STUDY ON PHENOTYPIC DETECTION OF INDUCIBLE CLINDAMYCIN RESISTANCE AMONG STAPHYLOCOCCUS AUREUS ISOLATES BY USING THE LOWER LIMIT OF RECOMMENDED INTER DISK DISTANCE.

MALWANCHAL UNIVERSITY

IN THE PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF

MASTER OF MEDICAL LAB TECHNICIAN

UNDER GUIDANCE OF

DR. RAMANATH KARICHERI,

PROFESSOR, DEPARTMENT OF MICROBIOLOGY,

INDEX MEDICAL COLLEGE HOSPITAL &RESEARCH CENTRE, INDORE

INDEX DEPARTMENT OF PHYSIOTHERAPY AND PARAMEDICAL SCIENCES

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I hereby declare that the project entitled '**STUDY ON PHENOTYPIC DETECTION OF INDUCIBLE CLINDAMYCIN RESISTANCE AMONG STAPHYLOCOCCUS AUREUS ISOLATES BY USING THE LOWER LIMIT OF RECOMMENDED INTER DISK DISTANCE' i**s a bonafide and genuine research work will be done by me under the guidance of **DR. RAMANATH KARICHERI.**

DATE: - **MR.DURGESH KUMAR YADAV**

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Examiner Sign.

Name

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Study on phenotypic detection of inducible clindamycin resistance among Staphylococcus aureus isolates by using the lower limit of recommended inter disk distance.

By

LIST OF ABBREVIATIONS

S. aureus – *Staphylococcus aureus*

iMLS$_B$ – inducible Macrolide Lincosamide Streptogramin B.

cMLS$_B$ – constitutive Macrolide Lincosamide Streptogramin B.

erm - erythromycin ribosome methylase

CLSI – Clinical and Labarotary Standard Institute

ER/CS – erythromycin resistant and Clindamycin sensitive

ER/CR - erythromycin resistant and Clindamycin resistant

AST – Antibiotic Sensitivity Testing

Introduction

Staphylococcus aureus is one of the most common pyogenic bacteria infecting man.[1] *S. aureus* is known for acquiring antimicrobial resistance promptly after the introduction of new antibiotics.[2]

Staphylococcus aureus and coagulase-negative staphylococci (CNS) are recognized to be causing nosocomial and community-acquired infections in every region of world. The increasing prevalence of methicillin resistance among staphylococci is an increasing problem.[3] Once these organisms are recognized to be causing infections in a region, it is of interest to determine which of the alternatives to vancomycin are suitable for therapy.[4,5,6] The Macrolide-lincosamide-Streptogramin B (MLS_B) family of antibiotics is commonly used in the treatment of staphylococcal infections.[7]

Clindamycin has been used to treat serious infections caused by S. aureus strains in children for more than 30 years. It remains effective for many more infections. Clinamycin resistance is common among healthcare-associated MRSA strains and Macrolide-inducible resistance to clindamycin was first recognised in the early 1960s. [8]

Clindamycin, a protein synthesis inhibitor, is a frequent therapeutic option for staphylococcal infections, particularly for skin and soft tissue infections and as an alternative for penicillin allergic patients.[7] It remains effective for many infections caused by community-acquired methicillin-resistant *S. aureus* (CA-MRSA).[9, 10]

Clinical isolates resistant to clindamycin were first recognized in 1968.[11] Relapse of *S. aureus* infection in a rabbit model of endocarditis during clindamycin therapy was observed in the early 1970s.[12] Clinical and bacteriologic relapse in a

patient with *S. aureus* endocarditis during the fourth week of clindamycin therapy after initial improvement was reported in 1976.[13]

Staphylococcal resistance to clindamycin may be inducible ($iMLS_B$ – inducible Macrolide – Lincosamide – Streptogramin resistance) or constitutive. It is noted that treatment of patients harbouring $iMLS_B$ staphylococci with clindamycin leads to the development of constitutive resistance, subsequently leading to therapeutic failure.[14]

Macrolide resistance in Staphylococcus is mainly mediated by two mechanisms, Macrolide-Lincosamide-Streptogramin type B (MLSb) or efflux mechanism phenotypes. The MLSb carries the ***erm*** gene encoding rRNA methylase that alters the antimicrobic binding site on the 23S rRNA, resulting in macrolides, lincosamides, and streptogramin B resistance. The expression of MLSb phenotype can be further classified as inducible MLSb or constitutive MLSb.

When an inducible MLSb strain is exposed to an inducer (such as a low-level of erythromycin), the level of rRNA methylase expression increases resulting in an increased resistance to antimicrobics of the MLS class (such as clindamycin). The efflux mechanism encoded by the msrA gene confers resistance only to macrolides and streptogramin type B. Detection of macrolide-lincosamide-streptogramin (MLS) resistance among Staphylococcus has recently drawn more attention in the clinical laboratories. [15]

The National Committee for Clinical Laboratory Standards (NCCLS, USA; currentlty CLSI) recently published recommendations for the detection of macrolide-lincosamide streptogramin (MLSb) resistance in Staphylococcus using the D-test (D-zone test) in order to differentiate between inducible MLSb and efflux mechanisms.

These statements provide guidelines for laboratorians to detect the MLSb resistance phenotypes in macrolide resistant staphylococci.[15]

Inducible MLS_B resistance can be detected by a simple test known as Disk approximation test or D-test.[16] The ideal inter-disk distance between the antibiotics is yet not clear and Clinical Laboratory Standards Institute (CLSI) recommends a range of 15 to 26 mm disk separation.[17]

Aims and Objectives

The study was conducted in the department of microbiology, Index Medical College and General and Super Speciality Hospital, Indore, from April 2020-April 2021.

The aims and objectives of the study were

1. To know the prevalence of MRSA strains, among the clinical isolates of Staphylococcus *aureus*.
2. Phenotypic detection of the inducible clindamycin resistance among clinical isolates of *Staphylococcus aureus* (both MSSA and MRSA) by disk approximation test-D-test.
3. To know the resistance pattern of clinical isolates of *Staphylococcus aureus* to various antibiotics being used routinely.

Review Of Literature

STAPHYLOCOCCUS AUREUS:

History:

Staphylococcus was first reported in pus by Robert Koch in 1878. Pasteur obtained liquid culture of the cocci from pus in 1880 and produced abscesses by inoculating them in to rabbits. In 1881 Sir Alexander Ogston, a Scottish surgeon, established conclusively the causative role of the coccus in abscesses and other suppurative lesions. In 1884 Rosenbach after through study adopted the name Staphylococcus as proposed by Ogston in 1881 (Staphyle – Greek – bunch of grapes, kokkos – berry) due to the typical occurrence of the cocci in grape – like clusters in the pus and in culture. Rosenbach divided Staphylococcus in to two groups – Staphylococcus pyogenes aureus and Staphylococcus pyogenes albus. Passet (1885) described a third variety – Staphylococcus citreus. Staphylococcus pyogenes aureus was finally named Staphylococcus aureus in 1980.[18]

Classification:

Staphylococcus aureus belongs to the family *Micrococcaceae*. It falls under Group I of Baird Parker classification based its properties to ferment glucose with production of acid, both aerobically and anaerobically, to ferment lactose, mannitol and maltose, producing acids, to produce coagulase, phosphatase and give a positive Voges Proskauer's test. [18,19]

Morphology:

Staphylococci are gram-positive cocci, spherical or ovoid in shape, each individual coccus has a diameter of 0.7 to 1.2 µm. They are arranged characteristically in grape like clusters. Cluster formation is because of cell division in 3 perpendicular planes and daughter cells remaining in close proximity due to incomplete separation after division. They may also occur singly, in pairs or in short chains of 3 to 4 cells especially when examined from liquid culture. They are non motile, non flagellated, asporogenous with G+C content of DNA being 30 – 39 mol%.[18]

Habitat:

Staphylococcus is a ubiquitous organism. Natural population of Staphylococci is associated with skin, skin glands and mucous membranes of warm blooded animals. It is also isolated from environment sources e.g. fomites, soil, sewage, water and from animal products like eggs, meat and milk. Of these the primary source are man and animals. Man harbours *Staphylococcus aureus* in the anterior nares and also elsewhere in body surfaces. Staphylococcus also survives in the hospital environment and develops resistance to antibiotics and disinfectants which are commonly used against it.[18]

Cell Wall Composition: [18]

The wall of *Staphylococcus aureus* has a polysaccharide capsule covering the rigid cell wall matrix. The basic component of this matrix which confers shape and stability to the organisms and represents 50% of the cell wall weight is the peptidoglycan.

The peptidoglycan is a polysaccharide polymer composed of unbranched β – linked 1- 4 chains of altering subunits of N-acetyl muramic acid and N-acetyl glucosamine. Pentapeptide side chains are linked to the muramic acid residue of peptide subunit and C-terminal D-alanine residue of neighbouring subunits. This peptidoglycan has important biologic properties. It elicits the production of interleukin – 1 from human monocytes, induces a local Swartzmann reaction, is capable of attracting polymorphonuclear neutrophils, has endotoxin like activity, activates complement and elicits the production of opsonic antibodies.

Other important cell wall constituents include a group of phosphate containing polymers called teichoic acids which constitute about 40% of cell wall weight. Some of these are covalently bound to peptidoglycan while others are linked to the lipid of bacterial cell membrane. The backbone of cell wall teichoic acid is an alternating sequence of ribitol phosphate. In the most strains, a unique substance protein A is also part of the cell wall, which has high affinity for Fc fragment of immunoglobulin subgroups of IgG.

The presence or absence of capsule varies from strain to strain. Encapsulated strains in tissue are protected from the complement mediated attack by polymorphonuclear leucocytes. The presence of capsule also increases the ability of the organism to spread through the tissue and is therefore undoubtedly an important factor enhancing the virulence of the organism is tissue infections.

Cultural Characteristics: [18]

Staphylococci grow readily on ordinary media within a temperature range of 10 - 42˚C, the optimum temperature being 37˚C and a pH of 7.4 to 7.6. They are aerobes and facultative anaerobes.

On nutrient agar, after incubation for 24 hours, the colonies are large being 2-4mm in diameter, circular, convex, smooth, shiny, opaque and easily emulsifiable with most strains producing a golden yellow non diffusible pigment and other strains producing white, yellow pigment. On nutrient agar slope, confluent growth gives characteristic oil – paint appearance. On blood agar, colony morphology is similar as that on nutrient agar, with most strains being haemolytic (while others are non-hemolytic), especially when incubated under 20 – 25% carbon dioxide. Hemolysis is usually of β type and is predominantly seen on sheep or rabbit blood and weakly on horse blood agar. For primary isolation, sheep blood agar is recommended. Human blood should not be used as it may contain antibodies or other inhibitors. On MacConkey agar, smaller colonies which are pink are produced due to lactose fermentation.

Several selective media that can be used for the isolation of the organism are:

Salt milk agar (8 – 10% Nacl), Salt broth (8 – 10% Nacl), Ludlam's medium (containing lithium chloride and potassium tellurite), Robertson cooked meat medium containing 10% Nacl, Mannitol salt agar, Potassium tellurite agar on which black colonies are seen due to reduction of tellurium.

Pigment production occurs optimally at 22˚C and only in aerobic cultures. It is enhanced when 1% glycerol monoacetate or milk is incorporated in the medium. The

pigment is a lipoprotein allied to carotene. In liquid media, uniform turbidity is produced.

Biochemical Reactions:[18]

Staphylococcus aureus produce Catalase enzyme that decomposes hydrogen peroxide to water and nascent oxygen, thereby protecting them from the lethal effect of hydrogen peroxide that is accumulated as an end product of carbohydrate metabolism. Catalase may be absent in respiratory deficient mutants.

Various sugars are fermented aerobically or anaerobically with the production of acid but no gas.

- Glucose fermented with acid only (aerobically or anaerobically)
- Lactose – acid only (aerobically)
- Maltose – acid only (aerobically)
- Mannitol – acid only (aerobically or anaerobically)
- Glycerol – acid only (aerobically)
- L - arabinose, D – cellobiose, dulcitol, D – fucose, D – melezitose, raffinose, salicin, starch, D – xylose and xylitol – acid only (aerobically)

Other Biochemical Reactions: [18]

- Gelatin is liquefied due to gelatinase production.
- Nitrates are reduced to nitrites by nitrate reductase enzyme.
- Ammonia is produced from arginine by arginine dihydrolase.
- Urea is hydrolysed due to urease production.
- Methyl red (MR) and Voges – Proskauer's (VP) test are positive.
- Indole is not produced.
- Citrate is not utilized as the sole source of carbon.
- Phosphatase is produced.
- Thermostable DNase is produced.
- Small amount of hydrogen sulphide may be formed from cysteine.

