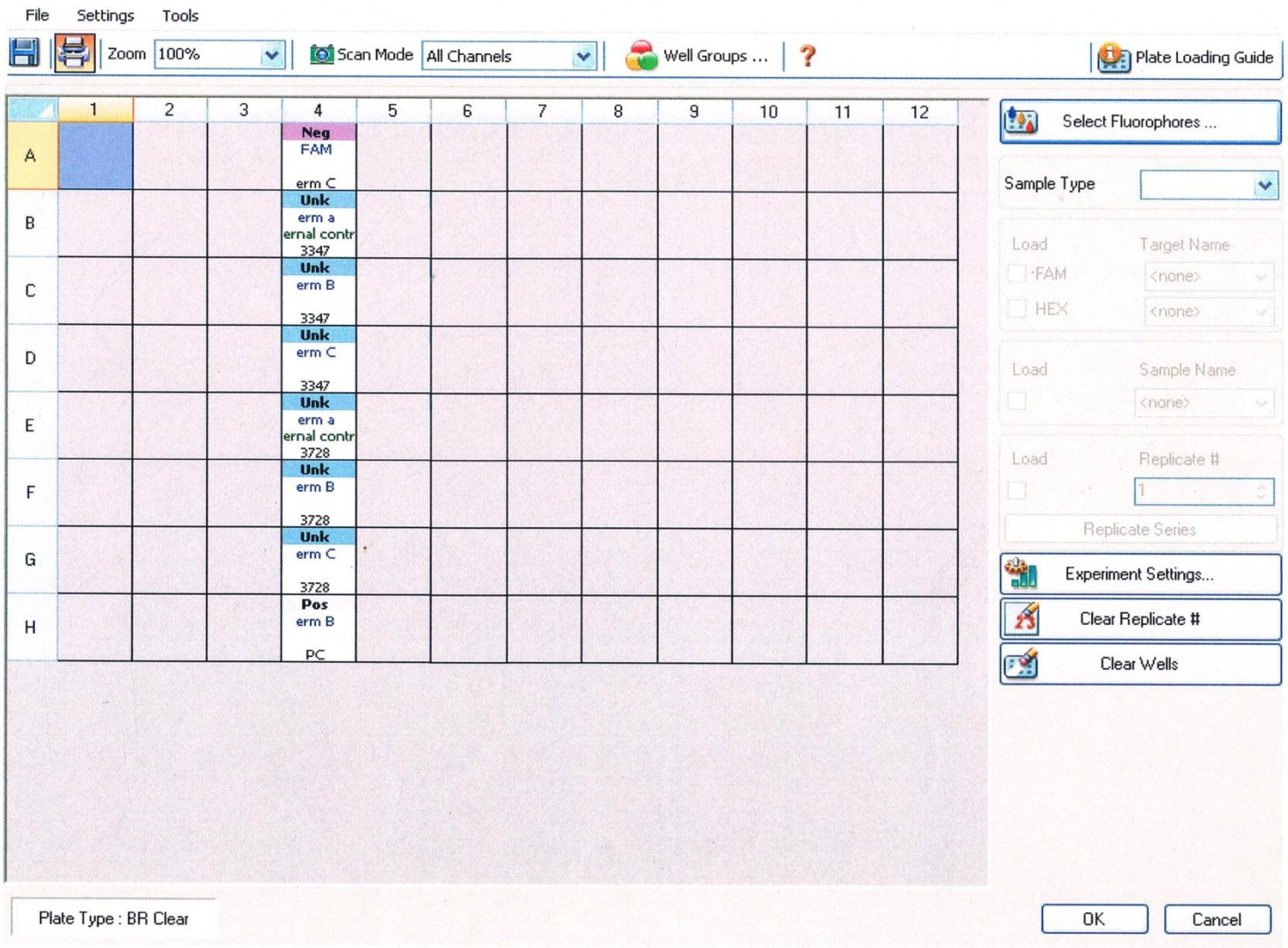
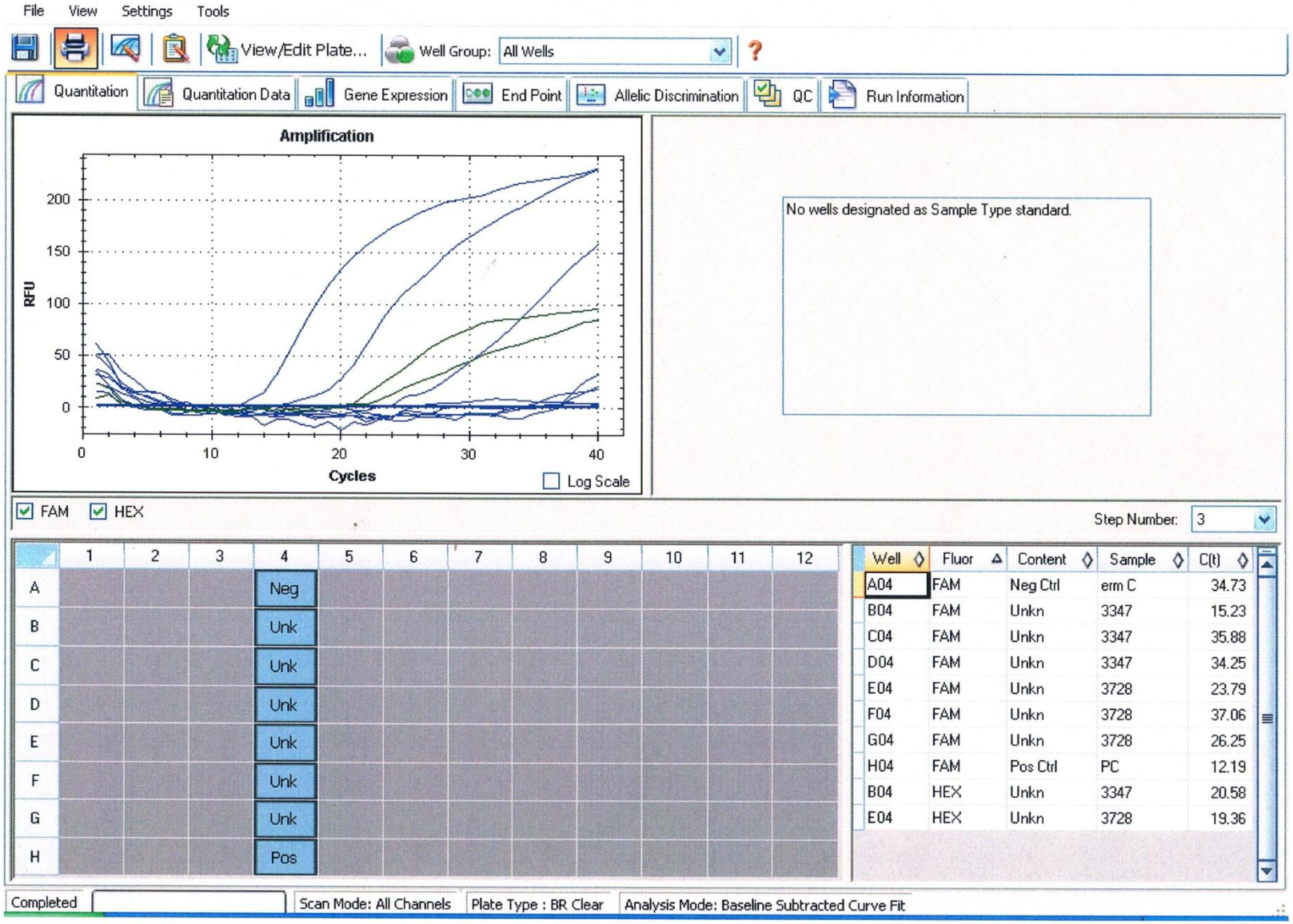


Fig.17 PCR Graph 3



5. RESULTS

The study was conducted in the Department of Microbiology, Tirunelveli Medical College, Tirunelveli , during the period April 2013 to May 2014. Clinical samples of sample size -100 *Staphylococcal* isolates which was isolated from the collected samples from out-patient and hospitalised patients of various departments.

The *Staphylococcal* strains isolated from the study samples were 80% *Staphylococcus aureus* and 20 % *CONS*. These isolates were processed and further tested for antibiotic sensitivity patterns .They were screened for Methicillin sensitivity pattern by Cefoxitin disc method and inducible Clindamycin resistance pattern by D-test.

D-test was used to screen clinical isolates of *Staphylococci* which were erythromycin-resistant for resistance to Macrolides, Lincosamides and Streptogramins (MLSB) which may be constitutive (MLSBc) inducible (MLSBi) or MS phenotype . The erythromycin ribosome methylase *(erm*) genes responsible for this resistance among inducible Clindamycin resistance (MLSBi) pattern was detected by Real-Time PCR and was further analysed.

Statistical Analysis :

Data regarding the subjects were described in terms of percentages. The ages of the subjects were compared between the genders by percentages .Statistical software IBM SPSS statistics 20 was used for the statistical procedures . The ($P < 0.05$) was considered as significant in Chi square and Fisher exact test.

Table 4: Distribution of *Staphylococcus aureus and Coagulase Negative Staphylococcus.*

Total Staphylococcal isolates	***Staphylococcus aureus***	***Coagulase Negative Staphylococcus.***
100	**80**	**20**

The *Staphylococcal* strains isolated from the study samples were 80% *Staphylococcus aureus* and 20 % *CONS* (Table-1)

Figure 18: **Distribution of *Staphylococcus aureus and Coagulase Negative Staphylococcus.***

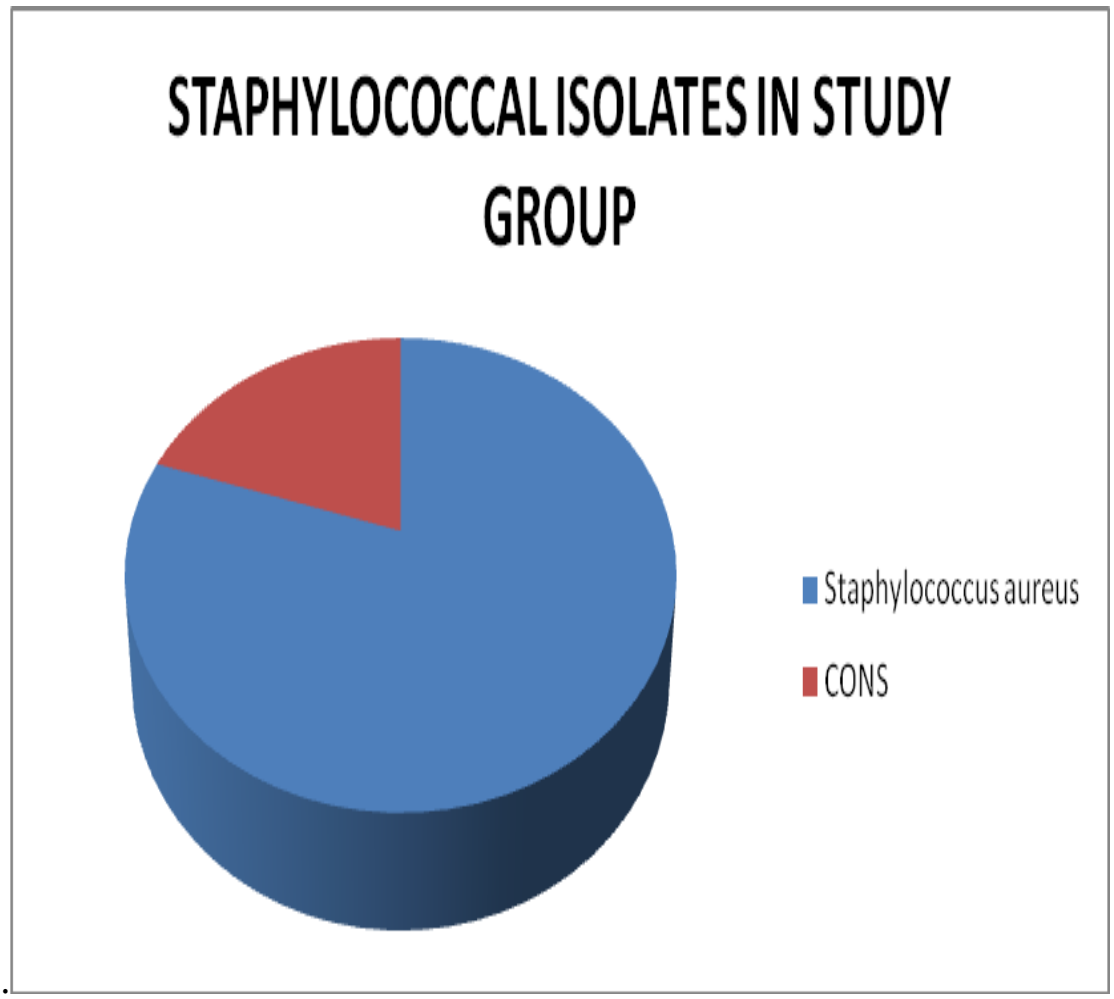


Table 5 : Age-sex wise distribution of the study group.

