Detection of Inducible Clindamycin Resistance in Methicillin-Resistant and -Susceptible *Staphylococcus aureus* Isolated from Clinical Samples in a Tertiary Care Hospital of Dhaka City

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DECLARATION OF STUDENT

I hereby declare that the thesis work entitled "Detection of Inducible Clindamycin Resistance in Methicillin-Resistant and Methicillin-Susceptible *Staphylococcus aureus* Isolated from Clinical Samples in a Tertiary Care Hospital of Dhaka City." has been authored and submitted by me, Syeda Adeeba Parvin with only using the mentioned sources. And this Bachelor's Thesis has never been submitted in the same or substantially similar version to any other examinations' office. All explanations that have been utilized verbatim or in a similar vein are clearly indicated. Any mention of contributions by external individuals or institutions, as well as any material sourced from other references, have been appropriately acknowledged and referenced.

The research work was carried out in the Department of Microbiology, Ibrahim Medical College, Dhaka for the partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology.

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APPROVAL

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ABSTRACT

A Gram-positive bacterium, *Staphylococcus aureus*, a microorganism present in the nasal passages of approximately 30% of individuals and on the skin of around 20% is responsible for a diverse range of clinical illnesses. Reputed as the most perilous among the numerous common types of staphylococcus aureus bacteria which often cause skin infections. One of the global public health issues is managing the infections of methicillin-resistant *S. aureus* (MRSA) is quite challenging for its resistance to many antibiotics. MLSB antibiotics, including macrolides, lincosamides, and streptogramin B, are frequently employed in the treatment of MRSA (Methicillin-resistant Staphylococcus aureus). The resistance to these MLSB antibiotics among MRSA is increasing which can occur through efflux mechanisms or through inducible or constitutive resistance mechanisms. The pathogen is often treated by clindamycin whereas overuse of these antibiotics can increase the likelihood of acquiring inducible clindamycin resistance, leading to therapeutic failure and D-test is a necessity to detect such resistance. The objective of this study was to identify inducible clindamycin-resistant Staphylococcus aureus strains among both Methicillin-resistant Staphylococcus aureus (MRSA) and Methicillin-sensitive Staphylococcus aureus (MSSA) isolates. Therefore, a total of 200 clinical samples (wound swabs, pus, urine, blood) were collected from October 2022 to July 2023, from a tertiary care hospital in Dhaka city, out of which 48% were MRSA both phenotypically and genotypically. Inducible resistance was found among 64 (32%) isolates, and constitutive resistance in 53 (26.5%). MRSA had higher inducible (50%) and constitutive resistance (31.2%) than MSSA strains (15.3%, and 22.1% respectively). Female patients exhibited a higher prevalence of inducible clindamycin resistance (82.8%), as well as inpatients (87%). All strains showing inducible clindamycin resistance demonstrated 100% sensitivity to vancomycin. The study indicates that implementing the D-test as a standard procedure in routine disc diffusion testing is essential to prevent treatment failures.

Keywords: MRSA, MSSA, Inducible clindamycin, D-test.

With gratitude to the Almighty Allah, my supervisor, my cherished family, and dear friends...

Learned that,

“Never give up no matter what…keep trying”

“Always be confident and be independent”

“If something you desire from your heart truly; you will get it someday somehow”

“Time never stops for anyone, just go with the flow”

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin sensitive *Staphylococcus aureus*
MLS_B	Macrolide-lincosamide-Streptogramin B
$iMLS_B$	Inducible Macrolide-lincosamide-Streptogramin B
$cMLS_B$	Constitutive Macrolide-lincosamide-Streptogramin B
iCR	Inducible clindamycin resistant
MSA	Mannitol Salt agar
MHA	Muller Hinton Agar
D+	D-zone test positive
D-	D-zone test negative
MS	Macrolide-streptogramin B phenotype
CLSI	Clinical and Laboratory Standards Institute
PCR	Polymerase Chain Reaction
spp	Species
pH	Negative logarithm of hydrogen ion concentration
µm	Micrometer
µl	Microliter

Chapter I

Introduction

1.1 Introduction

1.1.1 Overview

Staphylococcus aureus, a bacterium having the ability to cause from no infection to serious infection in a wide range, is a gram-positive cocci pathogen, highly found in hospitals, carriers are largely the hospital staff, and the patients admitted are the most likely to catch the infection. The pathogen was first identified by the surgeon Sir Alexander Ogston in 1880 in Scotland, in pus from a surgical abscess in a knee joint, and was later named *Staphylococcus aureus* by Friedrich Julius Rosenbach. (Ogoston, 1984)