Staphylococcus aureus produces two types of coagulase namely bound and free coagulase**.** Bound coagulase or clumping factor is a heat stable constituent bound to the bacterial cell wall. It acts directly on fibrinogen and converts it to fibrin without the involvement of the substances in the plasma. This fibrin precipitates on the Staphylococci causing clumping when a bacterial suspension is mixed with plasma. Free coagulase is an extra cellular protein enzyme which causes the formation of a clot when Staphylococcus aureus is incubated in plasma. The mechanism involves the activation of a coagulase reacting factor (CRF) present in the plasma, which is a prothrombin like substance and after activation gets converted to thrombin like substance. This thrombin like substance reacts with fibrinogen to produce the fibrin

clot. The slide coagulase test correlates well with the tube coagulase test, whereas 12% of the positive tube coagulase show negative slide coagulase tests.

Pathogenicity: [18]

Staphylococcus aureus produces and secretes a number of enzymes and toxins which have been variously implicated as possible pathogenic factors.

Enzymes:

Catalase:

Hydrogen peroxide is produced by all Staphylococcal strains and is converted on to non-toxic H_2O and O_2 by the action of Catalase. Because Staphylococcal phagocytic killing is mediated by toxic O_2 radicals, produced by polymorphonuclear neutrophils, it has been proposed and shown that Catalase production by counteracting host defence mechanism, correlates with pathogenicity.

Coagulase:

There are two types – bound coagulase and free coagulase. They stimulate the conversion of fibrinogen to fibrin by binding to prothrombin with a 1:1 stoichometry. The complex becomes enzymatically active and initiates fibrin polymerization. The reaction is used to differentiate *Staphylococcus aureus* from coagulase negative Staphylococci.

Hyaluronidase:

This enzyme hydrolyses hyaluronic acids, a group of acid monopolysaccharides present in the cellular matrix of connective tissue. Its role in the

pathogenicity of Staphylococcus aureus is yet unsolved. It probably breaks down connective tissue leading to initiation and spread of infection.

Beta lactamase:

Production of these enzymes is responsible for antibiotic resistance among the Staphylococci. Most of these are plasmid – mediated. Their physiological role in the cellular metabolism in the absence of beta lactam antibiotics is unknown.

Other enzymes:

Staphylococcus aureus produces a thermo stable nuclease that is tested on a DNA substrate for taxonomic purpose, but in fact is a phosphodiesterase with both exo and endonuclease activity that cleaves nucleic acids in to three phosphomono – nucleotides. In addition Staphylococcus aureus produces several lipases that are thought to play a disseminating role during infection.

Toxins:

Staphylococcus aureus produces a variety of extra cellular products which are defined as toxins because they affect host cell function and / or morphology.

Haemolytic toxins: Alpha (α), Beta (β), Gamma (γ), Delta (δ)

These toxins contribute to the picture of local or systemic infections.

α - toxin:

Electrophoretically a heterogenous protein, it acts on wide variety of eukaryotic cell membranes (e.g. erythrocytes, leucocytes, platelets, fibroblasts and HeLa cells) but not on bacterial cytoplasmic membranes. Erythrocytes are the most sensitive target cells of the biologic action of *Staphylococcus aureus* and they display a species

– specific external binding capacity that correlates with their sensitivity to haemolysis. This toxin, when injected subcutaneously, is also dermonecrotic.

β -toxins:

This toxin displays its cytotoxic effect by degrading sphingomyelin. It is therefore active on a variety of cells, including human erythrocytes, leukocytes and fibroblasts. Its effects have been extensively investigated in erythrocytes, where the haemolytic activity depends on the sphingomyelin contents and / or distribution on the membrane surface of the erythrocytes.

γ - toxin:

It lyses erythrocytes from many species, including humans, by unknown mechanism.

δ - toxins:

This molecule is strongly surface active and disrupts biologic membranes by a detergent like action. Another interesting property of the δ toxin is its inhibition of water absorption and simultaneous stimulation of cAMP production in the rabbit and guinea pig ileum, which suggests a role in the pathogenesis of acute diarrhoea in some Staphylococcal infections.

Leukocidin:

This toxin, consists of two components apparently exerts an exclusive action on human and rabbit phagocytic cells. Its direct membrane damaging effect can be observed microscopically within 60 minutes. Both components of the toxin act

synergistically at the phagocyte membrane level by forming pores, which leads to increased permeability to cations.

Enterotoxin:

About half of all isolated strains of *Staphylococcus aureus* produce enterotoxins of which there are presently 8 antigenically distinct types causing gastroenteritis – A, B, C1, C2, C3, D, E, H. These remarkably heat stable toxins are major causes of food poisoning, by increasing dramatically intestinal peristalsis, possibly by symptomatic activation. A central nervous system effect is also suggested by the intensity of vomiting in food poisoning. They all exert their action by stimulating a subset of T lymphocytes and are therefore called bacterial superantigens.

Epidermolytic toxin or exfoliatin is a group of two serologically and biologically distinct proteins responsible for the major dermatologic findings of the Staphylococcal scalded skin syndrome.

Exfoliatins produce dramatic changes in the epidermis of neonates, which are characterized by extensive scalding. The histologic correlate is the formation of intra epidermal blisters at the granular cell layer.

Staphylococcal scald skin syndrome (SSSS) is an exfoliative skin disease in which the outer layer of epidermis gets separated from the underlying tissues. The severe form of SSSS is known as Ritter's disease in the newborn and toxic epidermal necrolysis in older patients.

Toxic Shock Syndrome Toxin (TSST – Type I) (formerly known as enterotoxin type F or pyogenic exotoxin C) causes potentially fatal multisystem disease characterized by fever, desquamative skin rash, hypotension and multi system

involvement. It occurs in patients who harbour Staphylococcus aureus strains that elaborate either toxic shock syndrome (TSS – 1) or other related enterotoxins. Other pyogenic exotoxins enhance the susceptibility to toxigenic shock.

Infections caused by *Staphylococcus aureus*:[18]

Skin and soft tissue infections:

Direct invasion through breaks in skin and mucous membranes is characteristic of diseases produced by Staphylococcus aureus resulting in a wide variety of soft tissue infections including folliculitis, furuncle (boil), local abscesses, lymphangitis and lymphadenitis, wound infections, carbuncle, impetigo, paronychia and less often cellulites.

Exfoliative diseases:

In this, stripping of the superficial layers of the skin from the underlying tissues occur due to action of epidermolytic toxins.

Musculoskeletal infections:

Osteomyelitis, arthritis, bursitis, pyomyositis.

Bacteremia:

Bacteremia with *Staphylococcus aureus* defined as seeding of the blood stream in the presence (secondary bacteremia) or absence (primary bacteremia) of a defined peripheral focus of infection. Peripheral focus of the primary lesion is usually a furuncle, carbuncle or infected wound or in the hospital setting an intravascular line, urinary catheter or other instrumentation procedures. Due to seeding, abscesses can

develop in multiple organs as in kidney, liver etc. Pyemia and suppurative thrombophlebitis can also develop.

Pyomyositis: Usually tropical in origin in which large abscesses develop in voluntary muscles, often multiple. Staphylococci reach the muscle through the blood stream but the factors that determine the site of abscess formation are unknown.

Respiratory infections:

Tonsillitis, pharyngitis, sinusitis, otitis, bronchopneumonia, lung abscess, empyema and rarely pneumonia.

Central nervous system infections:

Abscess, meningitis, intracranial thrombophlebitis

Urinary tract infection:

Primary urinary tract infection with *Staphylococcus aureus* are usually related to colonization of urinary catheters with ultimate access to the bladder, secondary Bacteremia from an initial urinary tract focus occurs; hence urinary isolates of Staphylococcus aureus are to be considered significant even with low colony count.

Endocarditis and pericarditis occur infrequently

Toxic shock syndrome: Develops due to TSST – I

Staphylococcal gastroenteritis: Occurs due to entero toxin A, B, C 1-3, D, E, and H.

Underlying conditions predisposing to infections:

Staphylococcal infections are particularly common in patients with immunocompromised status like immunosuppressive therapy, patients on anti-malignancy and systemic organ involvement like chronic liver failure, liver diseases and cardiovascular diseases.

INDUCIBLE MACROLIDE RESISTANCE:

Macrolides (e.g. erythromycin), lincosamide (e.g. clindamycin) and streptogramin B (e.g. Quinupristin-dalfopristin) antimicrobial agents are widely used in the treatment of staphylococcal infections.[20,7] However failures during therapy with MLS_B are more commonly reported.[21,14] Macrolides, lincosamides and streptogramin (MLS) antibiotics are structurally unrelated however related microbiologically because of their similar mode of action. MLS antibiotics inhibit bacterial protein synthesis by binding 23s rRNA. [22]

Erythromycin:

Erythromycin is the first member of macrolides, isolated from *Streptomyces erythreus* in 1952. It is bacteriostatic at low but cidal (for certain bacteria) at high concentrations. Cidal action depends on the organism concerned and its rate of multiplication.[23]

Clindamycin:

This potent lincosamide antibiotic inhibits protein synthesis by binding to 50s ribosomal RNA.[23] Clindamycin is used in the treatment of skin and soft tissue infections, caused by the staphylococci. It has good oral absorption property and important option in out patient therapy. It is also alternative for patients who are allergic to penicillin. It is a good alternative for the treatment of both methicillin resistant and

susceptible staphylococcal infections.[7] It has excellent tissue penetration except for the central nervous system.[24] The drug accumulates in abscesses and no renal dosing adjustments are needed [7], for the detection of macrolide-inducible Clindamycin resistance clinically. Since 1968,[11] the clinical importance has been increased to know the prevalence of such strains among the clinical isolates of *Staphylococcus aureus.* Clindamycin resistance may be inducible or constitutive. Inducible resistance cannot be detected by the routine antimicrobial susceptibility tests or by disc diffusion method.

According to various studies done on CLSI guidelines by different researchers, it is evident that, nearly 28% of MRSA showed inducible Clindamycin resistance by D-test.[25]

Clindamycin suppresses production of Panton-Valentine leukocidin, alpha hemolysin and toxic-shock syndrome toxin 1 by *S. aureus* invitro at the translational (ribosomal) level.[26] This is the basis for use of Clindamycin for toxin suppression in addition to a cellwall-active agent for treatment of life-threatening streptococcal and staphylococcal infections (toxic shock syndrome, necrotizing fasciitis).[27]

The MLS family antibiotics have three different mechanisms of resistance: target site modification, enzymatic antibiotic inactivation and macrolide efflux pumps.[28, 29a, b]

In target site modification, methylation of the A2058 residue, located in the conserved domain V of 23S rRNA, which leads to cross resistance and results in the formation of the phenotype of resistance pattern known as MLS_B.[30] This phenotype is encoded by erythromycin ribosome methylase (***erm***) genes.[31]

The expression of the MLS_B phenotype can be constitutive (MLS_{BC}) or inducible (MLS_{Bi}). Inducible resistance is observed when the inactive mRNA is produced by the production of methylase enzyme in the presence of an inducer, while active methylase mRNA is produced in strains where constitutive expression is seen.[22]

The strains carrying the inducible *erm* gene are resistant to the inducer and remain susceptible to non inducer macrolides and lincosamides. The *erm* gene product is a ribosome methylase whose expression is normally minimal. Low levels of erythromycin is an inducer of the MLS_{Bi} phenotype, which forms the basis of the D test.[32, 33]

Clindamycin susceptible, erythromycin resistant *Staphylococcus aureus* (Clindamycin-erythromycin discordant) may develop Clindamycin resistance.[29a, 22] These mutants are stably erythromycin and Clindamycin resistant. Since erythromycin resistance can occur with other mechanisms (e.g. efflux pumps and enzymatic modification), the D test identifies inducible resistance.[34]

Macrolide antibiotic resistance in *Staphylococcus aureus* and CONS may be due to an active efflux mechanism encoded by *msrA* (conferring resistance to macrolides and type B streptogramins only) or may be due to ribosomal target modification affecting macrolides, lincosamides, and type B streptogramins (MLS_B resistance).[7, 35, 36]

Mechanism:

The inducible methylase system is regulated at the level of mRNA translation (translational attenuation) rather than gene transcription. The gene is transcribed to

mRNA constitutively, but the mRNA cannot be read into protein without the presence of a translational inducer.