AGE	MALE	FEMALE
0-10	3(5.46%)	2(4.45%)
11-20	4(7.28%)	2(4.45%)
21-30	15(27.28%)	9(20%)
31-40	14(25.45%)	9(20%)
41-50	6(10.90%)	10(22.22%)
51-60	7(12.72%)	9(20%)
>60	6(10.90%)	4(8.88%)
TOTAL	55(100%)	45(100%)

Majority of the study group were found to be among the age group 21-30 yrs. The Male to female sex ratio was 1.22 :1 . Chi square P-value was 0.657 and statistically found not significant. There was no association between age group and gender wise distribution.

Figure 19 : Age-sex wise distribution of the study group.

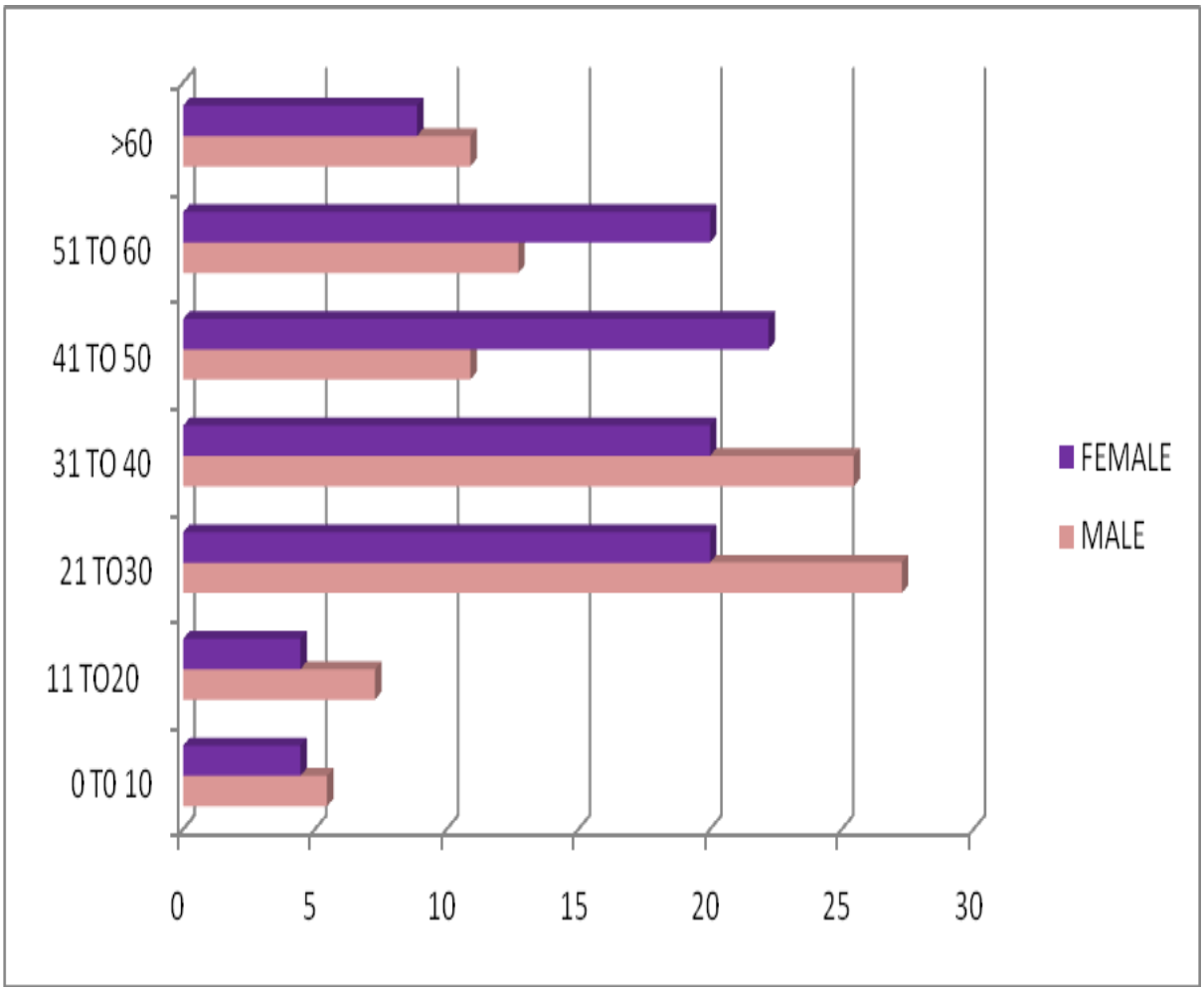


Table 6: Distribution of *Staphylococcus aureus* and *Coagulase Negative Staphylococcus* among samples.

Samples	***Staphylococcus aureus***	***Coagulase Negative Staphylococcus***
Pus	60(75%)	20(100%)
Wound swab	8(10%)	0
Aural swab	8(10%)	0
Vaginal swab	4(5%)	0
Total	80(100%)	20(100%)

Among the clinical samples collected 80% were pus, 8% wound swabs, 8% aural swabs and 4% vaginal swabs. Majority of the *Staphylococcus .aureus* about 75% and *CONS* 100% were isolated from pus.

Figure 20 : Distribution of *Staphylococcus aureus* and *Coagulase Negative Staphylococcus* among samples.

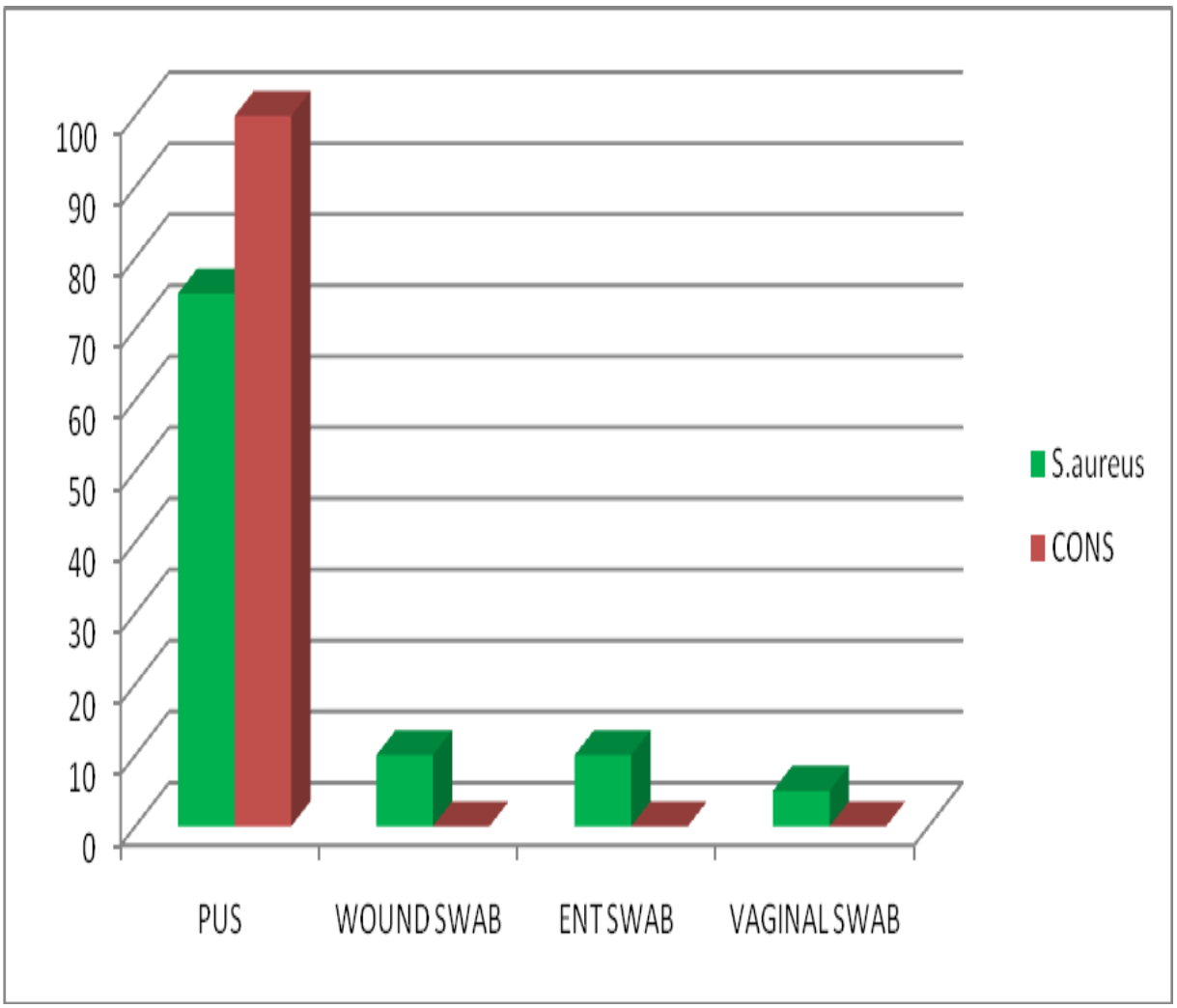


Table 7: Distribution of *Staphylococcus aureus* and *Coagulase Negative Staphylococcus.*

***Staphylococcal* isolates**	**Out patient department(OPD)**	**In patient department(IPD)**
S.aureus	16(80%)	64(80%)
CONS	4(20%)	16(20%)
TOTAL	20	80

Among the *Staphylococcal i*solates 80% were *Staphylococcus aureus* and 20% were *Coagulase Negative Staphylococcus* . *Staphylococcus aureus* was found pronounced than *CONS* in both OPD and IPD patients. Fisher's Exact p value was 1.00. There was no significant association between *Staphylococcal i*solates and hospitalization

Figure 21 : Distribution of *Staphylococcus aureus* and *Coagulase Negative Staphylococcus.*

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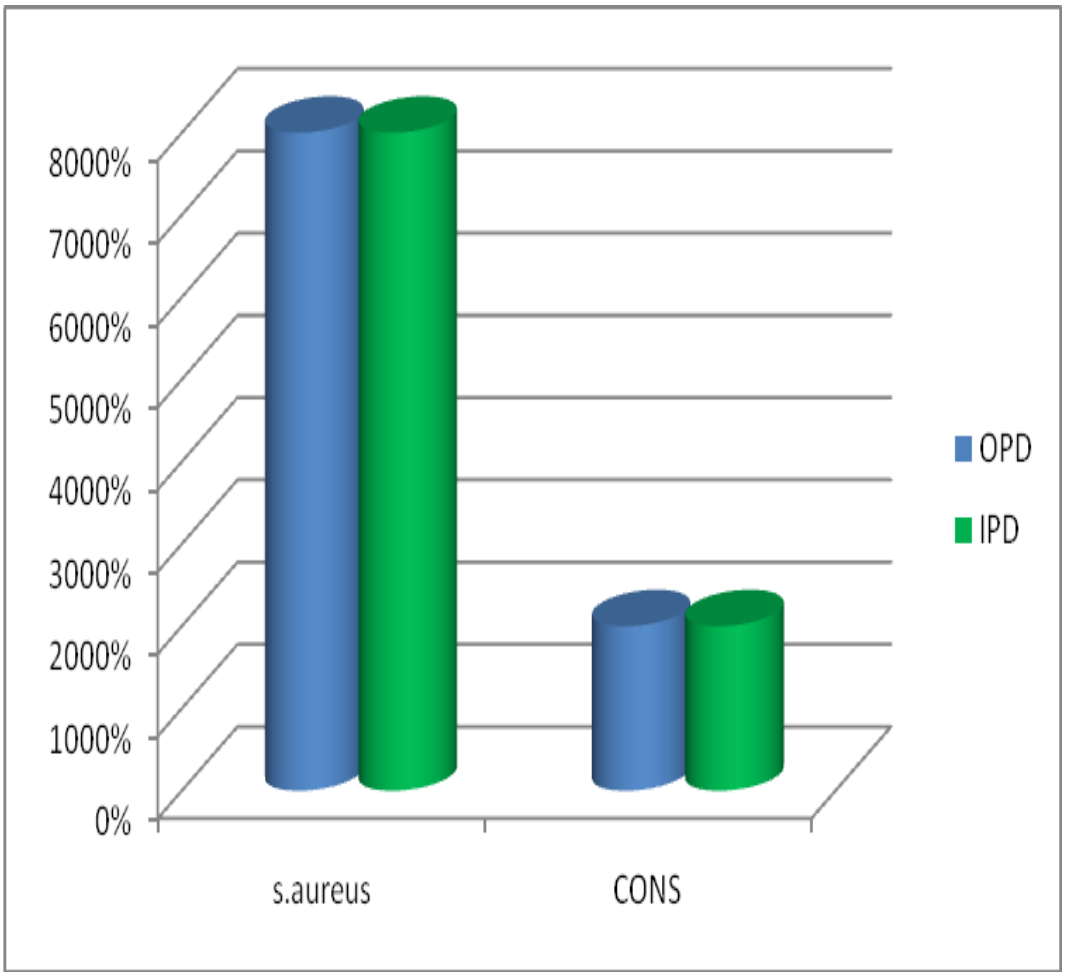

The *staphylococcal* isolates was found to be distributed about 80% in samples collected in in-patient department and 20% among out - patient department.