A facultative anaerobe, *Staphylococcus aureus* bacteria which often causes skin infection, is found in healthy women's lower reproductive tracts, and in the nostrils, however diseases like pneumonia, bacteremia, or sepsis, osteomyelitis bone infection, boils, folliculitis, scalded skin syndrome, abscesses, toxic shock syndrome, cellulitis, endocarditis the heart valve infections also can be caused by such pathogens. It is still one of the top five reasons for nosocomial infections and frequently results in infections of the wounds after surgery. Sneezing, coughing, using contaminated objects, or direct contact with an infected individual are the most common ways that it spreads. It can infect distant organs while also spreading through the circulation. The risk of a more serious staph infection is higher for hospital patients or personnel, even though it may be acquired in the community. (Bush et al., 2023)

Various infections are promoted by producing potent protein toxins and expressing cell-surface proteins that bind and inactivate antibodies by pathogenic strains. The bacteria are usually resistant to several types of antibiotics, including almost all antibiotics that are related to penicillin (called beta-lactam antibiotics). Strains of bacteria that are resistant to almost all beta-lactam antibiotics

are called methicillin-resistant *Staphylococcus aureus* (MRSA), a present worldwide emerging problem. MRSA, once primarily associated with hospitals and healthcare facilities, is now acknowledged as a prevalent source of infections within the general population. This community-associated MRSA spreads rapidly among healthy individuals. There is a matter of concern since MRSA frequently develops multiple antibiotic resistance, or can do so easily, restricting the range of available treatments. On the other hand, antibiotics such as clindamycin and erythromycin have been useful options in the treatment of infections caused by staph bacteria. Clindamycin is a bacteriostatic compound that inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit. Nevertheless, the drug effectiveness is decreasing or getting limited as there might be an expression of lincosamide-streptogramin B resistance (MLSB) (Timsina et al., 2020). Eventually as a result we can observe Methicillin-resistant Staphylococcus aureus (MRSA) isolates exhibiting inducible clindamycin resistance (iCR) may appear resistant to erythromycin and sensitive to clindamycin during routine testing. However, the presence of inducible clindamycin resistance can only be conclusively identified through the D-test. MRSA infections can start from skin infections, become severe, and cause sepsis a potentially fatal response to a serious infection in the body. In the community, it can spread creating a life-threatening situation as well and, in the hospitals, infecting staff members along with patients, who are highly at risk if left untreated.

1.1.2. Morphological Characteristics:

Staphylococcus aureus is a Gram-positive facilitative anaerobic cocci-shaped bacterium singly, in pairs, or in a short chain of 3-4 bacteria contained in grape-like clusters arrangement when they are viewed through a microscope. It produces colonies having circular shapes, convex elevation, and entire margins. When cultured usually round golden-yellow colonies are visible. *S. aureus* reproduces asexually by binary fission. It is a natural habitat found in the mammalian body surface,

they are normal flora of the skin and mucous membrane, present in the nose. The pathogen may survive on the dry skin. Thirty percent (30%) of the normal human healthy population is affected by *S. aureus* as it asymptomatically colonizes on the skin of the human host. On the Mannitol Salt Agar, the colonies are circular, 2–3 mm in diameter, with a smooth, shiny surface; colonies appear opaque and are often pigmented golden yellow. Carotenoid pigment staphyloxanthin is responsible for the characteristic golden color of *S. aureus* colonies. This pigment acts as a virulence factor. In different biochemical tests including catalase, coagulase, on Mannitol salt agar it gives positive result Through a small wound on the skin surface can enable the bacteria to infect easily, through proper hygiene maintenance it can be avoided.

Classification of *Staphylococcus aureus* (Cowan et al., 1954):

Domain: Bacteria

Kingdom: Eubacteria

Phylum: Firmicutes

Class: Bacilli

Order: Bacillales

Family: Staphylococcaceae

Genus: *Staphylococcus*

Species: *S. aureus*

1.1.3 Emergence and Transmission of MRSA:

From the early history, we get a clear idea about how Penicillin used to treat severe infections where it had quite good effectiveness on *S. aureus*, unfortunately with time the pathogen became resistant. By 1960, 80% of the hospitals were penicillin-resistant (Chambers, 2001). Methicillin was introduced in 1959 to address infections caused by penicillin-resistant Staphylococcus aureus (Enright, 2002).