In the inducible *erm* cassette, the open reading frame of the methylase gene and its dedicated ribosomal binding site is proceeded by DNA sequences that encode another ribosomal binding site, a short 19 amino acid peptide with a stop codon, and an additional sequence that includes 2 inverted repeats (IRs).[33, 37, 38]

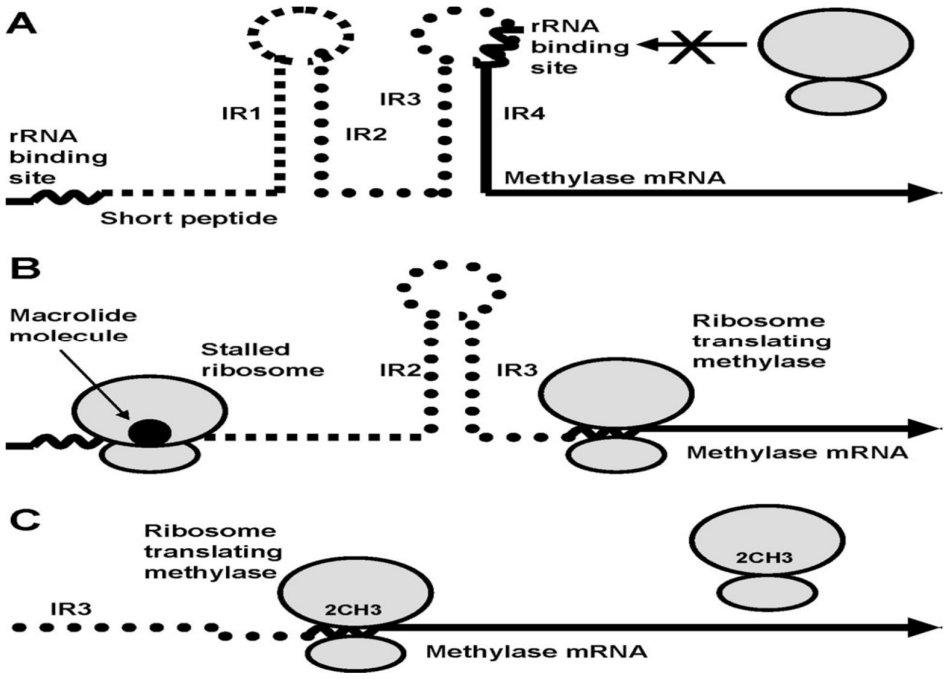


FIGURE 1. A, Shows the schematic representation of the secondary structure of the inducible methylase cassette in the absence of macrolides. Two stem loops are formed by the interactions of inverted repeat 1 (IR1) with IR2 and IR3 with IR4. IR4 consists of the ribosomal binding site and initial codons of the methylase mRNA. Ribosomes are unable to complex with the methylase binding site in this conformation. B. Shows the secondary structure after a macrolide-bound ribosome complexes with the first few codons of the short peptide and stalls. Other ribosomes not yet inhibited by macrolides then bind to and translate the methylase, which protects remaining

ribosomes from further macrolide binding. Panel C shows an example of mutation of the “inducible” mRNA sequences that can lead to constitutive methylase translation. The peptide and IR2 are deleted, and all mature ribosomes are methylated. Adapted from *Mol Cell*. 2008;30: 190-202.

D-test:

The combination of resistance to erythromycin with susceptibility to Clindamycin in *S. aureus* can be due to the $iMLS_B$ genotype or efflux pumps that remove macrolides but not clindamycin from the microbe.

The D-test, based on disk diffusion susceptibility testing, is recommended to determine if the $iMLS_B$ genotype is present.[39] In the D-test, disks containing erythromycin (15 µg) and clindamycin (2 µg) are placed 15 to 20 mm apart on an agar plate that has been inoculated with the clinical isolate (Fig. 2). A clindamycin-susceptible, erythromycin-resistant isolate should have a zone of inhibition 21 mm in diameter, with minimal if any inhibition of growth around the erythromycin disk.

The round zones of erythromycin and clindamycin that diffuse outward from the disks partially over lap in this configuration. Erythromycin molecules reach the outer region of the clindamycin zone prior to Clindamycin molecules. When the $iMLS_B$ genotype is present, this leads to methylase translation, permitting microbial growth in this region despite subsequent diffusion of inhibitory concentrations of clindamcyin. This growth blunts the expected round zone of growth-inhibition around the Clindamycin disk into a D-shape facing the erythromycin disk (Fig-2), which indicates

a positive D-test. A negative D-test (round zone) indicates efflux-mediated macrolide resistance with retained clindamycin susceptibility.[27]

NCCLS recommends placing the clindamycin and erythromycin disks anywhere from 15 to 26 mm apart for the D-test.[40] However, a 15-mm distance, in an edge to edge position, had a 100% sensitivity and specificity, while the 22-mm distance, in an edge to edge position, had a sensitivity of 87% and a specificity of 100% when compared with the presence of the *erm* gene as the gold standard for the detection of inducible clindamycin resistance.[17]

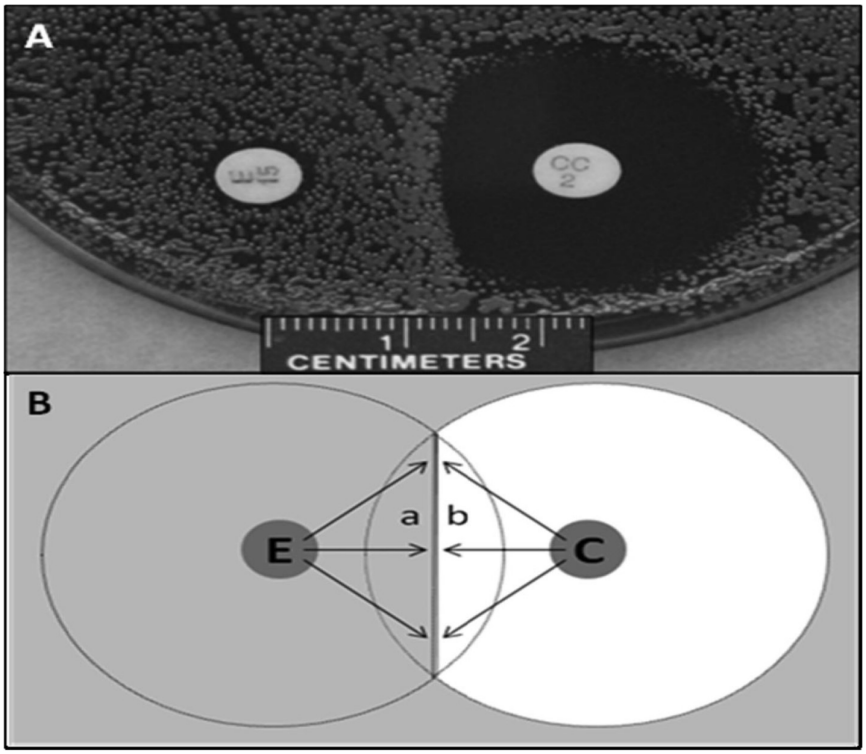


FIGURE 2. A, Is a photo of a positive D-test. The erythromycin and Clindamycin disks are on the left and right, respectively. The expected O-shape of the Clindamycin zone of growth inhibition, which would be seen in a negative D-test, is blunted on the side facing the erythromycin disk, resulting in a D-shaped zone. B, Is a stylized cartoon of the D-test. Erythromycin molecules diffuse into the region of the clindamycin zone

labeled “a” before clindamycin molecules, inducing the methylase, conferring resistance and allowing microbial growth despite subsequent arrival of otherwise inhibitory concentrations of clindamycin. Growth-inhibiting concentrations of clindamycin reach the region labeled “b” before erythromycin molecules can arrive to induce resistance. Arrows denote approximate equidistant diffusion of each antibiotic to a line bisecting the distance between the 2 antibiotic disks. The actual “intersection” of erythromycin versus clindamycin effects is often slightly bowed as in panel A. Gray areas indicate microbial growth on the agar surface; white areas, growth inhibition; E, erythromycin disk; C, clindamycin disk.[27]

The D test is acceptable for all Staphylococcus spp. including oxacillin-susceptible or oxacillin-resistant *S. aureus* or CONS. Many of the recently recognized methicillin resistant *Staphylococcus aureus* (MRSA) that cause community-associated infections have the *msr*A gene, and oral clindamycin may be a treatment option for these patients. In this case, these *S. aureus* strains are susceptible to clindamycin and do not present inducible resistance to this antimicrobial agent. Although clindamycin can be effective in some patients, it is not recommended to use it before conduct the D test.[41]

The disk diffusion based on the D-test produced four phenotypes of staphylococci, designated as D-positive, D-negative, resistant (R) and susceptible (S). A D-shaped zone around the Clindamycin disk is D-positive phenotype, indicating an inducible clindamycin resistance. Erythromycin resistant, clindamycin susceptible showing a circular shape zone around the clindamycin disc is D-negative phenotype. Clindamycin and erythromycin resistance indicating constitutive resistance (Resistant phenotype), and both are susceptible it is sensitive phenotype.[42]

Clinical implications of a positive D-test:

A positive D-test indicates the presence of $iMLS_B$ genotype so it is possible that microbes resistant to Clindamycin may lead to clinical failure.[10] Because of the clinical reports of Clindamycin failure associated with D-test positive strains, the clinical and CLSI recommend that laboratories report D-test positive isolates as resistant to Clindamycin.[41]

For sepsis, pneumonia, osteomyelitis and other serious invasive *S. aureus* infections, even the small risk of emergence of resistance indicated by a positive D-test result lead to avoidance of clindamycin, or prompt change from Clindamycin to another agent to which the isolate is susceptible.[11] When starting or continuing Clindamycin for less severe infections caused by D-test positive strains, close follow-up for potential failure and late relapse is needed. [27].

At the community level, relative frequency of constitutive MLS_B – related versus $iMLS_B$ – related clindamycin resistance is monitored to provide appropriate guidance for empiric therapy for clinical scenarios where community acquired S. aureus infections are suspected.

For infection by strains with the $iMLS_B$ genotype, suppression of toxin production by clindamycin can be expected during the critical first hours to days of therapy when this effect is clinically important.

Materials And Methods

This study was conducted from April-2020 to April 2021 in the Department of Microbiology, with the help of various clinical departments in Index medical college, Indore. A total of 613 samples were collected from Inpatients of all clinical departments in Index general and super specialty hospital, Indore.

Place of Study:

1. Department of Microbiology, Index medical college.
2. Index General and Super Speciality hospitals, Indore.

Period of study: April 2020 – April 2021

Clinical Samples:

The clinical samples were collected from patients with active infections like boils, folliculitis, wound infections, cellulitis, abscesses, osteomyelitis, bacteremia, and urinary tract infections.

Collection and transport of clinical specimens:

Data collection:

Complete data about the patients such as name, age, sex, OP/IP number, date, time of collection, source of specimen, and details about the clinical history is recorded before the specimen is processed.

PROFORMA for study group

Personal history:

Name :

Sex :

Age :

Occupation :

Address :

Date :

IP/OP no :

Ward/unit :

General history:

H/o skin and soft tissue infections:

H/o penicillin usage and response to the clinical condition:

H/o diabetes mellitus:

H/o recurrent respiratory tract infections:

Diagnosis:

Type of clinical sample collected:

Transport medium:

CLINICAL SAMPLE PROCESSING:

Gram staining:

Culture on

Nutrient agar :

Blood agar :

Mac conkey agar :

Mannitol Salt Agar:

Catalase test:

Tube coagulase test:

Mannitol Fermentation Test:

ANTIBIOTIC SENSITIVITY TESTING: (By Kirby-Bauer disc diffusion method)

Antibiotics	Resistant	Intermediate Sensitive	Sensitive
Oxacillin (1µg)			
Clindamycin (2µg)			
Penicillin (10µg)			
Ampicillin (10µg)			
Erythromycin (15µg)			
Vancomycin (30µg)			
Ciprofloxacin (30µg)			
Tetracycline (30µg)			
Cefotaxime (30µg)			
Amoxyclav (30µg)			

D-test result

PUS: Surface wounds are colonized with environmental bacteria, so before obtained culture the site is decontaminated with surgical soap and 70% ethyl alcohol.

When specimen is to be collected by a Dacron swab, the wound margins are separated by thumb and forefinger of one hand or a small opening is made in a closed

abscess with a sterile scalpel blade before extending the tip of the swab deeply in to the depths of the lesion. Care was taken not to touch the adjacent skin margins. Aspiration of the loculated fluid or pus from depths of pustular or vesicular wounds and abscesses with sterile needle and syringe.