Table 8 : Antibiotic resistance among *Staphylococcal* isolates.

Antibiotics	**Staphylococcus aureus**	***Coagulase Negative Staphylococcus***	**Total**
Ampicillin	35(87.5%)	5(12.55%)	40
Amoxy clav	38(77.55%)	11(22.45%)	49
Doxycyclin	34(82.93%)	7(17.07%)	41
Ciprofloxacin	31(75.60%)	6(14.63%)	41
Cotrimoxazole	24(80%)	6(20%)	30
Gentamycin	26(78.79%)	7(21.21%)	33
Cefoxitin	25(83.33%)	5(16.67%)	30
Erythromycin	34(80.95%)	8(19.05%)	42
Clindamycin	4(80%)	1(20%)	5

The antibiotic resistance among *Staphylococcus aureus* were found to be 87.5% Ampicillin%,77.55%Amoxyclav, 82.93% Doxycyclin, 75.6 % Ciprofloxacin, 80% Cotrimoxazole , 78.79% Gentamycin 83.33% Cefoxitin 80.95% Erythromycin and 80% Clindamycin.

The antibiotic resistance pattern among *CONS* were 12.55% Ampicillin, 22.45% Amoxy clav, 17.07% Doxycyclin,14.63 % Ciprofloxacin, 20% Cotrimoxazole and 21.21% Gentamycin,16.67% Cefoxitin 19.05% ,Erythromycin and 20% Clindamycin. Chi square P-value < 0.001 was found statistically significant. There was an association between antibiotic resistance with staphylococcal isolates.

Figure 22 : Antibiotic resistance among *Staphylococcal* isolates.

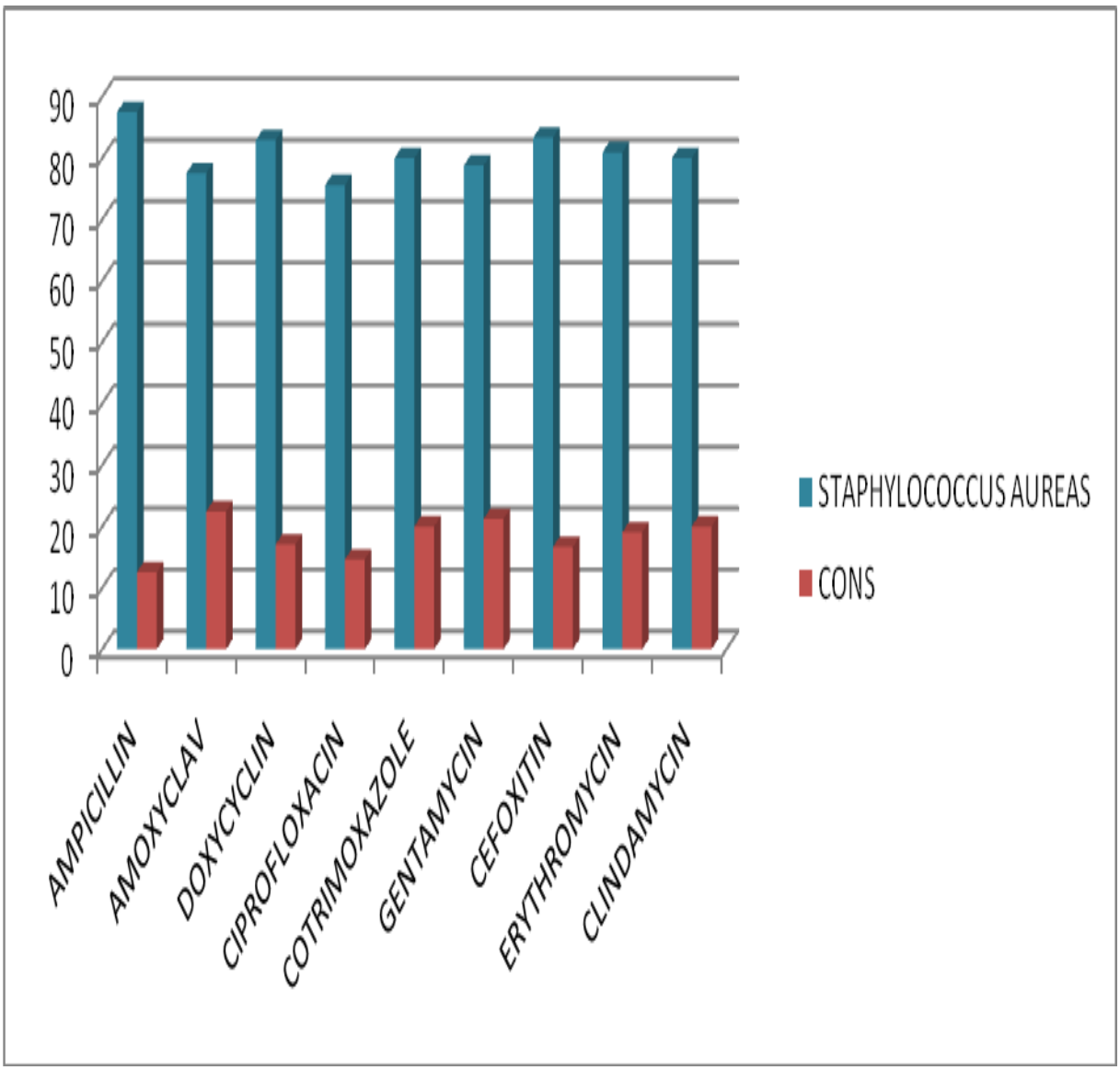

Table 9 : Distribution of *Staphylococcal* strains among OPD/IPD *patients.*

***Staphylococcal* isolates**	***MSSA***	***MRSA***	***MSCONS***	***MRCONS***	**Total**
Out patient department	10 (50%)	6 (30%)	4 (20%)	0	20
In patient department	45 (56.25%)	19 (23.75%)	11 (13.75%)	5 (6.25%)	80
Total	55	25	15	5	100

Among the *Staphylococcal i*solates obtained from out - patient department 50% were MSSA, 30% MRSA and 20% MSCONS. Samples from In-patient department showed 56.25 % MSSA, 23.75% MRSA, 13.75% MSCONS and 6.25 % MRCONS. . The Fishers Exact P-value was 0.546 .This was not statistically significant. There was no association between the distribution of staphylococcal isolates among the OPD/IPD patients.

Figure 23 : Distribution of *Staphylococcal* strains among OPD/IPD *patients.*

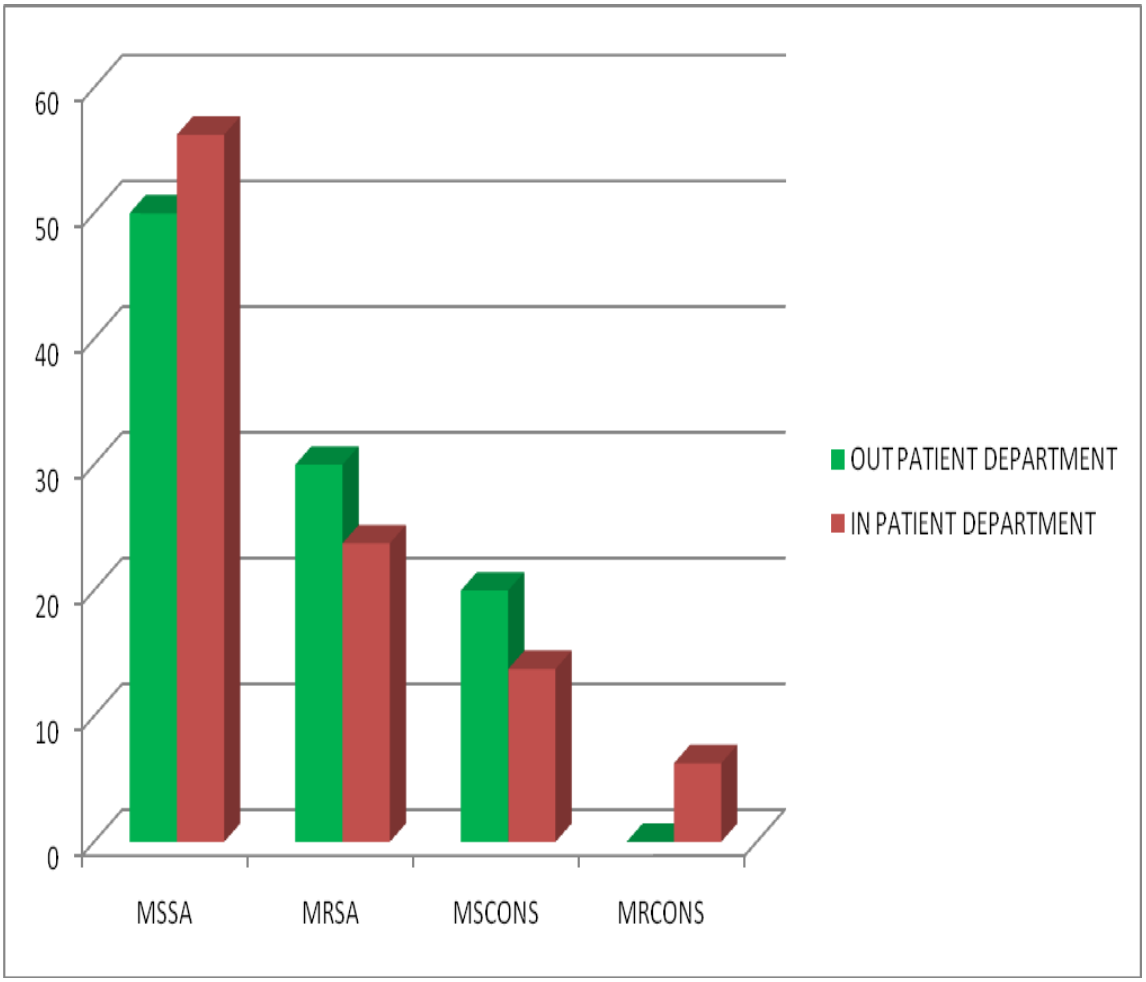

Table 10: Distribution of *MSSA* , *MRSA, MSCONS, MRCONS* among clinical samples.

Samples	***MSSA***	***MRSA***	***MSCONS***	***MRCONS***	**Total**
Pus	42(52.5%)	18(22.5%)	15(18.75%)	5(6.25%)	80
Wound swab	5(62.5%)	3(37.5%)	0	0	8
Aural swab	6(75%)	2(25%)	0	0	8
Vaginal swab	2(50%)	2(50%)	0	0	4

During the study 100 *Staphylococcal* strains were isolated from clinical samples collected from various departments which included 80 pus samples, 8 wound swabs, 8 aural swabs and 4 vaginal swabs. The majority of the samples collected from various wards were from pus (80%).Among the pus samples *MSSA* were 52.5%,*MRSA* (22.5%) *MSCONS* (18.75%) and *MRCONS* (6.25%).MSSA The chi-square P-value was 0.804 .This was not found to be statistically significant. There was no association between the distribution of staphylococcal isolates among the samples

Figure 24 : Distribution of *MSSA* , *MRSA, MSCONS, MRCONS* among clinical samples .

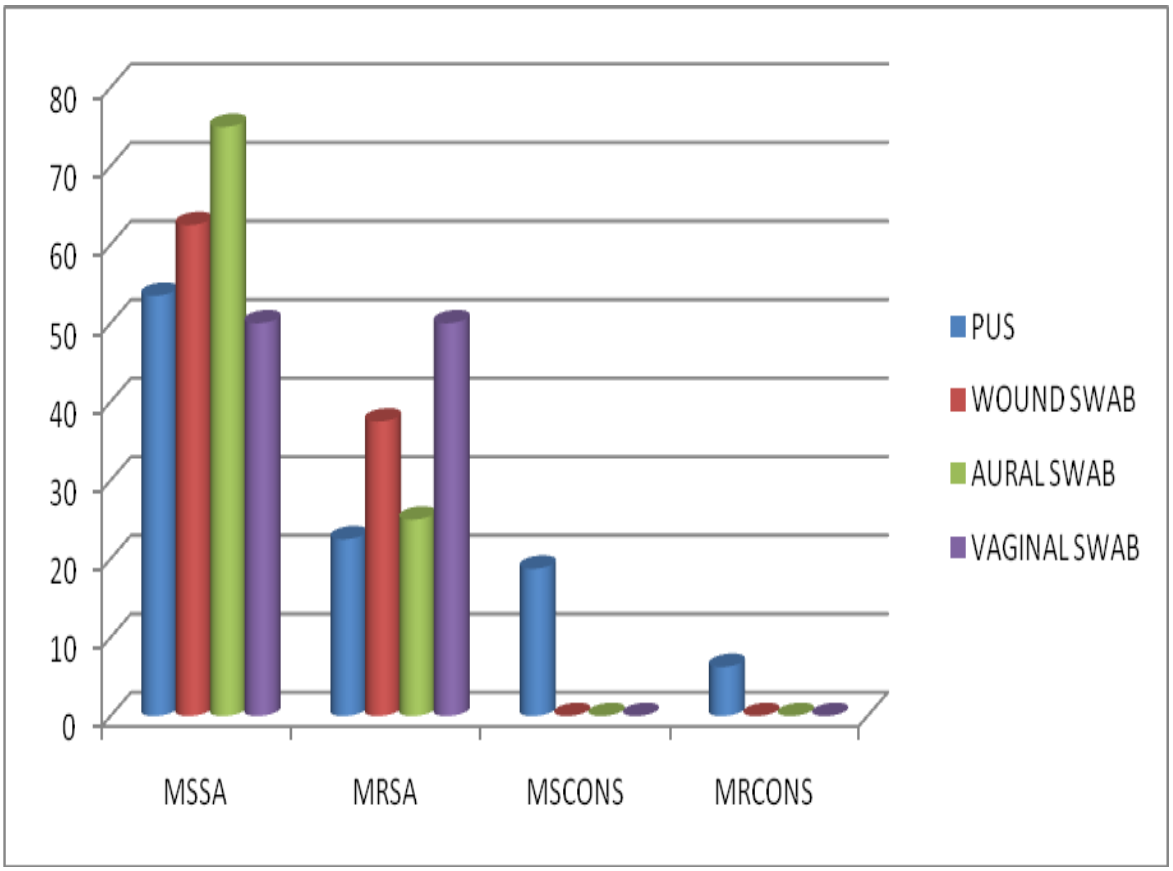

Table 11: Phenotypic distribution of iMLSB , cMLSB &MSP among Erythromycin resistant isolates.