As a result, an alternative came by the second half of the 20th century, where methicillin and vancomycin, beta-lactam antibiotics were used to treat *S. aureus* with successful results Eventually, MRSA strain of *S. aureus* have become resistant to the most β-lactam antibiotics. However, it has led to the emergence of multidrug-resistant MRSA strains. (Livermore, 2000)

S. aureus can develop resistance to methicillin and other β-lactam antibiotics by expressing a foreign penicillin-binding protein (PBP), specifically PBP2a. This protein is resistant to the action of methicillin but is capable of performing the functions of the host PBPs. The rise in the prevalence of MRSA in the environment is caused by changes in genes encoding target proteins and the acquisition of genes causing antibiotic resistance. After the increasing cases of methicillin-resistant *Staphylococcus aureus*, vancomycin is used to overcome MRSA where this antibiotic puts a stop to the formation of peptidoglycan cross-linkages. Nevertheless, the vancomycin-resistant strain of *S. aureus* became evident within different countries inclusive of Japan, the USA, and other countries (Hiramastsu et al., 1997). The U.S. Centers for Disease Control and Prevention (CDC) have formulated guidelines for the appropriate utilization of vancomycin, primarily in response to the elevated incidence of penicillin resistance and the potential for Methicillin-Resistant Staphylococcus aureus (MRSA) to acquire vancomycin resistance. Thus, the physicians can treat with certain antibiotics after confirming the identity of the infecting pathogen.

As there has been an increasing rate of the MRSA which are resistant to beta-lactam antibiotics, as a result physician have been using MLSB antibiotics as a better option. Among which Clindamycin has emerged as a crucial treatment option owing to its excellent pharmacokinetic properties. It is available in both intravenous and oral formulations, boasting a remarkable 90% oral bioavailability. Additionally, its affordability, potent tissue penetration, accumulation in deep abscesses, and ability to inhibit toxin production in *S. aureus* make it invaluable. However, it's important to note that excessive usage of clindamycin can contribute to the development of inducible clindamycin resistance, ultimately leading to treatment failures. During the treatment, resistance occurs when methylase is produced only when an inducing substance like erythromycin is present. During treatment, (Inducible type of MLSB resistance) iMLSB phenotypes can be mutated into (chromosomal resistance) cMLSB phenotypes (Mahalakshmi et al., 2021). However, to detect such a result D-test is done, where inducible clindamycin shows blunting of the clear circular area of no growth around the clindamycin disk on the side adjacent to the erythromycin disk and is designated D-test positive. *S. aureus* can become increasingly resistant if the clinician's rash use of the antibiotics without performing the D-test. This prompts some researchers to warn against the use of clindamycin for *S. aureus* isolates with $iMLS_B$-resistant phenotypes. (Mahfouz, 2023)

The MRSA is transmitted from the contact of any infection-sharing person as well as any items used by that individual such can include razors, towels, cloths, etc. Sometimes the infection may spread by certain things that are used by many people, for example, floors, sinks, door handles, tables, and chairs. Usually, MRSA cannot infect the body unless there are any wounds or cuts, or any skin breakage is present there. Even people having no health issues, especially adults, and children a lot of times may avoid any sort of skin scrapes which a lot of the times lead to MRSA,

as it may proliferate. Athletes, children in daycare and schools, military personnel living in barracks, and those who have just undergone inpatient medical treatment are all at increased risk. A person's risk of contracting MRSA can rise when they engage in activities or are in locations with high levels of human traffic, skin-to-skin contact, and shared supplies or equipment. Two out of every 100 persons carry MRSA, according to studies. (Kluytmans et al., 1997) Surgical wounds, chronic hepatic, pulmonary, or vascular illness, cancer, recent exposure to antibiotics, intravenous drug usage, ICU hospitalization, etc. are additional factors that contribute to the spread of MRSA. (Morb, 1981)

1.1.4 Antibiotic resistance mechanism in *S. aureus*:

The *S. aureus* organism first became resistant to penicillin where the resistance occurs by the production of an enzyme called penicillinase. Penicillin being in a group of β-lactam antibiotics contains a β-lactam ring, which is usually cleaved by the enzyme eventually making the antibiotic ineffective in the patient's body. After the penicillin, methicillin has turned resistant where the resistance is arbitrated through the *mec* operon, due to the expression of an additional penicillin-binding protein (PBP2a), containing a lower affinity of binding β-lactams similar to penicillin, etc. (Hachbath et al., 1995).

Although it can take over the transpeptidation or cross-linking processes of the host PBPs, it is resistant to the action of methicillin. PBP2a production is controlled and typically kept at a low level, but if mutations occur in the regulatory genes, the level of synthesis can be increased. This permits the development of resistance to all β-lactam antibiotics and forgoes their therapeutic use in MRSA infections.

On the other hand, MLS_B resistance phenotypes can be constitutive ($cMLS_B$) or inducible ($iMLS_B$). Inducible MLS_B resistance phenotypes are drawn out when rRNA methylase is produced in the presence of an inducer agent, such as erythromycin. Infections caused by $iMLS_B$ *S. aureus* isolates fail to respond to clindamycin therapy because the rRNA methylase enzyme secretion is active in the presence of erythromycin as an inducer, resulting in clindamycin inactivation and increasing antibiotic resistance. (Mahfouz, 2023)