URINE: Clean catch mid stream urine was collected. Patient was advised to clean the external genitals properly with soap and water and wipe with clean dry cloth and then collect mid stream urine in sterile wide mouthed screw capped universal container. Incase of hospitalized patients who had indwelling or Foley's catheter, urine was collected directly from the catheter using a number 28 needle and syringe. A portion of the soft rubber connector between catheter and catheter tubing was disinfected with alcohol, and punctured with sterile syringe and needle, and then urine was aspirated. All urine samples were processed immediately after collection.

BLOOD: Venipuncture site was prepared with thorough soap washing, then 2% povidone iodine was applied and allowed to dry for 1 or 2 minutes, removing the iodine with 70% alcohol wash. Povidone iodine and alcohol was applied on the skin by circular manner from inner to outwards. Collected blood samples were then inoculated into blood culture bottles and transported to laboratory immediately for incubation.

SPUTUM: Patient was instructed to a simple mouth wash in the early morning then deeply coughed sputum was collected in a sterile wide mouth universal container and not to collect the saliva.

ENDOTRACHEAL ASPIRATES: Endotracheal aspirates were collected by using sterile 12 Gauge endotracheal suction catheter tube. Suction catheter was connected to the suction pump and passed through endotracheal tube. In case of purulent secretion, the endotracheal suction catheter tips were collected using sterile scissors

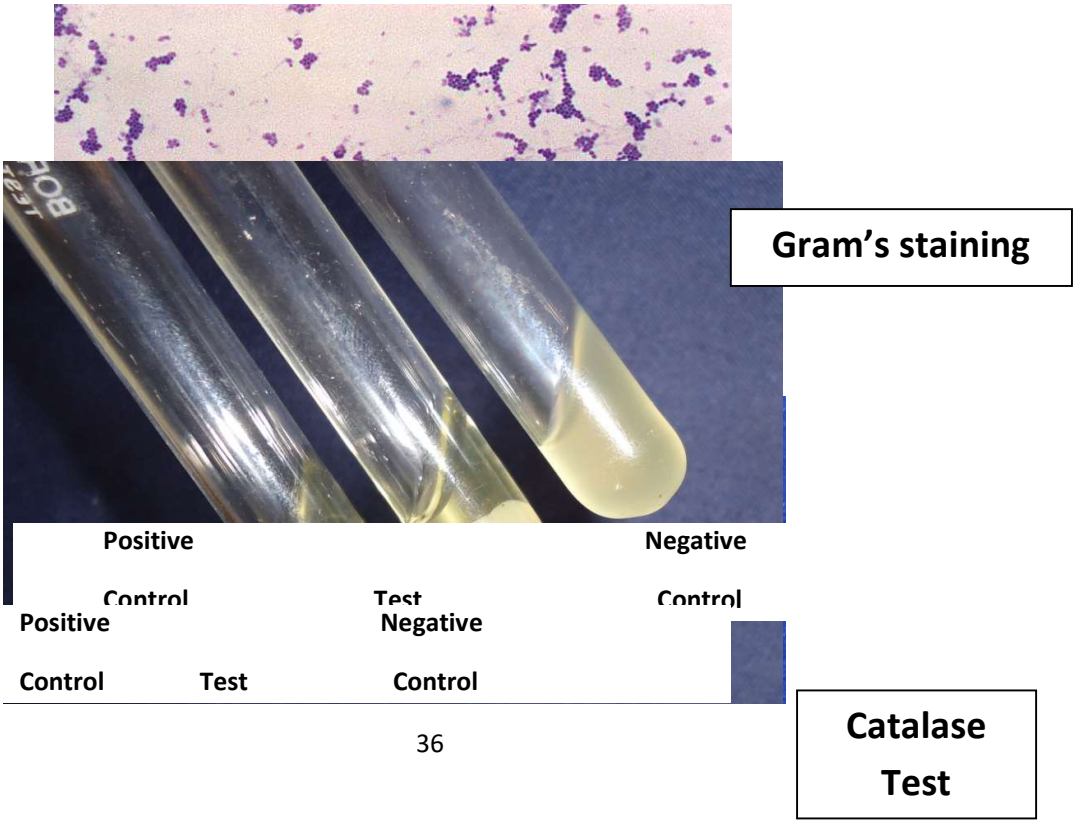
into screw capped sterile containers. In case of thick or dry secretions with clinical diagnosis of pneumonia, sterile normal saline is pushed into endotracheal tube and then suction was done for the aspirate. The samples were transported to microbiology laboratory within 15 min. specimens were processed as soon as they were obtained.

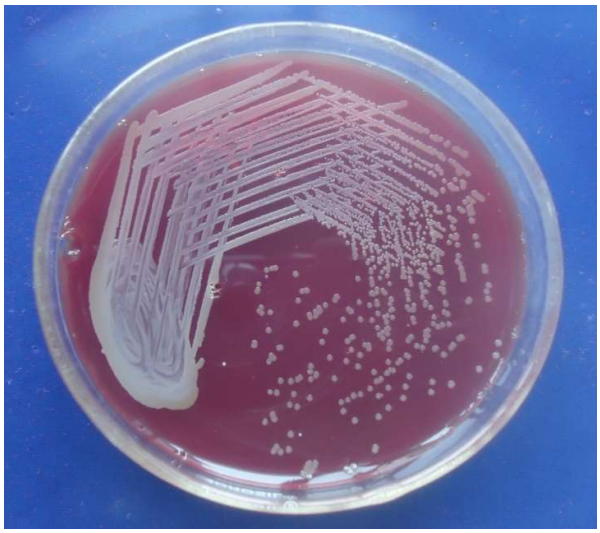
Processing of samples:

All the collected samples were transported to laboratory and processed immediately.

First, smears were prepared on sterile microscopic glass slides and Gram staining was performed and observed for grams reaction, size, shape, arrangement of the organisms, for the presence of any pus cells, squamous epithelial cells, yeast cells etc..(Annexure-II)

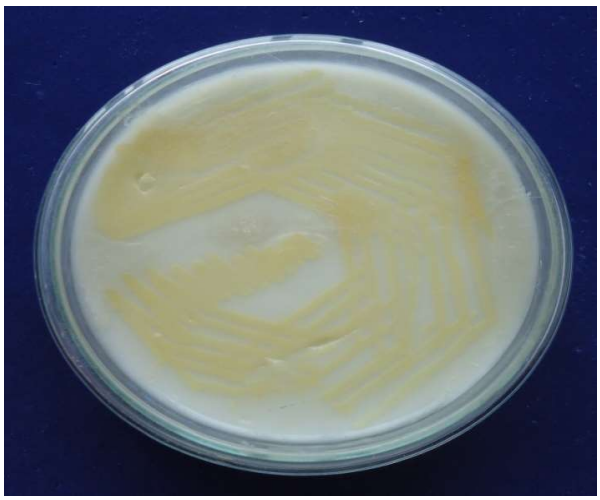
Then the samples were inoculated on to Nutrient agar, Blood agar, Mac conkey agar and Mannitol salt agar.(Annexure-I) Then the inoculated plates were incubated at 37^0c for 24-48 hrs.





Growth on Blood agar

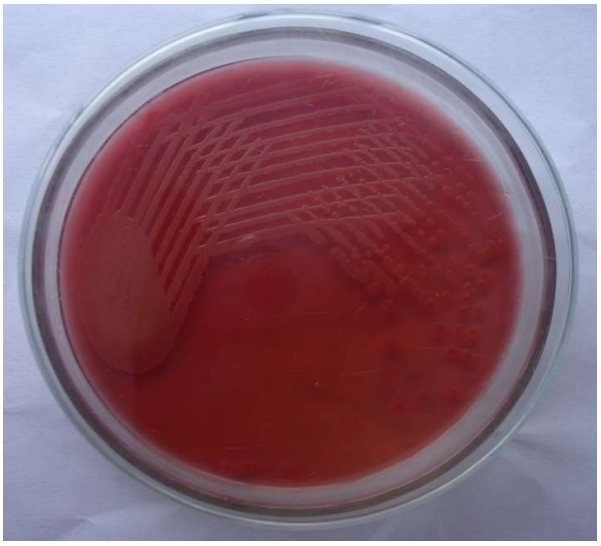
Growth on Mannitol Salt Agar



Growth on Salt Milk Agar

Mannitol fermentation Test

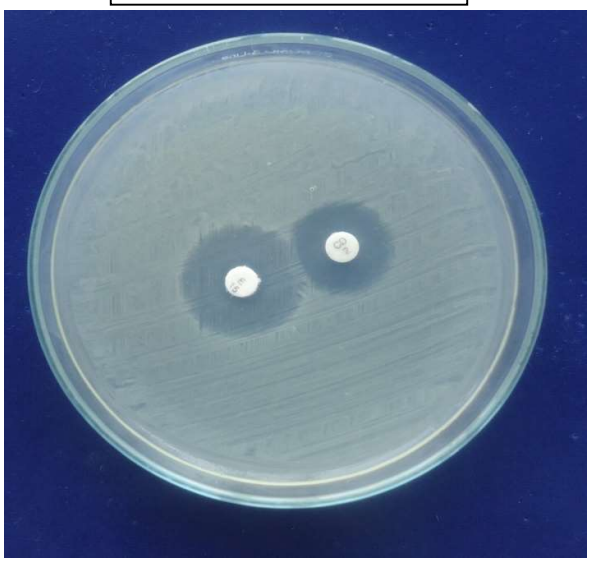
Growth on Nutrient agar



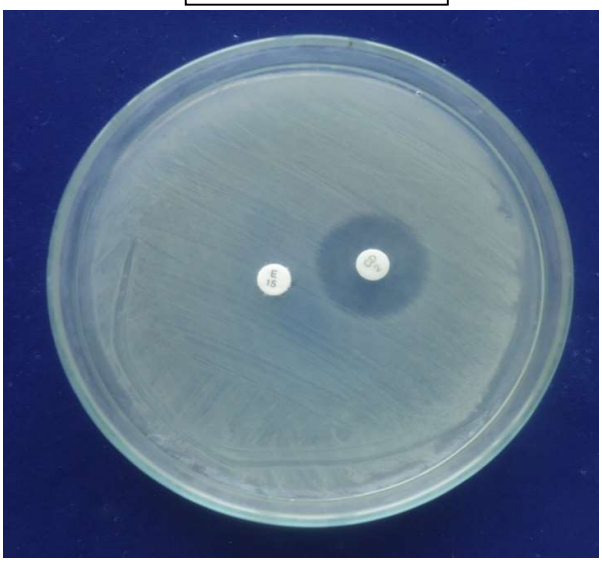
Growth on Mac Conkey agar

Phenotypes

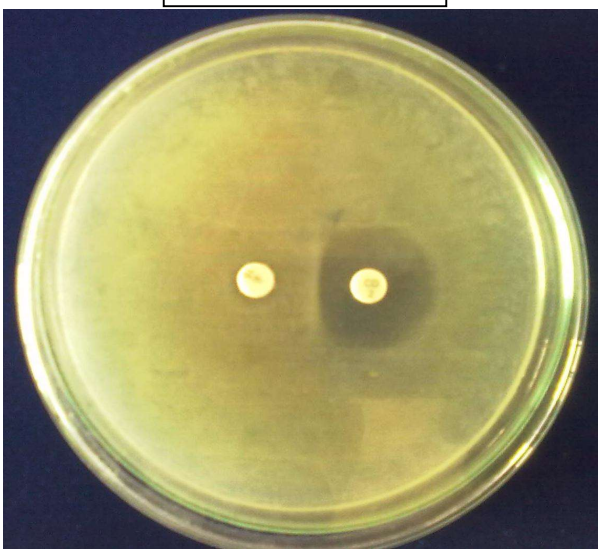
Erythromycin sensitive and clindamycin sensitive