Phenotypes	Number
iMLSB	14(35%)
cMLSB	5(12.5%)
MS	21(52.5%)
Total Erythromycin resistant isolates	40

Out of the 40 Erythromycin resistant isolates based on D-test the phenotypic distribution of iMLSB , cMLSB and MS were 35%, 12.5% and 52.5% respectively.

Figure 25 : Phenotypic distribution of iMLSB , cMLSB &MSP among Erythromycin resistant isolates

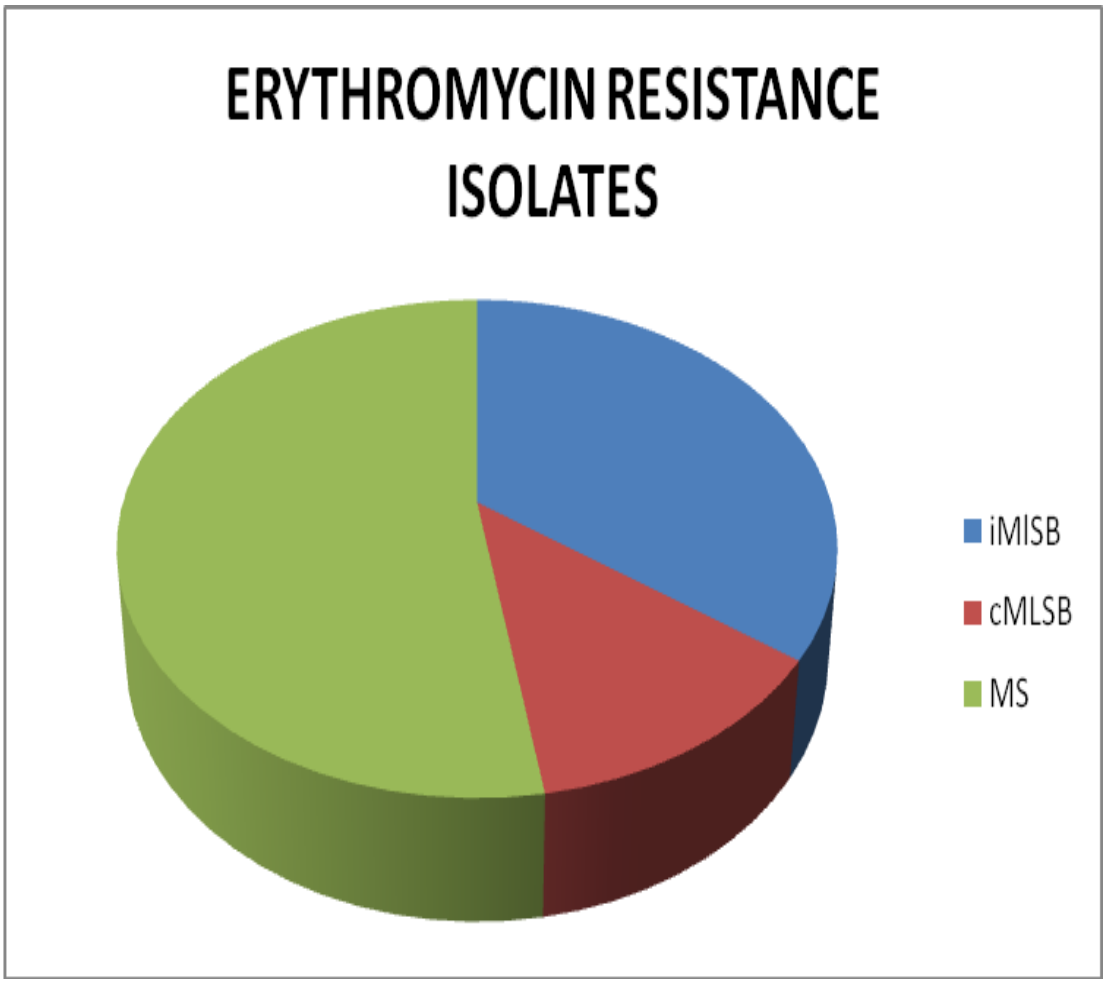

Table 12 : Distribution of iMLSB , cMLSB AND MS phenotypes among samples .

Samples	iMLSB	cMLSB	MSP
Pus	9(64.28%)	3(60%)	17(80.95%)
Wound swab	3(21.43%)	0	0
Aural swab	1(7.14%)	0	3(14.95%)
Vaginal swab	1(7.14%)	2(40%)	1(4.76%)
Total	14	5	21

The distribution of iMLSB phenotype among samples were found to be pus 64.29%, wound swabs 21.43%, aural swabs 7.14% and vaginal swabs 7.14%.The distribution of cMLSB phenotype among samples were pus 60%, and vaginal swabs 40%. The distribution of MS phenotype among samples were pus 80.95% , aural swabs 14 .29 % and vaginal swabs 4 .76 %. The chi-square P-value was 0.05 .This was found statistically significant. There was association between the phenotypic distribution and the samples.

Figure 26 : Distribution of iMLSB , cMLSB AND MS phenotype among samples .

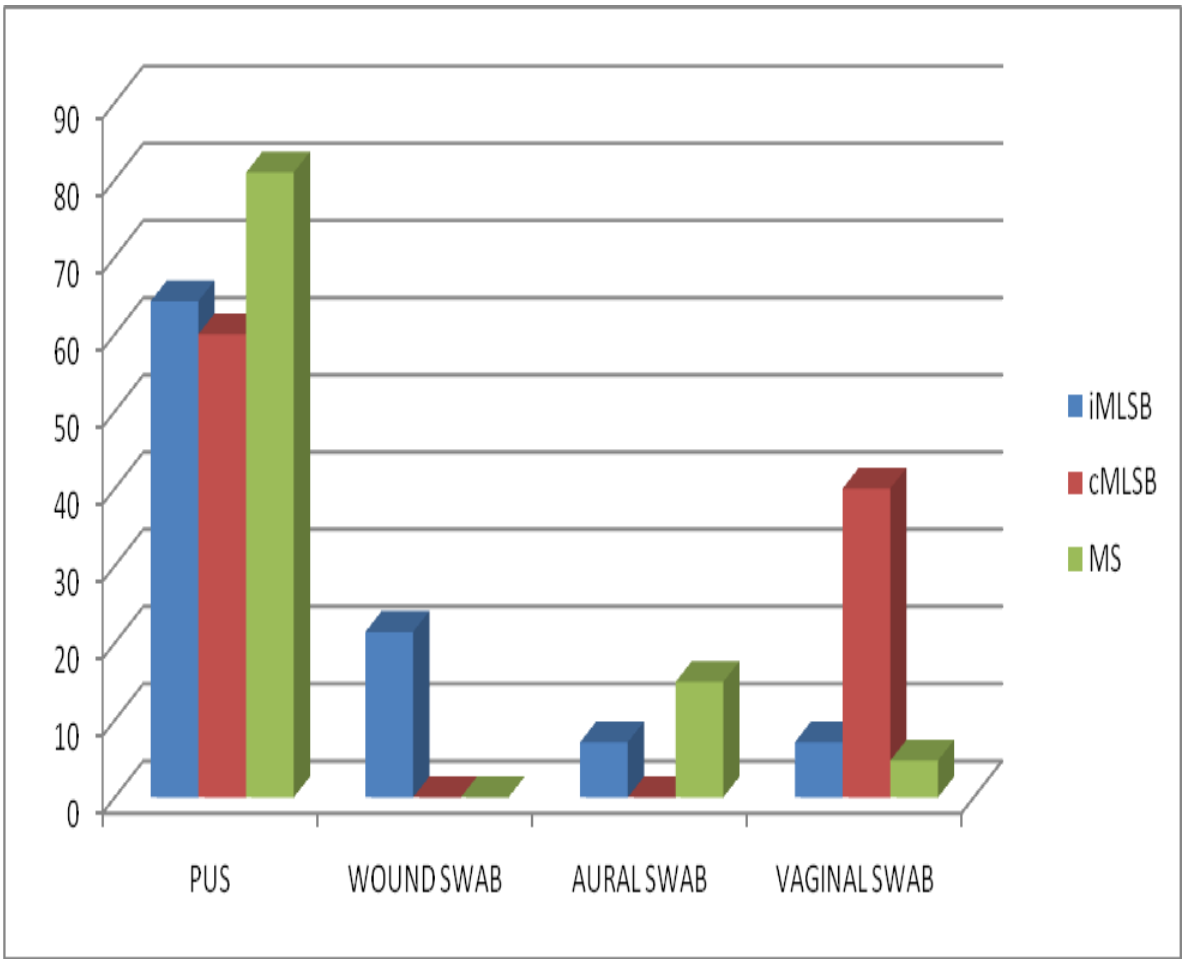

Table 13 :Phenotypic distribution of iMLSB , cMLSB &MSP among *Staphylococcal* isolates.

Phenotype	***S.aureus***	***CONS***
iMLSB	12(37.5%)	2(25%)
cMLSB	4(12.5%)	1(12.5%)
MSP	16(50%)	5(62.5%)
	32	8

The phenotypic distribution of iMLSB , cMLSB and MS among *S.aureus* were 37.5%, 12.5%, , 50% and *CONS* 25%, 12.5%, 62.5% respectively.

P-value 0.473 fe found not significant.

Figure 27 : Phenotypic distribution of iMLSB , cMLSB &MSP among *Staphylococcal* isolates.

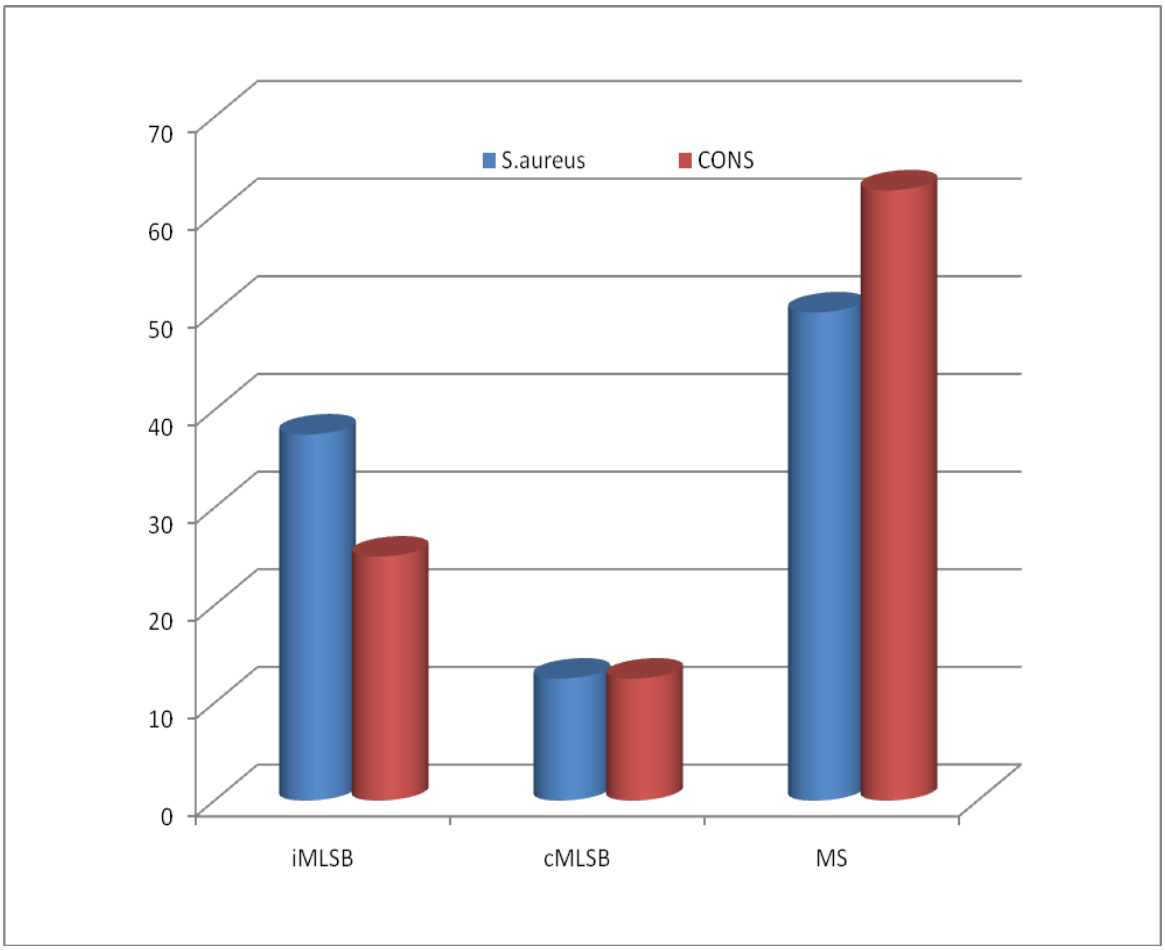

Table 14: The relation of *MSSA, MRSA ,MSCONS, MRCONS* IN MLSB PHENOTYPES.