D-test negative

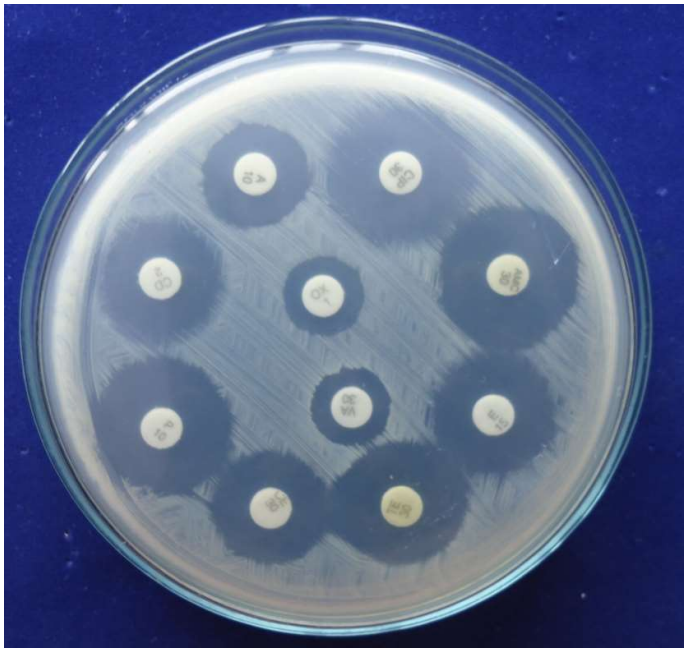


D-test positive or iMLS$_B$

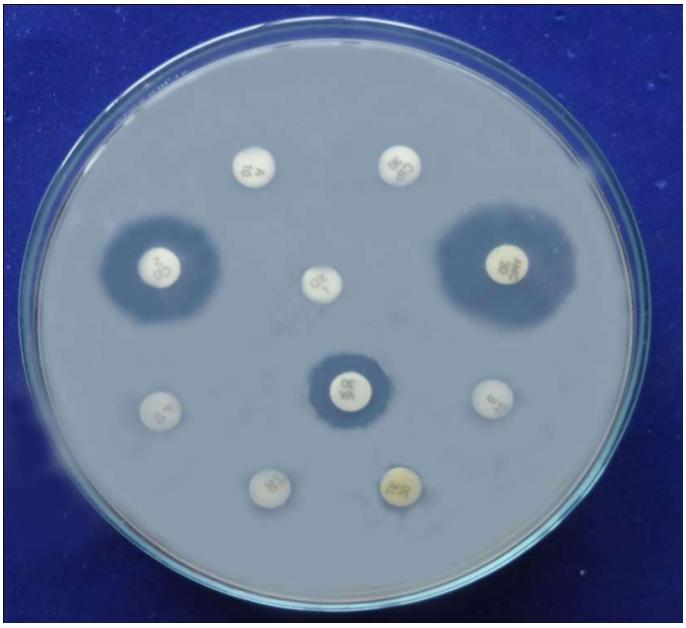


Erythromycin and clindamycin resistant or cMLS$_B$

Antibiotic Sensitivity Testing



***Staphylococcus aureus* ATCC 25923**



Staphylococcus aureus

After incubation with isolated colonies gram staining, Catalase and Oxidase tests were performed.(Annexure-II)

Staphylococcus aureus colonies were identified based on preliminary tests and subjected for confirmation tests.

Identification of *Staphylococcus aureus*:

Staphylococci were identified microscopically by their typical arrangement in gram stain in which they are arranged in grape like clusters and catalase test is done to differentiate between staphylococci and streptococci.

Further to differentiate between *Staphylococcus aureus* and other species of staphylococci, coagulase test (both slide and tube) and mannitol fermentation tests were performed and also observed for golden yellow pigmentation on nutrient agar.(Annexure-I, II)

Detection of methicillin resistance by disc diffusion method:

The suspension for inoculation was prepared from isolated colonies from an overnight growth on Nutrient agar plate. The growth was suspended in 0.5ml of sterile saline and turbidity was adjusted to 0.5 Mac Farlands opacity standard. A sterile swab was dipped into this suspension and excess of inoculum was removed by pressing it against the sides of test tube. This swab was used to inoculate Mueller Hinton agar supplemented with 4% NaCl and was allowed to dry for few minutes. 1µg Oxacillin discs obtained from Hi-media laboratories private limited, Mumbai, were applied within 15 minutes after the inoculation. The plates were incubated for 48 hours at 35^0c. The *S. aureus* standard strain ATCC 25923 was used as quality control. The diameter of zone of inhibition was measured with the help of scale and compared with CLSI zone size interpretative chart.

Detection of Erythromycin resistant and Clindamycin susceptible strains by disc diffusion method:

The suspension for inoculation was prepared from isolated colonies from an overnight growth on Nutrient agar plate. The growth was suspended in 0.5ml of sterile saline and turbidity was adjusted to 0.5 Mac Farlands opacity standard. A sterile swab was dipped into this suspension and excess of inoculum was removed by pressing it against the sides of test tube. This swab was used to inoculate Mueller Hinton agar plate and was allowed to dry for few minutes. 15µg Erythromycin discs and 2µg Clindamycin discs obtained from Hi-media laboratories private limited, Mumbai, were applied within 15 minutes after the inoculation. The plates were incubated for 24 hours at 37^0c. The *S. aureus* standard strain ATCC 25923 was used as quality control. The diameter of zone of inhibition was measured with the help of scale and compared with CLSI zone size interpretative chart.

Detection of Inducible Clindamycin resistance by recommended inter disc distance method (D-test):

This test was done to strains which were erythromycin resistant and clindamycin susceptible. The suspension for inoculation was prepared from isolated colonies from an overnight growth on Nutrient agar plate. The growth was suspended in 0.5ml of sterile saline and turbidity was adjusted to 0.5 Mac Farlands opacity standard. A sterile swab was dipped into this suspension and excess of inoculum was removed by pressing it against the sides of test tube. This swab was used to inoculate Mueller Hinton agar plate and was allowed to dry for few minutes. Disc approximation testing was performed using 2µg Clindamycin and 15µg Erythromycin discs. Discs were placed 15mm edge to edge distance. The *Staphylococcus aureus* standard

strain ATCC 25923 was used as quality control. The plates were incubated for 24 hours at 37^0c aerobically.

Detection of susceptibility to other antimicrobial agents by disc diffusion method:

Antimicrobial susceptibility testing of the isolated strains of *Staphylococcus aureus* to various antimicrobial discs was carried out according to Kirby-Bauer disc diffusion method. The *S. aureus* standard strain ATCC 25923 was used as quality control. All the antibiotic discs were obtained from Hi-media Pvt Limited, Mumbai, India. The following antibiotics were used and the diameter of zone of inhibition was measured with the help of scale and results were compared with CLSI zone size interpretative chart.

The antibiotic discs used were

Penicillin-G	-	10µg
Ampicillin	-	10µg
Oxacillin	-	1µg
Erythromycin	-	15µg
Clindamycin	-	2µg
Cefotaxime	-	30µg
Amoxyclav	-	30µg
Ciprofloxacin	-	30µg
Tetracyclin	-	30µg
Vancomycin	-	30µg

ANNEXURE I

Preparation of the media and reagents:

Media:

Nutrient agar medium:

Ingredients:

Peptone	-	10 g
Meat extract	-	10 g
Sodium chloride	-	5 g
Agar	-	20 g
Distilled water	-	1000 ml

From Hi media: Nutrient agar base powder is taken and dissolved in distilled water and autoclaved at 121^0c for 15 minutes at 15 lbs pressure, poured in to petri plates to a thickness of 4 mm and stored at 4^0c in the refrigerator.

Mac conkey agar:

Ingredients:

Peptone	-	20 g
10% aqueous solution of lactose	-	100ml
Agar	-	20 g
Neutral red	-	35 ml

Sodium taurocholate - 5 g

Distilled water - 1000ml

P^H - 7.5

From Hi media: Mac conkey's agar base powder is taken and dissolved in distilled water and autoclaved at 115^0c for 10 minutes at 10 lbs pressure, poured in to petri plates to a thickness of 4 mm and stored at 4^0c in the refrigerator.

Blood agar:

Ingredients:

Blood agar base - 44g

Distilled water - 1000ml

From Hi media: blood agar base powder is taken and dissolved in distilled water and autoclaved at 121^0c for 15 minutes at 15 lbs pressure. 100 ml of citrated human blood was added to 1000ml of blood agar base at 50^0c and media were poured to a thickness of 4 mm.

Mueller Hinton agar:

Ingredients:

Beef infusion - 30ml

Casein hydrolysate - 1.75g

Starch - 0.15g

Agar - 1g

P^H - 7.4

From Hi media: Mueller Hinton agar base powder is taken and dissolved in distilled water and autoclaved at 121^0c for 15 minutes at 15 lbs pressure, poured in to petri plates to a thickness of 4 mm and stored at 4^0c in the refrigerator.

Mannitol salt agar:

This is selective and indicator medium for isolating *S. aureus* from contaminated clinical specimens.

Requirements:

Peptone base - 10 g

Mannitol - 10 g

NaCl - 75 g

Agar - 20 g

Distilled water - 1000 ml

Phenol red is an indicator

Preparation:

Peptone and NaCl was dissolved in water by heating. Agar was added and allowed to dissolve. Final pH was adjusted to 7.2±0.2. Phenol red were added and mixed thoroughly. After sterilization, add sterile mannitol, after adding, the medium

was poured in to petri plates to a thickness of 4 mm and stored at 4^0c in the refrigerator.

Mannitol:

Peptone water is an all purpose growth medium and used as the base for mannitol fermentation test etc.

Requirements:

Peptic digest of animal tissue	-	10 gm
Mannitol	-	10 gm
Sodium chloride	-	5 gm
Andrades indicator	-	10 ml
Distilled water	-	1 litre

Preparation:

- Peptone and sodium chloride along with andrades indicator were dissolved in warm water and the final pH was adjusted to 7.2±0.2.
- It was sterilized by an autoclave at 15 lbs 'P', 121^0c for 15-20 min.
- After sterilization, 2 ml was poured into sterile test tubes under aseptic conditions.
- Then the test tubes were refrigerated for storage and use.

ANNEXURE-II

Gram staining:

The Gram staining differentiates between two major groups of bacteria i.e. Gram positive and Gram negative bacteria. The smears prepared from bacterial growth. Colonies were taken onto clean microscopic glass slide and emulsified with 0.85% saline to form a thin smear. The slide was heat fixed; allowed it to cool to the touch, before applying stain.

Crystal violet (primary stain) was applied to the smear and allowed to remain without drying for 30 seconds and rinsed with tap water.

The slide was then stained with iodine which acts as mordant, and allowed to remain without drying for 30 seconds and rinsed with tap water shaking off all excess. The slide was decolourised using alcohol-acetone mixture, rinsed immediately with tap water, the slide was allowed to react with safranin (counter stain) to remain without drying for 30 seconds. Rinsed with tap water, gently blotted between the folds of filter paper and air dried, examined microscopically under oil-immersion lens.

Catalase test:

This test demonstrates the presence of Catalase an enzyme that catalyzes the release of oxygen from hydrogen peroxide. A small amount of the culture to be tested was picked up from a nutrient agar slope with a clean sterile glass rod and this was inserted into the hydrogen peroxide solution (3% v/w), held in a small sterile test tube. The production of immediate effervescence indicates a positive reaction.

Coagulase test:

Both slide and tube tests were performed for identification of *Staphylococcus aureus*. Fresh sterile human plasma was used.

Slide coagulase test: A clean grease free slide was divided into two portions with glass marking pencil. A drop of normal saline was placed on each section and one loop full of staphylococcus growth was taken from the nutrient agar plate and was emulsified in each of the two drops to make smooth suspension. A drop of undiluted plasma was added to only one part of the staphylococcal emulsion and stirred gently with a sterile wire. Clumping of the organism resulted if the strain was coagulase positive. The control slide, to which no plasma was added, did not show any clumping as it was done to exclude spontaneous auto agglutination. Test identifies bound coagulase.

Tube coagulase test: The tube coagulase test was done to detect free coagulase. Fresh human plasma was diluted one in six (1:6) and 0.5 ml of it was taken in three narrow test tubes. To one tube approximately 0.1 ml of overnight broth culture suspension of test organism was added. To the second tube 0.1 ml of overnight broth culture suspension of known coagulase positive staphylococci was added, which acts as positive control. Diluted plasma alone in the third tube serves as negative control. The tubes were incubated at 37^0c in the water bath and examined at 1, 2, and 4 hours for clot formation by tilting a tube through 90^0. If positive the plasma that clotted did not flow when the tube was inverted. If negative the tubes were left at room temperature overnight and re examined. Test detects free coagulase.

Mannitol Fermentation test:

Mannitol sugar medium was inoculated with the organism and incubated at 37^0c for 18 to 24 hrs. Positve test was indicated by observing the colour change of the medium to pink colour.

ANNEXURE III

Antibiotic sensitivity chart according to CLSI Guidelines

Table shows antibiotics used for testing also.

Method: Kirby-Bauer Disc Diffusion method.

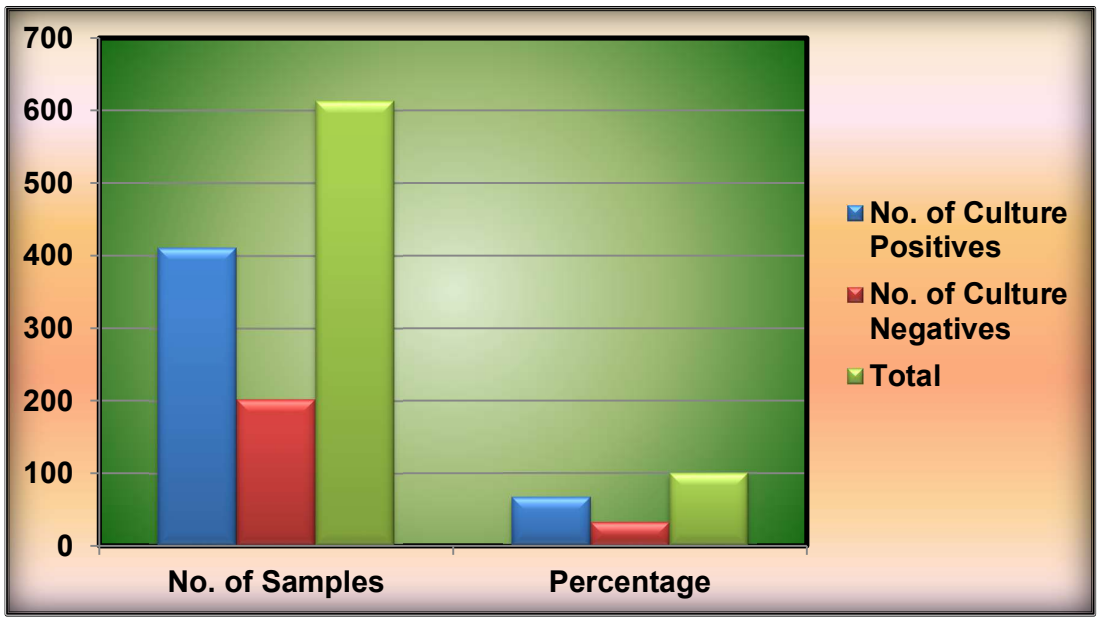
Antibiotics	Symbol	Disc potency	Sensitive	Intermediate sensitive	Resistant
Penicillin	P	10µg	≥29mm	------	≤28mm
Ampicillin	AMP	10µg	≥29mm	------	≤28mm
Oxacillin	OX	1µg	≥13mm	11-12mm	≤10mm
Clindamycin	CD	2µg	≥21mm	15-20mm	≤14mm
Erythromycin	E	15µg	≥23mm	14-22mm	≤13mm
Vancomycin	VA	30µg	≥15mm	------	------
Tetracyclin	TE	30µg	≥23mm	19-22mm	≤18mm
Ciprofloxacin	CIP	5µg	≥23mm	-----	≤18mm
Cefotaxime	CTX	30µg	≥26mm	15-22mm	≤14mm
Amoxyclav	AMC	30µg	≥20mm	-----	≤19mm

Results

Table – I

Analysis of total samples

Total samples	No. of samples	Percentage
No. of culture positives	411	67.04
No. of culture negatives	202	32.96
Total	613	100



able – II

Gender wise distribution of samples

Gender	No. of samples	Percentage
Males	368	60.03
Females	245	39.97
Total	613	100

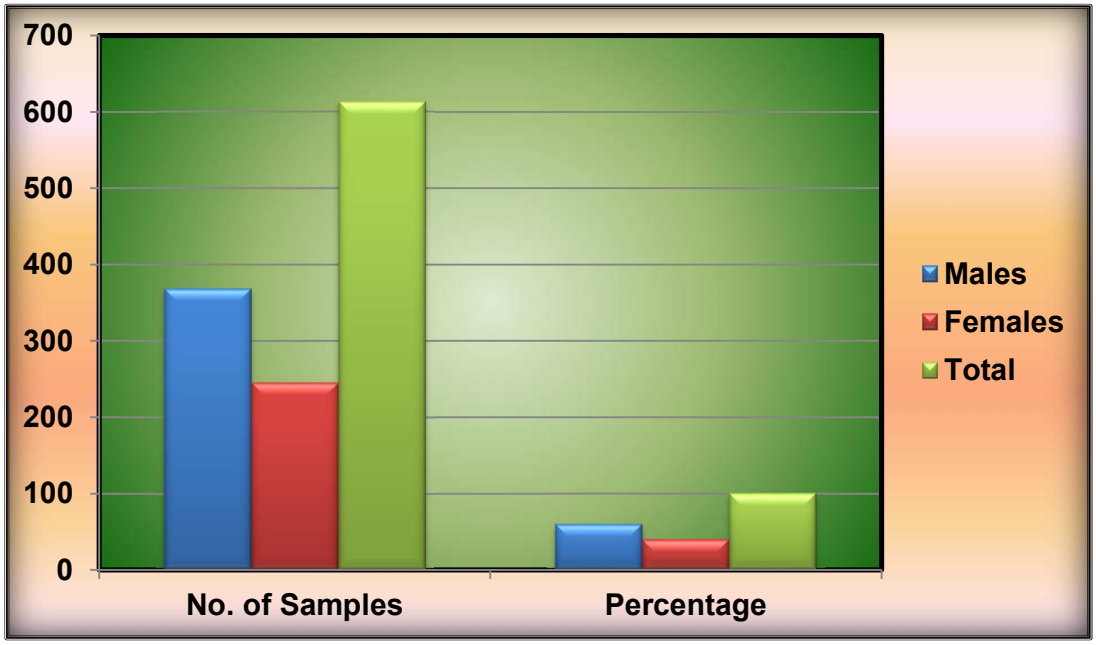


Table – III

Age wise distribution of total samples

Age	No. of samples	Percentage
0 – 10	24	3.92
11 – 20	63	10.27
21 – 30	112	18.27
31 – 40	134	21.86
41 – 50	98	15.98
51 – 60	134	21.86
61 – 70	24	3.92
71 – 80	24	3.92
Total	613	100

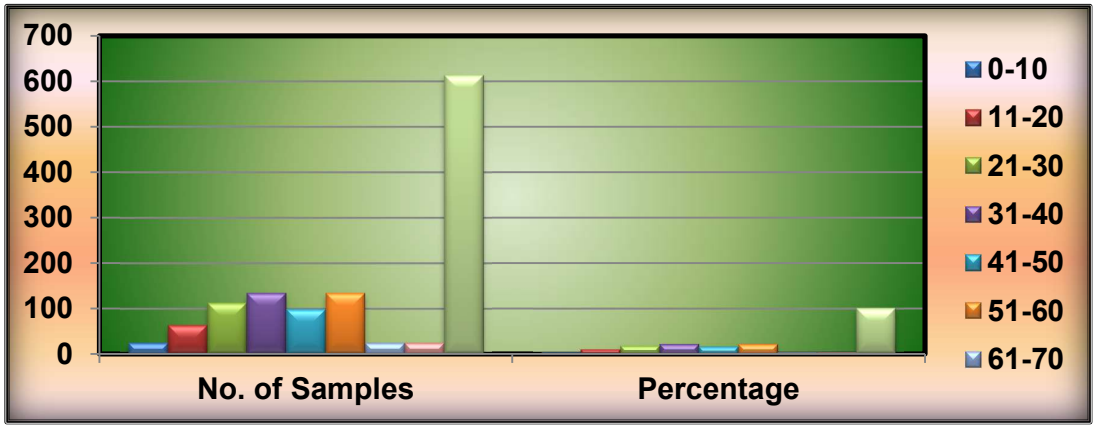

700
600
500
400
300
200
100
0
No. of Samples
Percentage
0-10
11-20
21-30
31-40
41-50
51-60
61-70

Table – IV

Distribution of different types of samples in the study

Type of sample	No. of samples	Percentage
Urine	74	12.07
Pus	**370**	**60.35**
Sputum and ET Aspirates	116	18.92
Blood	32	5.23
Other Body fluids	21	3.43
Total	613	100

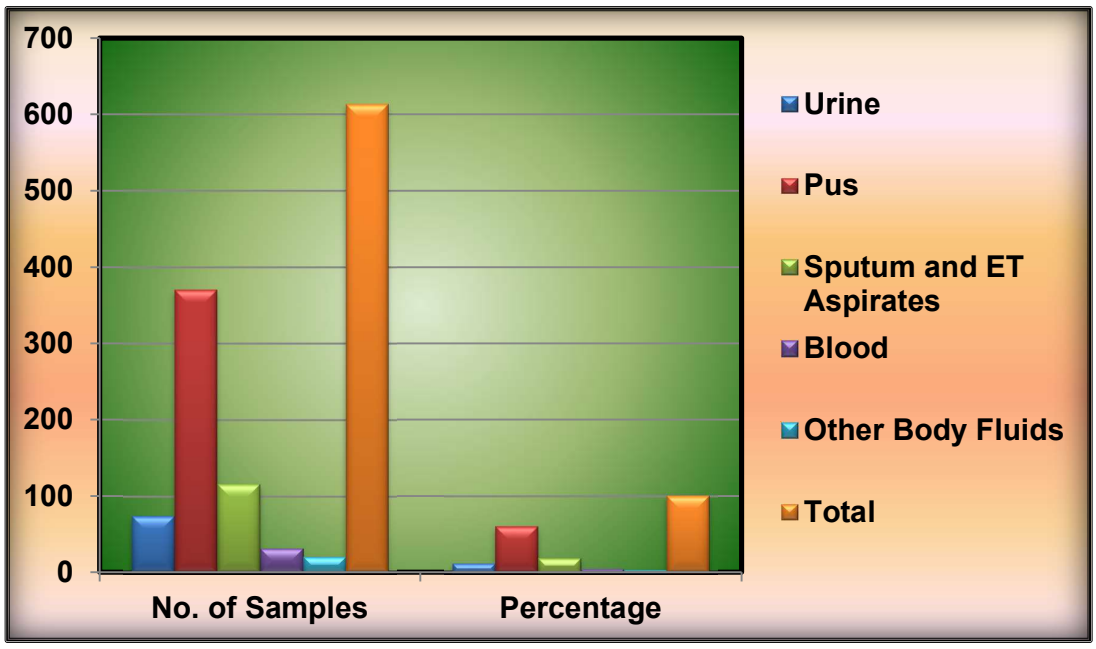


Table – V

Prevalence of *Staphylococcus aureus* and other isolates in the total no. of positive samples

Isolates	No. of isolates	Percentage
Pseudomonas spp	86	20.93
Escherichia coli	68	16.54
Klebsiella spp	41	9.98
Staphylococcus aureus	**150**	**36.50**
Proteus spp	33	8.03
Enterococcus spp	14	3.40
Citrobacter spp	07	1.7
CONS	08	1.94
Candida	04	0.98
Total	411	100

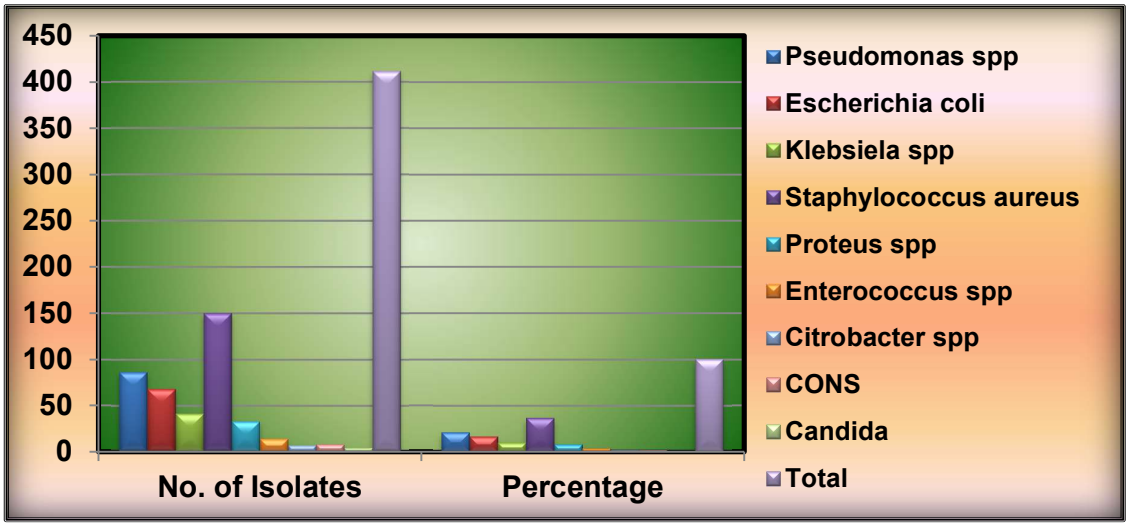


Table – VI

Prevalence of *Staphylococcus aureus* in different samples

Sample	No. of isolates	Percentage
Pus (285)	**112**	**27.26**
Sputum and ET Aspirates (59)	20	4.86
Other Body fluids (11)	04	0.98
Urine (46)	13	3.16
Blood (10)	01	0.24
Total (411)	150	36.50