MLSB Type	***MSSA***	***MRSA***	***MSCONS***	***MRCONS***	**Total**
iMLSB	6(42.85%)	6(42.85%)	0	2(14.28%)	14
cMLSB	3(60%)	1(20%)	1(20%)	0	5
MS	10(47.61%)	6(28.57%)	2(9.52%)	3(14.28%)	21

The phenotypic distribution of iMLSB , cMLSB and MS among MSSA were 42.85%, 60%, ,47.61% and MRSA 42.85%, 20%, 28.57% respectively

The phenotypic distribution of cMLSB and MS among MSCONS were 20%, 9.52% .The distribution of iMLSB and MS phenotype among MRCONS were 14.28% and 14.28% respectively

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Figure 28 : The relation of *MSSA, MRSA ,MSCONS, MRCONS* IN MLSB PHENOTYPES.

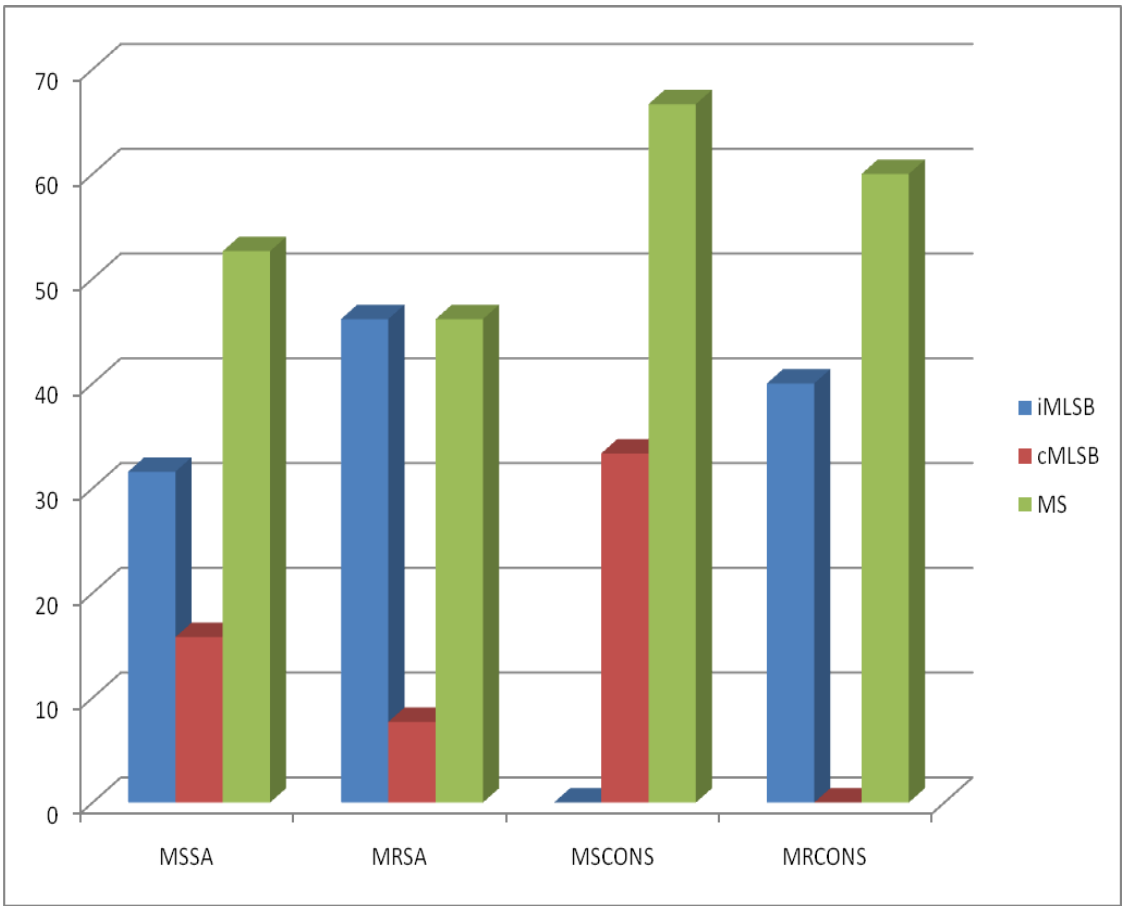


Table 15: Comparison of antibiotic resistance among iMLSB , cMLSB,MS phenotypes.

Antibiotics	iMLSB	cMLSB	MS
Ampicillin	13(32.5%)	2(5%)	7(17.5%)
Amoxy clav	13(26.53%)	2(4.08%)	9(18.36%)
Doxycyclin	8(19.51%)	2(4.87%)	6(14.63%)
Ciprofloxacin	4(9.75%)	3(7.31%)	10(24.39%)
Cotrimoxazole	5(16.66%)	1(3.33%)	7(23.33%)
Gentamycin	6(18.18%)	0	4(12.12%)
Cefoxitin	6(20%)	1(3.33%)	9(30%)

Among iMLSB phenotype antibiotic resistance were found in 32.5% Ampicillin,26.53% Amoxyclav,19.51% Doxycyclin,9.75% Ciprofloxacin,16.66% Cotrimoxazole , 18.18% Gentamycin and Cefoxitin 20%..Among cMLSB phenotype antibiotic resistance pattern were 5% Ampicillin,4.08 % Amoxy clav 4.87% Doxycyclin,7.31% Ciprofloxacin, 3.33% cotrimoxazole and 3.33% Cefoxitin. Chi-square P-value was 0.677 .This was found not statistically significant. There was no association between the antibiotic resistance and the different phenotypic isolates.

Figure 29 : Comparison of antibiotic resistance among iMLSB , cMLSB, MS phenotypes.

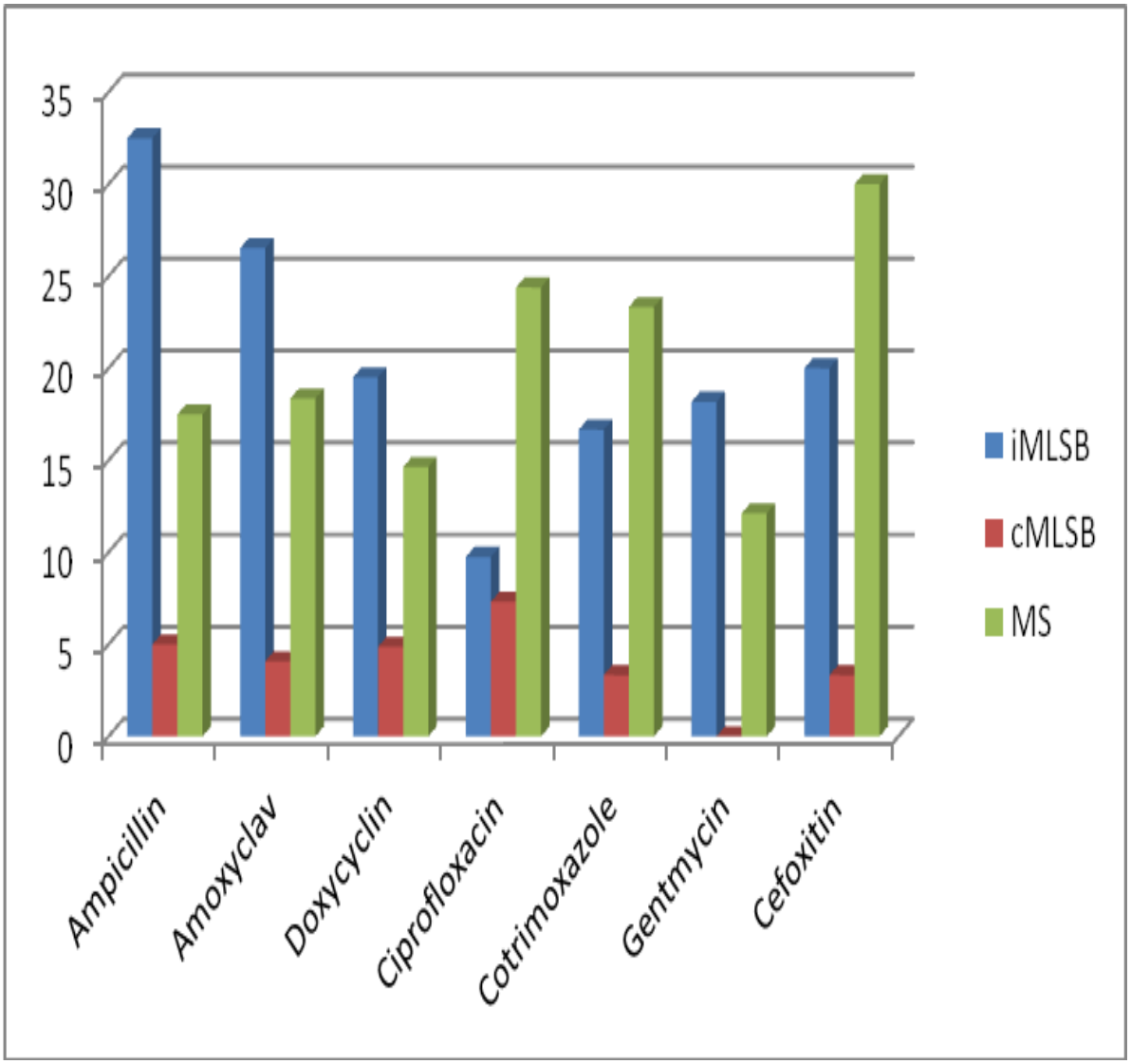


Table 16: Distribution of *erm* GENE IN Real Time PCR among inducible MLSB (iMLSB) phenotype.

Genotype-PCR	Number	Percentage
***erm* A only**	3	21.42
***erm* B only**	1	7.14
***erm* C only**	2	14.3
***erm* A & *erm* C**	7	50
***erm* B & *erm* C**	1	7.14
Total	14	100

Real time PCR detected 21.42% *erm* A,7.14% *erm* B,14.3% *erm* C,50% both *erm* A and *erm* C and 7.14% both *erm* B and *erm* C among the inducible MLSB (iMLSB) phenotype .