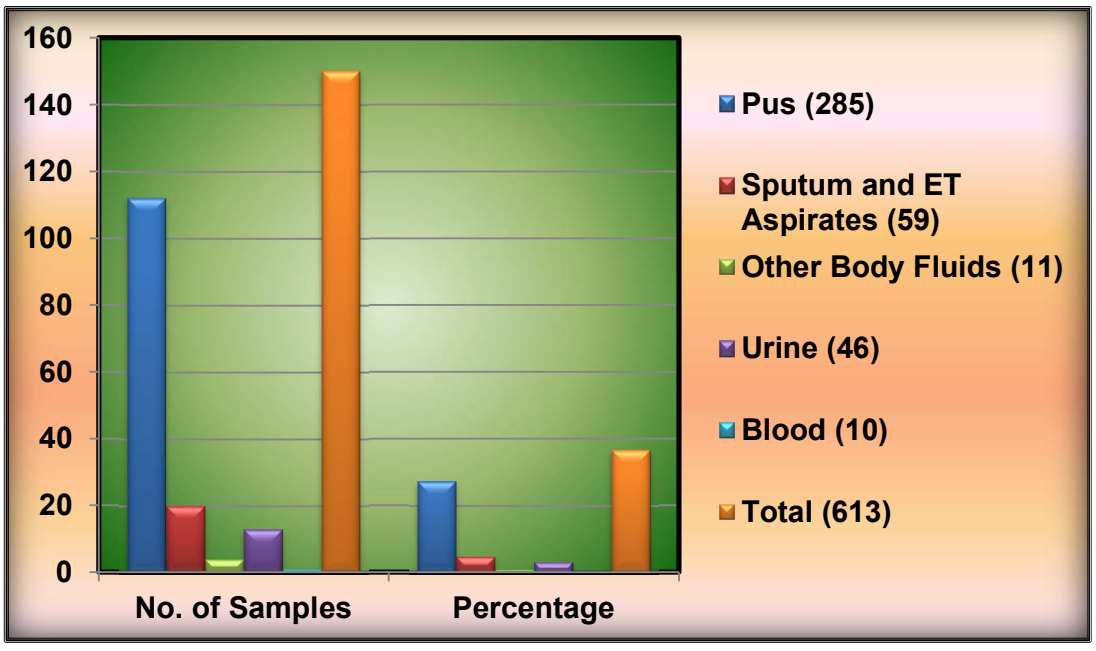


Table – VII

Antimicrobial resistance profiles of *Staphylococcus aureus* isolates by disk-diffusion method (n=150)

Antibiotics	Resistance	Percentage
Penicillin	**111**	**74**
Ampicillin	108	72
Oxacillin	77	51.33
Clindamycin	**24**	**16**
Erythromycin	81	54
Tetracyclin	48	32
Ciprofloxacin	48	32
Cefotaxime	72	48
Amoxyclav	60	40
Vancomycin	**0**	**0**

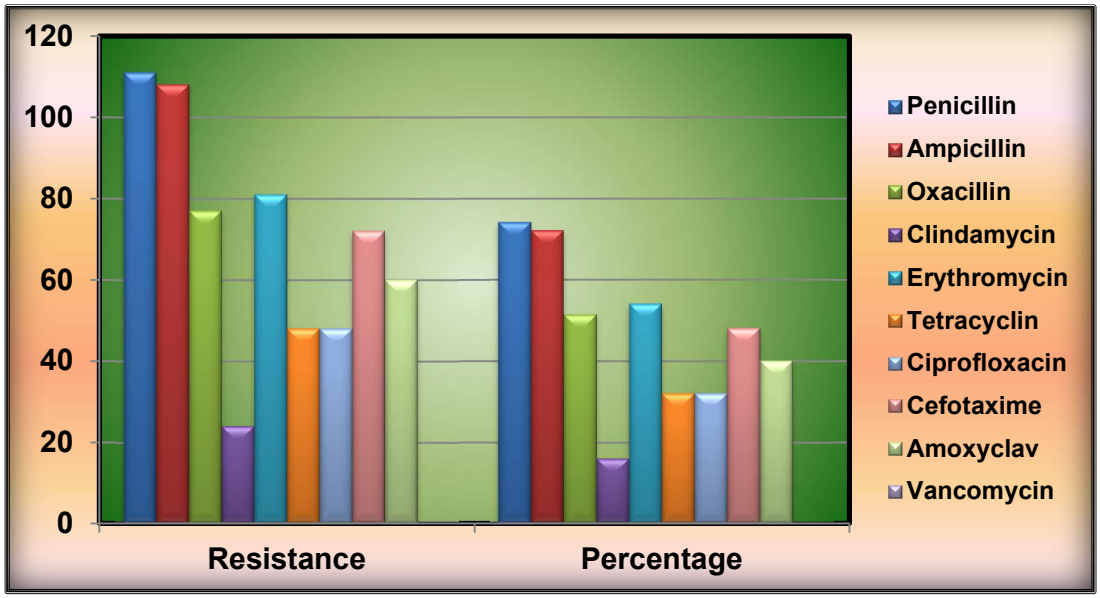


Table – VIII

Prevalence of methicillin resistance in *S. aureus* (n=150)

	No. of isolates	Percentage
MSSA	73	48.67
MRSA	77	51.33
Total	150	100

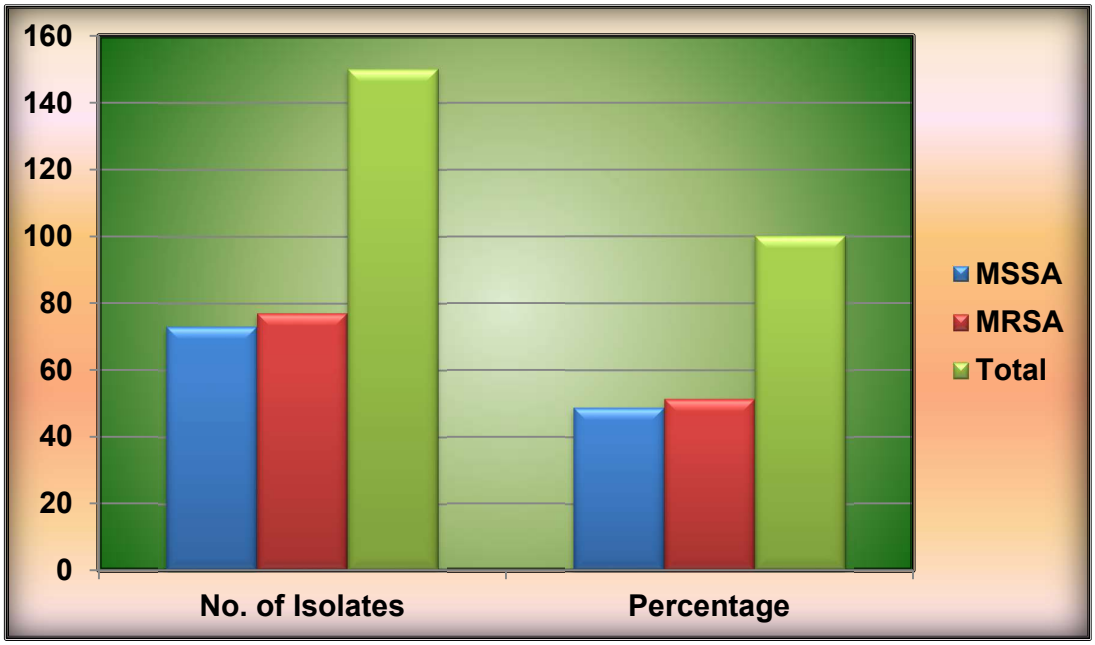

160
140
120
100
80
60
40
20
0
No. of Isolates
Percentage
MSSA
MRSA
Total

Table – IX

Inducible MLS_B among *S. aureus*

	Total	ER / CS	*iMLS$_B$*
S. aureus	150	57 (38%)	36 (63.15%)
MSSA	73 (48.67%)	27 (36.98%)	15 (55.5%)
MRSA	77 (51.33%)	30 (38.96%)	21 (70%)

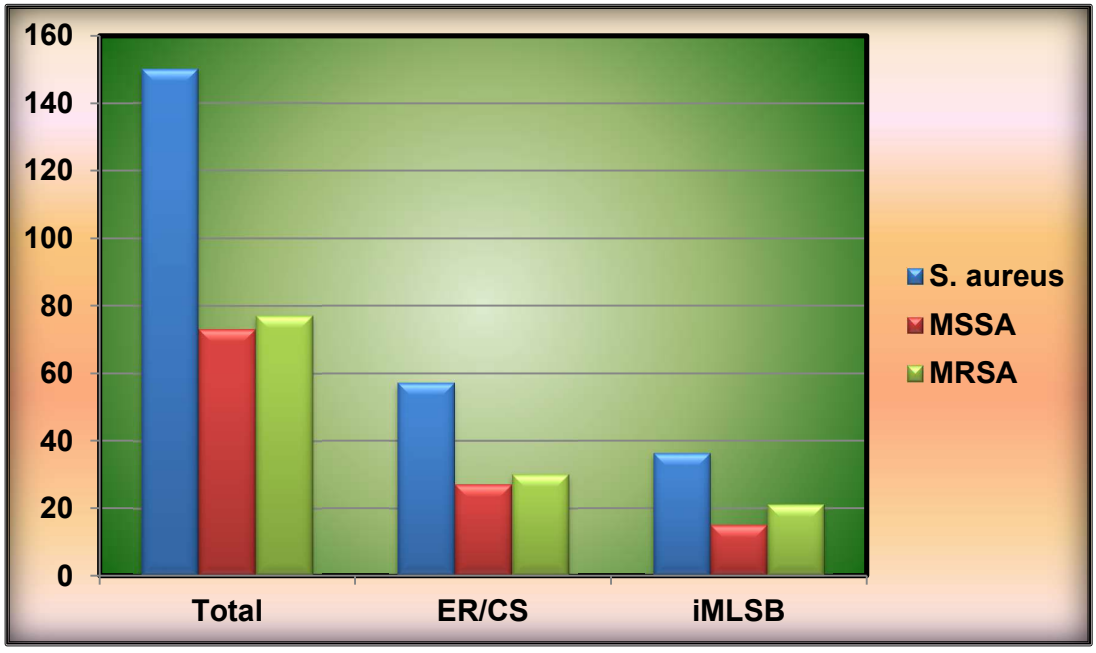
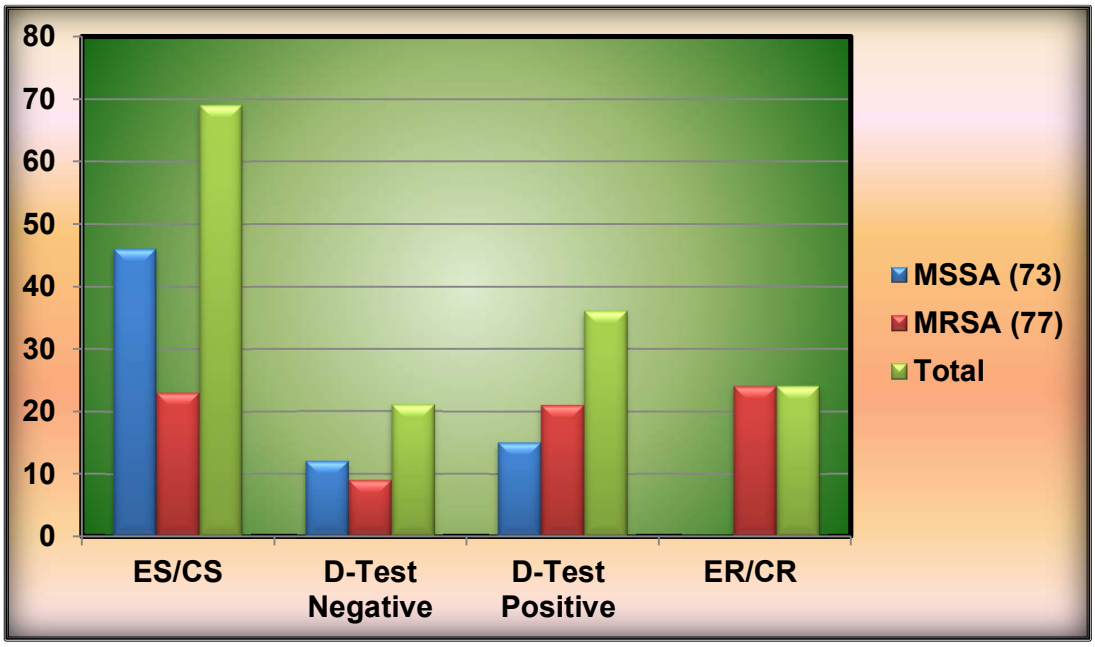


Table – X

Prevalence of four phenotypes among *S. aureus* isolates (n=150)

	Phenotypes			
	ES/CS	**D-test Negative**	**D-test Positive (or) $iMLS_B$**	**ER/CR (or) $cMLS_B$**
MSSA (73)	**46** (63.01%)	**12** (16.43%)	**15** (20.54%)	0
MRSA (77)	**23** (29.80%)	**09** (11.68%)	**21** (27.27%)	**24** (31.16%)
Total	**69** (46%)	**21** (14%)	**36** (24%)	**24** (16%)


80
70
60
50
40
30
20
10
0
ES/CS
D-Test Negative
D-Test Positive
ER/CR
MSSA (73)
MRSA (77)
Total

Discussion

Staphylococcus aureus is one of the most common pyogenic bacteria infecting man. It is known for acquiring antimicrobial resistance promptly after the introduction of new antibiotics. Clindamycin, a protein synthesis inhibitor, is a frequent therapeutic option for staphylococcal infections, particularly skin and soft tissue infections and as an alternative in penicillin-allergic patients. Staphylococcal resistance to clindamycin may be inducible or constitutive which can be detected by routine disk diffusion test using lower limit of inter disk distance.

The present study documents the phenotypic detection of inducible clindamycin resistance among *Staphylococcus aureus* isolated from a total of 613 samples collected from inpatients admitted to various wards with different types of infections over a period of one year (2010 – 2011) in Index General Hospital, Indore.

Among the total 613 samples processed, 411 (67.04%) were positive for culture and 202 (32.96%) showed no bacterial growth aerobically.

Of the 613 samples collected, 368 (60.03%) were from males and 245 (39.97%) were from females.

In the total 613 samples collected from patients of all age groups admitted in different wards, maximum no. of samples were collected from the age group of 31-40 i.e., 134 (21.86%) and 51-60 (21.86%).

Distribution of different types of samples collected was urine 74 (12.07%), sputum and ET aspirates 116 (18.92%), blood 32 (5.23%), other body fluids 21 (3.43%) and a major number of samples were pus 370 (60.35%).

Among all the isolates from total no. of samples collected and processed, higher number were *Staphylococcus aureus* 150 (36.50%), followed by Pseudomonas spp.

86 (20.93%), *Escherichia coli* 68 (16.54%), *Klebsiella spp*. 41 (9.98%), *Proteus spp*. 33 (8.03%), *Enterococcus spp*. 14 (3.40%), CONS 08 (1.94%), *Citrobacter spp*. 07 (1.7%) and *Candida* 04 (0.98%).

More prevalent isolates from pus sample were *Staphylococcus aureus* which constitutes 112 (27.26%) from 285 samples collected from patients with skin and soft tissue infections, followed by Sputum and ET aspirates 20 (4.86%), urine 13 (3.16%), other body fluids 04 (0.98%) and blood 01 (0.24%).

Kirby-Bauer disc diffusion method was performed to 150 Staphylococcus aureus isolates, Penicillin resistance was 74% which was alarmingly high, followed by Ampicillin 72%, Erythromycin 54%, Oxacillin 51.33%, Cefotaxime 48%, Amoxyclav 40%, Tetracyclin and Ciprofloxacin 32% each, Clindamycin 16% and none of the isolates have shown resistance towards Vancomycin.

In our study, MRSA strains were detected by disc diffusion method using oxacillin 1µg disc. Oxacillin disc has been used in most developing countries to detect MRSA.

Out of 150 *S. aureus*, 77 (51.33%) were MRSA strains and 73 (48.67%) were MSSA. These results correlated with the study done by ***Zorgani et al***.[43] who reported 65 (54.16%) MRSA and 55 (45.83%) MSSA from a total of 120 *S. aureus* isolates.

Out of 150 *S. aureus*, 57 (38%) were erythromycin resistant and clindamycin sensitive. Among the 57 erythromycin resistant clindamycin sensitive strains, 27 were MSSA, among which 15 (55.5%) were $iMLS_B$ phenotypes. Accordingly out of 30 ER/CS strains of MRSA isolates, 21 (70%) were $iMLS_B$ phenotypes.

From the total 150 S. aureus isolates 73(48.67%) were identified as MSSA strains and 77 (51.3%) were confirmed as MRSA strains. This study is correlating with the study done by ***S.E Mshana et al***.[44] who reported 61% and 32% of MRSA and MSSA respectively. The present study showing 36 isolates out of 57 which are ER/CS are $iMLS_B$ phenotypes (D-test positive) comprising around 63%. Results of present study is being correlated with the study done by ***MR Angel et al***.[45]

Prevalence of the four phenotypes in our study is as follows. Out of 73 MSSA strains, 46 (63.01%) were ES/CS, 12 (16.43%) were D-test Negative, 15 (20.54%) were $iMLS_B$ (D-test positive) and none of the isolates were ER/CR which are considered as $cMLS_B$ strains. Out of 77 MRSA strains isolated, 23 (29.80%) were ES/CS, 09 (11.68%) were D-test Negative, 21 (27.27%) were $iMLS_B$ and 24 (31.16%) were $cMLS_B$ phenotypes. Out of total 150 *S. aureus* strains 69 (49%) are ES/CS, 21 (14%) were d-test negative, 30 (24%) were D-test positive and 24 (16%) were ER/CR. Our results correlated with other studies like ***K. M. Mohanasoundaram***[25] who reported 19% $cMLS_B$, 28% $iMLS_B$, 30% D-test Negative and 23% ES/CS among 47 MRSA isolates. 10% $cMLS_B$, 11% $iMLS_B$, 12% D-test Negative and 67%ES/CS among 73 of MSSA isolates. ***Zorgani. A et al***[43], ***Nuran delialioglu et al***[46], ***S. E. Mshana et al***[44], reported 0% ER/CR i.e., $cMLS_B$ among MSSA isolates.

Accurate drug susceptibility data of the infecting microbe is an essential factor in making appropriate therapeutic decisions. The multiplicity of mechanisms, which confer resistance to MLS antibiotics, reflects the complexity of the resistant phenotypes as well as the clinical situation. The most widespread and clinically important resistance mechanisms encountered with gram positive organisms are the production of methylases and efflux proteins. The clinical failure of clindamycin therapy has been reported before.

In Staphylococci, invitro susceptibility testing for clindamycin by disc diffusion method with erythromycin and clindamycin discs in non adjacent positions may indicate false susceptibility. Failure to identify inducible clindamycin resistance by the macrolide antibiotics may lead to clinical failure when clindamycin is used therapeutically. So, the D-test can be made a routine test while performing the antibiotic sensitivity for routine clinical *S. aureus* isolates. D-test while treating the patients would be a deciding factor to use the clindamycin. Clindamycin in the presence of macrolide (erythromycin) antibiotic can be safely and effectively used in those patients where D-test is negative. There is an increasing interest in assessing the prevalence of inducible clindamycin resistant strains in hospitals as well as community.

While performing the D-test, 15 mm edge to edge distance between the Erythromycin and Clindamycin disks increased the sensitivity of detection of $iMLS_B$ strains. The authors confirmed the results with multiplex PCR using detection of atleast one *erm* gene is a gold standard and have reported 15 mm disk to disk distance to be 100% specific and sensitive.[17]

The 2004 NCCLS guidelines recommended use of the double disc test and suggest that isolates with the $iMLS_B$ phenotype should be reported as clindamycin resistant.[47] However, the clinical efficacy of clindamycin treatment for infections caused by $iMLS_B$ phenotypes of Staphylococci remains unclear. The few cases reported so far have presented conflicting results, and the elimination of a potentially useful drug such as clindamycin is undesirable, especially for the treatment of MRSA infections.[48]

Summary

Staphylococcus aureus has been recognized worldwide as an important causative agent of nosocomial and community acquired infections. The emergence of resistance to antimicrobial agents among *S. aureus* is an increasing problem. Methicillin resistant staphylococcus aureus (MRSA) is a notorious nosocomial pathogen which is prevalent in many countries.

Keeping in mind the above mentioned facts the present study is aimed to know the prevalence of MRSA strains among the clinical isolates of *Staphylococcus aureus,* their antibiotic resistance profile and to detect the phenotypes of *Staphylococcus aureus* showing the inducible clindamycin resistance with macrolide antibiotics (erythromycin).

A total of 613 samples were collected from the patients admitted in the various departments of NGH Indore over a period of one year and processed according to CLSI guidelines. *S. aureus* were isolated 150 in number from total culture positive samples in which the prevalence rate is estimated as 36.5%.

The highest percentage of isolation of *S. aureus* is from pus sample, again confirming its role as the first important pathogen causing skin and soft tissue infections.

Invitro suseptiility testing is done by Kirby-Bauer disc diffusion method. Kirby-Bauer disc diffusion method was performed against penicillin, ampicillin, erythromycin, oxacillin, cefotaxime, amoxyclav, ciprofloxacin, tetracycline, clindamycin, vancomycin. The resistance pattern of these drugs are 74%, 72%, 54%, 51.3%, 48%, 40%, 32%, 32%, 16% and 0% respectively.

From the total 150 S. aureus isolates 73(48.67%) were identified as MSSA strains and 77 (51.3%) were confirmed as MRSA strains.

Out of 150 isolates of *S. aureus*, 57 (38%) were ER/CS. D-test was performed for the detection of $iMLS_B$ phenotype on ER/CS type of *S. aureus* strains. Erythromycin (15µg) and Clindamycin (2µg) discs were placed at a distance of 15mm edge to edge on Mueller-Hinton agar.

Among the 57 number of ER/CS strains, 27 were MSSA, among which 15 (55.5%) were $iMLS_B$ phenotypes. Accordingly out of 30 ER/CS strains of MRSA isolates, 21 (70%) were $iMLS_B$ phenotypes. A total of 36 strains are $iMLS_B$ phenotypes (D-test positive) comprising around 63%.

Out of 73 MSSA strains, 46 (63.01%) were ES/CS, 12 (16.43%) were D-test Negative, 15 (20.54%) were $iMLS_B$ (D-test positive) and none of the isolates were ER/CR which are considered as $cMLS_B$ strains. Out of 77 MRSA strains isolated, 23 (29.80%) were ES/CS, 09 (11.68%) were D-test Negative, 21 (27.27%) were $iMLS_B$ and 24 (31.16%) were $cMLS_B$ phenotypes. Out of total 150 *S. aureus* strains 69 (49%) are ES/CS, 21 (14%) were d-test negative, 30 (24%) were D-test positive and 24 (16%) were ER/CR.

Due to the emergence of resistance to antimicrobial agents, the accurate drug susceptibility data of the infecting microbe is essential for deciding the therapeutic option.

The Erythromycin-Clindamycin disc approximation test or the D-test is a simple, reliable method to detect clindamycin resistance among the erythromycin resistant isolates.

The D-test is an easy test to perform, which can enable us in guiding the clinicians regarding the judicious use of clindamycin while treating skin and soft tissue infections.

Since it is likely that the prevalence of $iMLS_B$ and MRSA strains differs from region to region, it is recommended that clinical microbiology laboratories should routinely include disc diffusion induction tests by placing erythromycin and clindamycin discs 15 mm edge to edge to facilitate detection of inducible clindamycin resistance.

Early detection of inducible MLSB resistance will be a measure to enable the clinicians to save time. Consequently, treatment using clindamycin can be omitted in patients with infections caused by inducible resistant strains, and therapeutic failures may thus be avoided.

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