Figure 30 : Distribution of *erm* gene among iMLSB phenotype

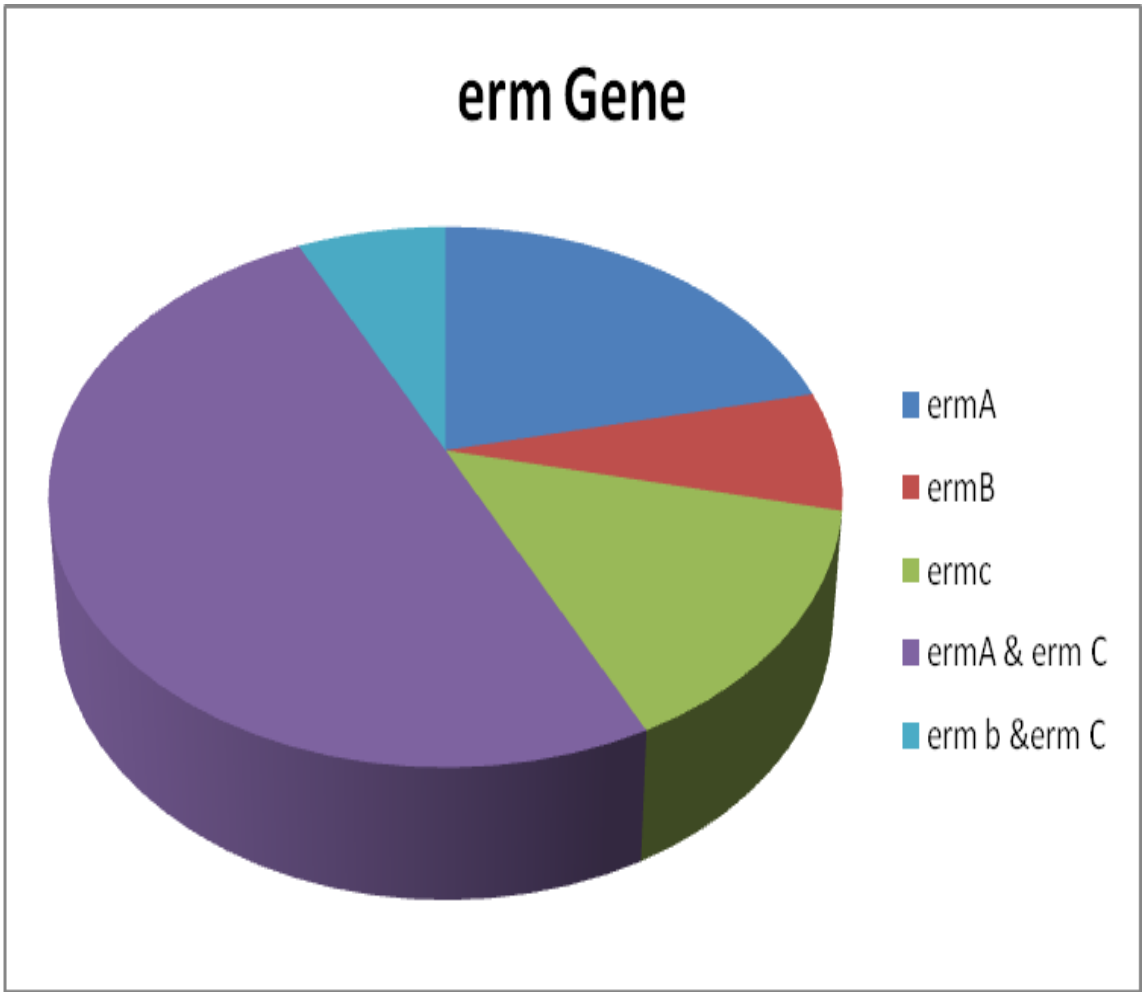


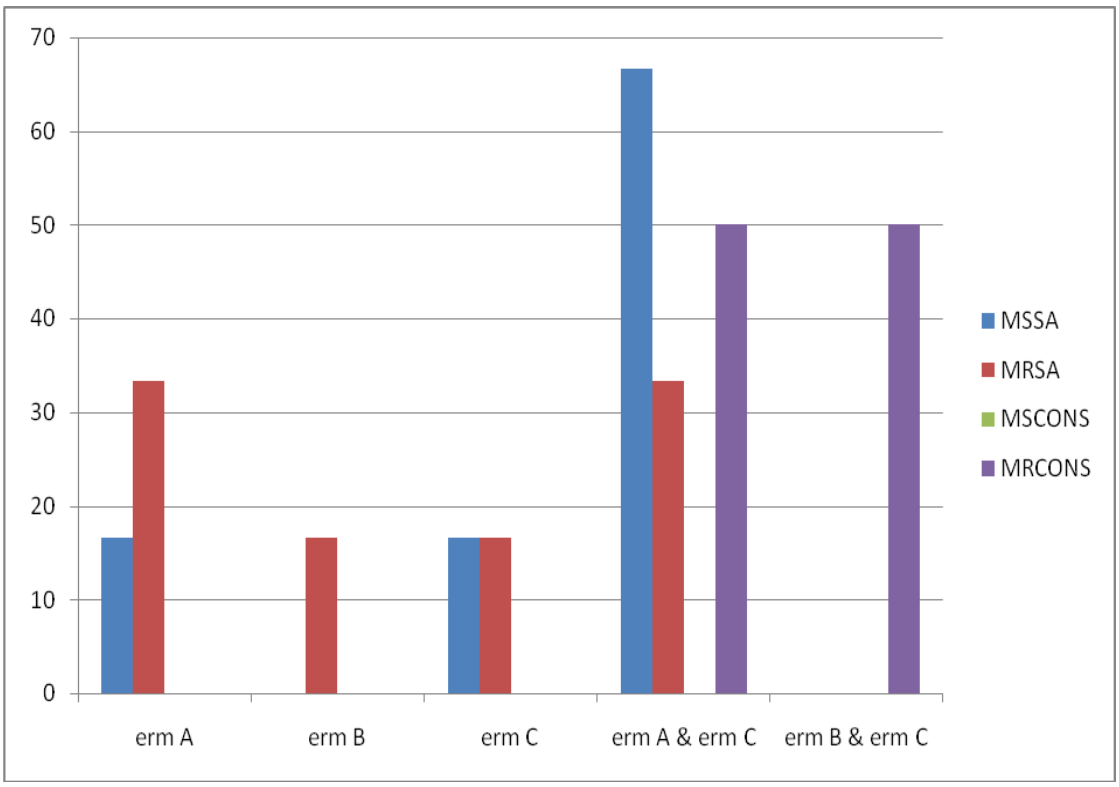
Table 17: Distribution of *erm* gene among *Staphylococcal* isolates.

Staphylococcal isolates	***Staphylococcus aureus***		***Coagulase Negative Staphyloccus***	
iMLSB	***MSSA***	***MRSA***	***MSCONS***	***MRCONS***
erm A	1(16.66%)	2(33.33%)	0	0
erm B	0	1(16.66%)	0	0
erm C	1(16.66%)	1(16.66%)	0	0
ermA &erm C	4(66.66%)	2(33.33%)	0	1(50%)
erm B &ermC	0	0	0	1(50%)
Total	6	6	0	2

Among *erm* A 16.66% were *MSSA* and 33.33% *MRSA*. In *erm* B 16.66% were *MRSA* , *erm* C was expressed among 16.66%- *MSSA* and 16.66%-*MRSA*. Both *erm* A & *erm* C were expressed in 66.66% *MSSA* ,33.33% *MRSA* and 50% *MRCONS* . Both *erm* B & *erm* C were expressed in 50% of *MRCONS*.

Chi-square P-value was 0.141 .This was found not statistically significant. There was no association between the erm gene distribution among the iMLSB phenotype.

Figure 31: Distribution of *erm* gene amongStaphylococcal isolates



In all the *Staphylococcal* D-test positive inducible Clindamycin resistance (iMLSB) phenotype *erm* gene was detected by real time PCR.

The sensitivity and specificity of D-test was 100% compared with the gold standard PCR.

DISCUSSION

6. DISCUSSION

Staphylococci producing life threatening infections in hospital and in community develops resistance towards antimicrobials. The development of antimicrobial resistance towards beta-lactam antibiotics and development of *MRSA* has become an increasing problem . Clindamycin is considered as an alternative drug of choice with good *in vitro* and *in vivo* activity[1]

The indiscriminate usage of antibiotic can be avoided by testing on clinical isolates the antimicrobial susceptibility as empirical treatment with antibiotics may lead to multi drug resistance mainly towards *MRSA.* [5]

Clindamycin, is an antibiotic of choice against *MRSA.* Which in the presence of Erythromycin produces resistance due to the induction by Erythromycin.This resistance is due to induction of cross- resistance between members of the Macrolide, Lincosamide, Strepto-gramin B (MLSB) group. The empirical therapy of *Staphylococcal* infections with antibiotics is not advisable because of the development of *MRSA* infections that is increasing in the community setting . The production of resistance to Clindamycin limits the effect of this drug.

Double Disk diffusion agar inhibitory assay or D – test is used to demonstrate MLSBi phenotype.[30]

In the present study, inducible resistance to Clindamycin (iMLSB) among Methicilln sensitive as well as resistant *Staphylococcal* strains at our institute was detected by D-test and *erm* gene responsible for iMLSB was detected by real time PCR.

In the present study out of 100 *Staphylococcal* strains isolated from clinical samples from various patients of different departments 80% were *Staphylococcus aureus* and the remaining 20% C*coagulase Negative Staphylococcus* .

In the present study 55% were males and 45% females Male and female were found to be in the ratio of 1.22 : 1.The gender association in this study showed no association and majority were found in age group of 21-30 yrs .

In this study *Staphylococcal* isolates were obtained 80% from pus, 8% from wound swab, 8% aural swab and 4% vaginal swab. Majority of the *Staphylococcus aureus* 75% and *CONS* 100% were isolated from pus.

In the present study among the isolated *Staphylococcal* strains 80% were from hospitalized patients and 20% were OPD patients. In this study out of the *Staphylococcal* isolates among 80% *Staphylococcus*

aureus 55% were *MSSA* and 25% *MRSA* .Among the 20% *CONS* 15% were *MSCONS* and 5% *MRCONS*. Samples obtained from OPD 50% were *MSSA*, 30% *MRSA* and 20% *MSCONS*. Samples from IPD showed 56.25% *MSSA*, 23.75*% MRSA*, 13.75% *MSCONS* and 6.25% *MRCONS* .

Out of the 40 Erythromycin resistant isolates based on D-test the phenotypic distribution of iMLSB , cMLSB and MS were 35%, 12.5% and 52.5% respectively

In a study by Vidhya et al [42] among the inducible Clindamycin resistance (D-test positive) was 13.33% constitutive resistance was 40% and MS phenotype (D-test negative) was 35.43%

In this study the distribution of iMLSB phenotype among samples were pus 64.28%, wound swab 21.43%, aural swab 7.14% and vaginal swab 7.14%.The distribution of cMLSB phenotype among samples were pus 60%, and vaginal swab 40%.The distribution of MS phenotype among samples were pus 80.95% , aural swab 14 .95 % and vaginal swab 4 .76 %.

The phenotypic distribution of iMLSB , cMLSB and MS among *S.aureus* were 37.5%, 12.5%, , 50% and CONS 25%, 12.5%, 62.5% respectively. Sureet et al [49] showed *Staphylococcus aureus* 9.9% and *CoNS* (5.5%) were inducible CL resistance. Matthew et al [8] in his study

showed a high prevalence (96.3%) of iMLSB resistance among *S. aureus* isolates .

The phenotypic distribution of iMLSB , cMLSB and MS among *MSSA w*ere 42.85%, 60%, ,47.61% and *MRSA* 42.85%, 20%, 28.57% respectively. This showed that *MSSA* was found more among cMLSB and *MRSA a*mong iMLSB.

The phenotypic distribution of cMLSB and MS among *MSCONS* were 20%, 9.52% respectively .The distribution of iMLSB and MS phenotype among *MRCONS* were 14.28% and 14.28% respectively

This is in concordance with few studies reported in India.

Deotale et al[39] found 27.6% iMLSB in *MRSA* and 1.6% in *MSSA.*

Gupta et al showed it to be 20% in *MRSA* and 17.33% in *MSSA.*

Prabhu et al showed 20% in *MRSA* and 6.15% in *MSSA.*

Vidhya et al [42] showed inducible clindamycin resistance is more in *MRSA* 23.07% compared to *MSSA* 3.52%.

Sureet et al [49] showed *MRSA* more than in *MSSA*, 35.9% and 4.7% respectively in iMLSB.

Matthew et al [8] showed high rate of iMLSB among both Methicillin sensitive and resistant isolates, obtained from both community- and hospital-acquired infections.

Antibiotic sensitivity: Among iMLSB phenotype antibiotic resistance were found in 32.5% Ampicillin,26.53% Amoxyclav,19.51% Doxycyclin,9.75% Ciprofloxacin,16.66% Cotrimoxazole , 18.18% Gentamycin and Cefoxitin 20%..Among cMLSB phenotype antibiotic resistance pattern were 5% Ampicillin,4.08 % Amoxy clav 4.87% Doxycyclin,7.31% Ciprofloxacin, 3.33% cotrimoxazole and 3.33% Cefoxitin. There was no association between the antibiotic resistance and the different phenotypic isolates.

In this study None of the isolates were resistant to Vancomycin. In a study by Tiwari et al [2] all the iMLS phenotype B *Staphylococcus aureus* strains were sensitive to Vancomycin and Linezolid (100%).

In this study Real time PCR showed 21.42% ermA,7.14% erm B,14.3% erm C,50% erm A and ermC and 7.14% ermB and ermC . Among erm A 16.66% were MSSA and 33.33% MRSA. In erm B 16.66% were MRSA , erm C was expressed among 16.66%- MSSA and 16.66%-MRSA. Both erm A & erm C were expressed in

66.66%MSSA,33.33% MRSA and 50% MRCONS .Both erm B and erm C were expressed in 50% of MRCONS

Study by Feibelcon et al [37] showed *S. aureus* isolates among 19 samples with iMLSB for genetic analysis, 18 contained *erm A* and remaining 1 showed *ermC*. Out of 4 CONS isolates showed ; 3 *ermC* , and one contained *ermA msrA*, but not *ermA* or *ermC was* detected among Clindamycin susceptible isolates with negative test . Only one resistance mechanism among the *S. aureus* isolates ; five (6%) possessed an *erm* gene plus *msrA*. 12 (12%) CONS isolates possessed two or more resistance genes

Matthew et al [8] showed out of 28 iMLSB among *S. aureus* isolates8 genotyped harbored *ermA*, and the remainder harbored *ermC*

The observations made in this study suggest routine D-test in all clinical laboratories to detect inducible Clindamycin resistance when *Staphylococcal* isolates showed Erythromycin resistant and Clindamycin susceptible pattern. Failure to identify them lead to therapeutical failure in the usage of Clindamycin for treatment.

In this present study D-test is an easy test which can be performed in every laboratory . It is also found inexpensive test which can be used for practical purpose than PCR which is the gold standard .This test can be used to detect an inducible Clindamycin resistance in

staphylococci as a routine test in laboratories where PCR is not available This test help us to provide confident laboratory reports and Clindamycin can be omitted in patients with infections caused by inducible Clindamycin resistance staphylococci, and therapeutic failures may be thus avoided.

SUMMARY

7. SUMMARY

This study was undertaken in Tirunelveli Medical College, Tirunelveli for a period of one year from 100 *Staphylococcal* isolates 80% were *Staphylococcus aureus* and rest 20% were *coagulase negative staphylococcus.* Out of strains isolated 80% were from hospitalized patients . The study group contained 55% males and 45% females and Male to female sex ratio was 1.2 2:1. Majority of the study group were found among the age group 21-30 yrs.

Majority of the *Staphylococcal aureus* 75% and CONS 100% were isolated from pus and majority of the MSSA 76.36% and 72% MRSA were also from pus.

Out of the 40 Erythromycin resistant isolates based on D-test the phenotypic distribution of iMLSB , cMLSB and MS were 35%, 12.5% and 52.5% respectively

In the phenotypic distribution of iMLSB *Staphylococcus .aureus* was 37.5% and *CONS* 25%,. MSSA 31.58% and MRSA 46.15%.

Majority of the iMLSB was susceptible to Ciprofloxacin Cotrimoxazole and Gentamycin. while cMLSB and MS phenotype were resistant to Ciprofloxacin when compaired to iMLSB .There was

high rate of resistance exhibited by cMLSB towards Cefoxitin when compaired to iMLSB.. No resistance was observed to Vancomycin.

Genetic analysis by real time PCR performed on iMLSB showed 21.42% *erm* A,7.14% *erm* B,14.3% *erm* C,50% *erm* A and *erm*C and 7.14% *erm*B and *erm* C.

In all the *Staphylococcal* D-test positive inducible Clindamycin resistance (iMLSB) phenotype *erm* gene was detected by real time PCR. The sensitivity and specificity of D-test was 100% compared with the gold standard PCR.

CONCLUSION

8 . CONCLUSION

- The present study "**DETECTION *OF erm* GENE AMONG INDUCIBLE CLINDAMYCIN RESISTANT STAPHYLOCOCCAL ISOLATES IN CLINICAL SAMPLES** '' was conducted in the Department of Microbiology, Tirunelveli Medical College, Tirunelveli during the period April 2013 to May 2014 .
- Cefoxitin disc diffusion test was used to detect *MRSA* and *MSSA,* which revealed among Staphylococcal isolates that *MSSA* was found pronounced than *MRSA.*
- D-test revealed iMLSB was found more among the *MRSA* than *MRSA* .
- Majority of the iMLSB was susceptible to Ciprofloxacin , Cotrimoxazole and Gentamycin. while cMLSB and MS phenotype were resistant to Ciprofloxacin when compaired to iMLSB .There was high rate of resistance exhibited by cMLSB towards Cefoxitin when compaired to iMLSB
- Genetic analysis by Real time PCR detected *erm* gene in all iMLSB phenotype which was responsible for the resistance .

- Since, failure in the therapy with Clindamycin used against *Staphylococcal* isolates had been frequently met, the Inducible resistance due to *erm* gene can be detected by D-test and can be used as a routine test in all microbiology laboratory, which helps the clinicians in avoiding treatment failure with Clindamycin.

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ANNEXURE-I

ANNEXURE-I

McFarland Standard (0.5)

Composition and preparation 1 % (V/V) solution of chemically pure (0.36N)Sulphuric acid and 1.175 % (W/V) solution of chemically pure (0.048M) barium chloride was prepared in two separate sterile flasks. Then 9.9 ml of sulphuric acid and 0.1 ml of barium chloride were added to the clean screw capped test tube and sealed. To make the turbidity standard of cell density to one half(0.5) of the McFarland standard tube No.1 which correspond to cell density of 1.5 × 108 organism/ml for determination of antibiotic sensitivity by Kirby-Bauer inoculated technique 0.5 ml of 1.7 %(W/V) barium chloride (Bacl2 2H 2O) was added to 99.5 ml of 1 % (V/V) Sulphuric acid(0.36N), mixed well and 5- 10 ml was distributed in sterile capped test tubes and sealed. .

ANNEXURE-II

ANNEXURE-II

PROFORMA

NAME:

AGE:

SEX:

ADDRESS:

WARD:

IP NO:

DATE:

SPECIMEN:

CLINICAL DIAGNOSIS:

RISK FACTORS:

1)DURATION OF HOSPITALISATION:

2)INSTRUMENTATION: CVC/IV LINE

3)ANTIBIOTICS ADMINISTERED:

a)CLASS:PENCILLINS/CEPHALOSPORINS/AMINOGLYCOSIDES MACROLIDES/CARBAPENEMS.

b)DURATION OF ANTIBIOTICS ADMINISTERED: <7 DAYS/>7 DAYS

4)ANY CHRONIC DISORDERS:DM/HT/RENAL

FAILURE/HART/OTHERS.

5) ICU STAY :YES/NO

INVESTIGATIONS

1)SLIDE COAGULASE

2)TUBE COAGULASE

3)ANTIBIOGRAM: CEFOXITIN (30µg)

ERYTHROMYCIN (15µg)

AMIKACIN/GENTAMYCIN(30µg)

CIPROFLOXACIN(5µg)

CLINDAMYCIN(2µg)

4)D-TEST: ERYTHROMYCIN

CLINDAMYCIN

5)PCR- erm[A],

erm[B],

erm[C].

ANNEXURE-III

ANNEXURE-III

Detection of *erm* gene among inducible Clindamycin Resistant *Staphylococcal* isolates in clinical samples

AIM:

The present study is aimed to detect erm gene in inducible Clindamycin resistant *Staphylococcal* isolates and to study the relationship between Clindamycin and Methicillin resistance .

MATERIALS AND METHODS:

A total of 100 *Staphylococcal* clinical isolates will be collected from the department of Microbiology, Tirunelveli Medical College, Tirunelveli. *Staphylococcal* isolates from pus-wound swab and aspirates will be identified using conventional bacteriological methods and their susceptibility tested by standard disk diffusion method on Muller Hinton agar[MHA] according to the standards of clinical and laboratory standards institute[CLSI].Detection of inducible Clindamycin resistence will be performed by D-test. The presence of methylase genes *erm*[A], *erm*[B], *erm*[C] will be determined by PCR.

JUSTIFICATION OF THE STUDY:

The emergence of resistance to most antimicrobial agents in *Staphylococci* indicates the need for new effective agents in the treatment of *Staphylococcal* infection. Among the alternatives available Clindamycin is

considered to be safe, effective and less costly agent. In vitro routine tests for Clindamycin susceptibility may fail to detect inducible Clindamycin resistance due to *erm* genes, resulting in treatment failure, thus necessitating the need to detect such resistance by a simple D-test on routine basis.

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MASTER CHART

S.NO	MICRO NO	OP/IP	AGE	SEX	SAMPLE	DIAGNOSIS	HOSPITAL STAY	SLIDE COAGULASE	TUBE COAGULASE	S.aureas	MSSA	MRSA	CONS	MSCONS	MRCONS	AMPICILLIN	AMOXYCLAV(30mcg)	DOXYCYCLINE(30mcg)	COTRIMOXAZOLE(25mcg)	CIPROFLOXACIN(5mcg)	GENTAMYCIN(10mcg)	CEFOXITIN(30mcg)	VANCOMYCIN	ERYTHROMYCIN(15mcg)	CLINDAMYCIN(2mcg)	D-TEST	PCR-ERM-A GENE	PCR-ERM-B GENE	PCR-ERM-C GENE	MISCELLANEOUS	MORTALITY
1	590	IP	7	F	SWAB	W.INFEC	10	P	P	P	P	N	N	N	N	R	R	S	R	R	S	S	S	S	S	SS	N	N	N	N	N
3	638	IP	20	M	PUS	ABSCESS	7	P	P	P	P	N	N	N	N	R	R	S	R	R	R	S	S	S	S	SS	N	N	N	N	N
6	664	IP	40	M	PUS	ABSCESS	20	P	P	P	P	N	N	N	N	S	S	S	S	S	S	S	S	**R**	**D**	**I**	N	N	**P**	N	N
10	895	IP	53	M	PUS	ABSCESS	7	P	P	P	P	N	N	N	N	S	S	S	S	R	S	S	S	R	S	MS	N	N	N	N	N
12	906	IP	12	F	PUS	ABSCESS	8	P	P	P	P	N	N	N	N	S	S	S	S	S	S	S	S	S	S	SS	N	N	N	N	N
13	911	IP	27	M	PUS	ABSCESS	10	N	N	N	N	N	P	N	P	S	S	S	S	R	R	R	S	R	S	MS	N	N	N	N	N
14	918	IP	54	M	SWAB	EAR	15	P	P	P	P	N	N	N	N	R	R	R	R	S	R	S	S	**R**	**D**	**I**	**P**	N	**P**	N	N
15	919	IP	8	M	PUS	ABSCESS	8	P	P	P	P	N	N	N	N	R	R	S	S	S	R	S	S	S	S	SS	N	N	N	N	N
16	921	IP	29	M	PUS	ABSCESS	5	P	P	P	P	N	N	N	N	R	R	S	S	S	S	S	S	S	S	SS	N	N	N	N	N
17	923	IP	55	M	PUS	ABSCESS	6	P	P	P	P	N	N	N	N	R	R	S	S	R	R	S	S	S	S	SS	N	N	N	N	N
20	929	OP	28	M	PUS	ABSCESS	0	P	P	P	P	N	N	N	N	S	S	R	R	S	R	S	S	S	S	SS	N	N	N	N	N
25	1269	IP	32	M	PUS	ABSCESS	8	P	P	P	P	N	N	N	N	S	R	S	S	S	R	S	S	R	S	MS	N	N	N	N	N
27	1349	IP	23	M	SWAB	W.INFEC	15	P	P	P	P	N	N	N	N	R	R	S	S	S	R	S	S	**R**	**D**	**I**	**P**	N	**P**	N	N
28	1381	IP	43	M	PUS	ABSCESS	5	P	P	P	P	N	N	N	N	S	R	R	S	S	S	S	S	S	S	SS	N	N	N	N	N
35	1628	IP	33	M	PUS	ABSCESS	16	P	P	P	P	N	N	N	N	R	R	R	S	S	S	S	S	**R**	**D**	**I**	**P**	N	**P**	N	N
36	1640	IP	28	M	PUS	ABSCESS	3	P	P	P	P	N	N	N	N	S	S	S	S	S	S	S	S	S	S	SS	N	N	N	N	N
37	1659	IP	32	M	PUS	ABSCESS	5	P	P	P	P	N	N	N	N	R	R	S	S	S	S	S	S	S	S	SS	N	N	N	N	N
38	1662	IP	54	M	PUS	ABSCESS	5	P	P	P	P	N	N	N	N	R	R	S	S	S	S	S	S	S	S	SS	N	N	N	N	N
40	1865	IP	10	M	SWAB	EAR	5	P	P	P	P	N	N	N	N	S	S	S	S	S	S	S	S	S	S	SS	N	N	N	N	N
48	2293	IP	14	M	PUS	ABSCESS	5	P	P	P	P	N	N	N	N	S	S	S	R	S	S	S	S	S	S	SS	N	N	N	N	N
49	2393	IP	41	M	PUS	ABSCESS	8	P	P	P	P	N	N	N	N	S	S	R	S	S	S	S	S	R	S	MS	N	N	N	N	N
50	2456	IP	22	M	SWAB	EAR	2	P	P	P	P	N	N	N	N	S	S	R	S	S	S	S	S	S	S	SS	N	N	N	N	N
52	2862	IP	40	M	PUS	ABSCESS	7	P	P	P	P	N	N	N	N	R	R	S	S	S	R	S	S	S	S	SS	N	N	N	N	N
54	2978	IP	43	M	PUS	ABSCESS	5	P	P	P	P	N	N	N	N	R	R	R	S	S	R	S	S	R	S	MS	N	N	N	N	N
55	2992	OP	9	F	PUS	ABSCESS	5	P	P	P	P	N	N	N	N	S	S	S	R	R	S	S	S	R	S	SS	N	N	N	N	N
60	3068	IP	42	M	PUS	ABSCESS	7	P	P	P	P	N	N	N	N	R	R	S	S	S	S	S	S	R	S	MS	N	N	N	N	N
61	3195	IP	22	M	PUS	ABSCESS	14	P	P	P	P	N	N	N	N	R	R	R	S	R	R	S	S	**R**	**D**	**I**	**P**	N	N	N	N
63	3204	IP	27	M	PUS	ABSCESS	5	N	N	N	N	N	P	P	N	S	S	R	S	R	S	S	S	S	S	SS	N	N	N	N	N
67	3256	IP	32	M	pus	VAGINAL	8	P	P	P	P	N	N	N	N	S	S	S	R	R	S	S	S	S	S	SS	N	N	N	N	N
69	3286	IP	56	M	SWAB	EAR	2	P	P	P	P	N	N	N	N	S	S	S	S	R	R	S	S	S	S	SS	N	N	N	N	N
76	3512	OP	34	M	PUS	ABSCESS	8	N	N	N	N	N	P	P	N	S	R	R	S	R	S	S	S	S	S	SS	N	N	N	N	N
78	3540	IP	58	M	SWAB	VAGINAL	6	P	P	P	P	N	N	N	N	S	S	R	R	S	R	S	S	S	S	SS	N	N	N	N	N
80	3542	IP	25	M	PUS	ABSCESS	7	N	N	N	N	N	P	P	N	S	S	S	S	R	S	S	S	S	S	SS	N	N	N	N	N

82	3546	OP	35	M	PUS	ABSCESS	5	N	N	N	N	N	P	P	N	S	R	R	R	S	R	S	S	S	S	SS	N	N	N	N	N
83	3547	IP	15	M	PUS	ABSCESS	6	N	N	N	N	N	P	P	N	S	R	S	S	S	R	S	S	S	S	SS	N	N	N	N	N
88	3641	IP	50	M	PUS	ABSCESS	7	P	P	P	P	N	N	N	N	S	S	R	R	R	S	S	S	S	S	SS	N	N	N	N	N
89	3674	OP	42	M	PUS	ABSCESS	7	P	P	P	P	N	N	N	N	S	S	R	S	R	S	S	S	**S**	S	SS	N	N	N	N	N
90	3675	IP	33	M	PUS	ABSCESS	6	N	N	N	N	N	P	P	N	S	S	S	S	R	S	S	S	S	S	SS	N	N	N	N	N
95	4028	OP	16	M	SWAB	W.INFEC	12	P	P	P	P	N	N	N	N	R	R	S	S	R	S	S	S	**R**	**D**	**I**	**P**	N	**P**	N	N
96	4178	IP	54	M	SWAB	EAR	3	P	P	P	P	N	N	N	N	S	R	S	R	S	S	S	S	R	S	MS	N	N	N	N	N
98	4209	IP	8	M	PUS	ABSCESS	8	P	P	P	P	N	N	N	N	S	S	R	R	R	S	S	S	S	S	SS	N	N	N	N	N
2	598	IP	29	M	PUS	ABSCESS	8	P	P	P	P	N	N	N	N	R	R	R	S	S	S	S	S	S	S	SS	N	N	N	N	N
4	656	IP	32	M	PUS	ABSCESS	6	P	P	P	P	N	N	N	N	S	S	R	R	R	R	S	S	S	S	SS	N	N	N	N	N
5	660	IP	30	F	SWAB	W.INFEC	10	P	P	P	P	N	N	N	N	S	S	S	R	S	S	S	S	S	S	SS	N	N	N	N	N
7	892	OP	52	F	PUS	ABSCESS	0	P	P	P	N	P	N	N	N	R	R	S	S	S	S	R	S	S	S	SS	N	N	N	N	N
8	893	IP	23	F	PUS	ABSCESS	5	N	N	N	N	N	P	P	N	S	S	S	R	R	S	S	S	R	S	MS	N	N	N	N	N
9	894	IP	43	F	PUS	ABSCESS	6	N	N	N	N	N	P	N	P	R	R	S	R	S	S	R	S	R	S	MS	N	N	N	N	N
11	897	IP	43	F	PUS	ABSCESS	5	P	P	P	P	N	N	N	N	S	S	S	S	S	S	S	S	S	S	SS	N	N	N	N	N
18	924	IP	42	F	PUS	ABSCESS	5	P	P	P	N	P	N	N	N	S	S	R	S	R	R	R	S	S	S	SS	N	N	N	N	N
19	927	IP	20	F	PUS	ABSCESS	4	P	P	P	N	P	N	N	N	R	R	S	S	R	S	R	S	S	S	SS	N	N	N	N	N
21	931	IP	25	F	SWAB	EAR	3	P	P	P	N	P	N	N	N	S	S	R	R	S	R	R	S	S	S	SS	N	N	N	N	N
22	932	OP	53	F	PUS	ABSCESS	0	P	P	P	N	P	N	N	N	S	S	R	R	R	R	R	S	R	S	MS	N	N	N	N	N
23	1271	IP	62	M	PUS	ABSCESS	7	P	P	P	N	P	N	N	N	S	S	S	S	S	R	R	S	R	S	MS	N	N	N	N	N
24	1273	IP	66	F	PUS	ABSCESS	16	P	P	P	N	P	N	N	N	R	R	R	R	S	R	R	S	**R**	**D**	**I**	N	N	**P**	N	N
26	1284	IP	30	F	SWAB	W.INFEC	18	P	P	P	N	P	N	N	N	R	R	S	S	S	R	R	S	**R**	**D**	**I**	**P**	N	**P**	N	N
29	1393	IP	40	M	SWAB	W.INFEC	7	P	P	P	N	P	N	N	N	R	R	R	S	S	S	R	S	S	S	SS	N	N	N	N	N
30	1398	OP	40	F	PUS	ABSCESS	0	P	P	P	P	N	N	N	N	R	R	R	S	S	R	S	S	S	S	SS	N	N	N	N	N
31	1434	IP	36	F	PUS	ABSCESS	4	P	P	P	N	P	N	N	N	S	S	S	R	R	R	R	S	R	S	MS	N	N	N	N	N
32	1525	IP	32	F	PUS	ABSCESS	8	P	P	P	P	N	N	N	N	S	S	R	R	R	S	S	S	R	S	MS	N	N	N	N	N
33	1625	IP	64	M	PUS	ABSCESS	6	N	N	N	N	N	P	P	N	S	R	R	S	S	S	S	S	S	S	SS	N	N	N	N	N
34	1627	IP	52	F	PUS	ABSCESS	7	N	N	N	N	N	P	N	P	S	R	R	S	S	R	R	S	R	S	MS	N	N	N	N	N
39	1763	OP	63	F	PUS	ABSCESS	0	P	P	P	P	N	N	N	N	R	R	S	S	S	S	S	S	S	S	SS	N	N	N	N	N
41	1866	IP	22	M	SWAB	W.INFEC	4	P	P	P	N	P	N	N	N	S	S	S	S	S	S	R	S	S	S	SS	N	N	N	N	N
42	1867	IP	25	M	PUS	ABSCESS	4	P	P	P	N	P	N	N	N	S	S	S	S	S	S	R	S	R	S	MS	N	N	N	N	N
43	1881	IP	43	F	PUS	ABSCESS	17	P	P	P	N	P	N	N	N	R	R	R	R	S	S	R	S	**R**	**D**	**I**	**P**	N	**P**	N	N
44	1886	IP	62	F	PUS	ABSCESS	4	P	P	P	N	P	N	N	N	R	R	S	S	S	S	R	S	S	S	SS	N	N	N	N	N
45	1893	IP	44	F	PUS	ABSCESS	6	P	P	P	P	N	N	N	N	R	R	S	S	R	R	S	S	R	S	MS	N	N	N	N	N
46	1956	OP	35	F	PUS	ABSCESS	4	P	P	P	P	N	N	N	N	R	R	R	S	R	R	S	S	S	S	SS	N	N	N	N	N
47	1909	IP	38	M	PUS	ABSCESS	7	P	P	P	P	N	N	N	N	S	S	R	S	R	S	S	S	S	S	SS	N	N	N	N	N
51	2678	OP	28	F	SWAB	W.INFEC	3	P	P	P	P	N	N	N	N	S	S	R	S	S	S	S	S	S	S	SS	N	N	N	N	N
53	2948	IP	56	F	SWAB	VAGINAL	9	P	P	P	N	P	N	N	N	R	R	R	S	S	S	R	S	R	R	C	N	N	N	N	N
56	3013	IP	53	F	PUS	ABSCESS	6	P	P	P	P	N	N	N	N	S	S	S	R	R	S	S	S	R	S	SS	N	N	N	N	N
57	3023	IP	42	F	PUS	ABSCESS	5	P	P	P	P	N	N	N	N	R	S	R	S	R	R	S	S	R	S	MS	N	N	N	N	N
58	3031	IP	22	M	PUS	ABSCESS	6	P	P	P	P	N	N	N	N	S	S	S	R	R	S	S	S	S	S	SS	N	N	N	N	N
59	3067	IP	46	F	PUS	ABSCESS	7	P	P	P	N	P	N	N	N	R	R	S	R	S	R	R	S	S	S	SS	N	N	N	N	N
62	3200	IP	26	F	PUS	ABSCESS	6	P	P	P	P	N	N	N	N	R	R	S	S	R	S	S	S	R	R	C	N	N	N	N	N

64	3216	IP	33	M	PUS	ABSCESS	5	P	P	P	P	N	N	N	N	S	S	R	S	R	S	S	S	S	S	SS	N	N	N	N	N
65	3217	OP	38	F	PUS	ABSCESS	4	N	N	N	N	N	P	P	N	S	S	S	S	S	R	S	S	S	S	SS	N	N	N	N	N
66	3254	IP	62	M	PUS	ABSCESS	6	N	N	N	N	N	P	N	P	R	R	R	R	S	R	R	S	**R**	**D**	**I**	N	**P**	**P**	N	N
68	3280	IP	63	M	PUS	VAGINAL	7	P	P	P	N	P	N	N	N	S	S	S	R	R	S	R	S	R	S	MS	N	N	N	N	N
70	3318	IP	43	F	PUS	ABSCESS	6	N	N	N	N	N	P	P	N	S	S	S	S	R	R	S	S	S	S	SS	N	N	N	N	N
71	3347	OP	23	M	SWAB	VAGINAL	15	P	P	P	N	P	N	N	N	R	R	R	S	S	S	R	S	**R**	**D**	**I**	**P**	N	N	N	N
72	3417	IP	34	F	PUS	ABSCESS	7	N	N	N	N	N	P	P	N	S	S	R	R	R	S	S	S	R	R	C	N	N	N	N	N
73	3418	IP	54	F	PUS	ABSCESS	16	P	P	P	N	P	N	N	N	R	R	R	S	S	S	R	S	**R**	**D**	**I**	N	**P**	N	N	N
74	3499	IP	50	F	PUS	ABSCESS	7	N	N	N	N	N	P	P	N	R	R	S	S	S	S	S	S	R	S	MS	N	N	N	N	N
75	3505	IP	38	F	PUS	ABSCESS	3	N	N	N	N	N	P	P	N	R	R	S	S	R	S	S	S	S	S	SS	N	N	N	N	N
77	3529	IP	42	F	SWAB	EAR	3	P	P	P	N	P	N	N	N	S	S	R	R	S	S	R	S	S	S	SS	N	N	N	N	N
79	3541	IP	23	M	PUS	W.INFEC	8	P	P	P	N	P	N	N	N	S	S	R	S	R	R	R	S	S	S	SS	N	N	N	N	N
81	3543	IP	36	F	PUS	ABSCESS	5	N	N	N	N	N	P	P	N	S	S	S	S	S	S	S	S	S	S	SS	N	N	N	N	N
84	3617	IP	60	F	PUS	ABSCESS	7	N	N	N	N	N	P	N	P	R	R	S	R	R	S	R	S	**R**	**D**	**I**	**P**	N	**P**	N	N
85	3621	IP	57	F	SWAB	EAR	4	P	P	P	P	N	N	N	N	R	R	S	S	R	S	S	S	R	S	MS	N	N	N	N	N
86	3624	OP	66	M	PUS	ABSCESS	7	P	P	P	N	P	N	N	N	S	S	S	S	S	S	R	S	R	S	MS	N	N	N	N	N
87	3627	OP	62	M	SWAB	VAGINAL	6	P	P	P	P	N	N	N	N	S	S	S	S	S	S	S	S	R	R	C	N	N	N	N	N
91	3677	OP	38	F	PUS	ABSCESS	4	N	N	N	N	N	P	P	N	S	R	S	S	S	S	S	S	S	S	SS	N	N	N	N	N
92	3681	IP	26	F	PUS	ABSCESS	4	P	P	P	P	N	N	N	N	S	R	S	S	S	S	S	S	S	S	SS	N	N	N	N	N
93	3728	OP	29	F	PUS	ABSCESS	14	P	P	P	N	P	N	N	N	R	R	S	S	R	S	R	S	**R**	**D**	**I**	**P**	N	N	N	N
94	3879	IP	30	F	PUS	VAGINAL	7	P	P	P	P	N	N	N	N	S	S	R	S	S	S	S	S	S	S	SS	N	N	N	N	N
97	4208	OP	64	F	PUS	ABSCESS	3	P	P	P	P	N	N	N	N	S	S	S	S	S	S	S	S	R	R	C	N	N	N	N	N
99	4256	OP	57	F	PUS	ABSCESS	7	P	P	P	N	P	N	N	N	S	S	R	S	S	S	R	S	S	S	SS	N	N	N	N	N
100	4268	IP	38	M	PUS	ABSCESS	8	P	P	P	N	P	N	N	N	S	S	S	S	R	S	R	S	S	S	SS	N	N	N	N	N