1.1.5 Epidemiology of MRSA and inducible clindamycin:

About 2-53 million people carry MRSA, predicted according to a conservative estimation based on Dutch and US prevalence data. *Staphylococcus aureus* is the number one cause of hospital-associated infections and a high proportion of these are caused by MRSA. Recently, due to the higher rate of antibiotic resistance, MRSA is also found in communities. The mortality rate associated with invasive MRSA infections is estimated to be 20% but varies considerably between studies in different settings. Since the 1940s evolution of MRSA of penicillin has changed a lot. Recently MRSA has emerged differently with having resistance in Macrolide-lincosamide-streptogramin B. As a result, multidrug resistance is an increasing problem in *Staphylococcus aureus.* The prevalence of MRSA was 80.5%, D-test positive has been reported as 20.4% in MRSA strains and 13.3% in MSSA in India (Bala et al., 2021) on the other hand, in Nepal, it was found that. 39.5% were MRSA, and 36.5% were iCR.

1.1.6 Spread Prevention

Standard precautions mentioned in the following might be helpful to control the spreading:

Protecting eye, nose, mouth

While treating any patient or caring for any patient the mucous membrane of the eyes, nose, and mouth must be protected from any sort of caregiving activity that may generate splashes or sprays of blood, body fluids, pus, or any sort of secretion. The mask needs to be worn and googles, face shields must be used according to the necessity while performing such task.

Hygiene

Whether a person wears a glove or not, must wash their hands after any touching of blood, body fluids, pus,, or any secretion immediately. If necessary wash hands in between all tasks one should anticipate.

Proper way to handle patient care equipment

Equipment that is used for patient care purposes might often get contaminated as it is soiled with blood, pus, secretions, and body fluids. These might transfer microorganisms to other patients, too the environment, might infect any hospital staff as well. Without appropriately cleaning the reusable equipment it should not be used for caring for other patients and it must be ensured. It is similarly crucial to discard any single-used items that will later not contaminate the environment. Hospital sheets, patients' clothing, bed rails, tables, etc. need to be cleaned on a frequent basis. Any sort of place or surface such as door knobs, must be cleaned and disinfected.

1.2 Literature review

Shameem et al. (2012) investigated to determine Inducible clindamycin among clinical isolates of staphylococci collected from wound swabs and pus samples. 20% of isolates were inducible clindamycin resistance, 5% were constitutive clindamycin resistance and 75% were clindamycin

sensitive. D-test was done, and out of 200 isolates 43.5% were MRSA and 35% were MSSA. Inducible clindamycin resistance was recorded 44 in number in total out of which 42 were MRSA as well. Coagulase-negative Staphylococci along with Inducible clindamycin was found in two isolates of MSSA that had zero percent of iCR. All the clindamycin-resistant strains were 100% sensitive to vancomycin and linezolid whereas 28.8% ciprofloxacin, 42% gentamycin, 42.3% tetracycline. Overall, the study demonstrates that a significant quantity of Staphylococci at their tertiary care hospital had inducible resistance to clindamycin.

Bala R et al. (2021) conducted their study to assess the importance of the D-test to avoid treatment failures in terms of detecting the inducible clindamycin by analyzing the percentage of resistant inducible clindamycin among methicillin-resistant and sensitive staphylococci. The samples were collected from a hospital, and all of them were pus samples from all age groups having skin and soft tissue infections. Along with the antibiotic susceptibility test and D-test, molecular confirmation of MRSA was done. Out of 235 isolates, 80.5% were MRSA where 69.5% were hospitalized patients and 19.15% were MSSA. Both MRSA-MSSA had 0 resistance in vancomycin. Inducible clindamycin resistance was found 22.1% within MRSA and 13,3% among MSSA. Other than this, constitutive clindamycin resistance was also inspected and that was 51.6%, and 13.3% respectively in MRSA and MSSA.

Devi Thapa et al. (2021) investigated to determine the occurrence of methicillin resistance and inducible clindamycin-resistant *S. aureus* at a tertiary care hospital, where they tested 1027 clinical isolates from samples included urine, pus, blood, sputum, body fluids. An antibiotic susceptibility test was done along with phenotypic detection of MRSA and inducible clindamycin resistance which is a D-test. All of the isolates of *S. aureus* were susceptible to vancomycin, and 67% were multidrug-resistant. Out of the 1027 isolates, 39.5% were MRSA and 36.5% were inducible

clindamycin-resistant, all isolates were resistant to penicillin-G and ampicillin. Most of the isolates were pus (50%) in MRSA. Outpatients (38%) and male patients (50%) were higher in MRSA. Inducible MLSB phenotype was 40%, and 34.9% within MRSA and MSSA consecutively on the other hand 20% and 17.4% were constitutive MLSB phenotype. From all the assessments the study demonstrates that the increasing number of MRSA and inducible clindamycin-resistant phenotypes need methicillin test and D-test to be included in routine susceptibility testing for managing the infections spread by *S. aureus* effectively.

Mallick S K et al. (2009) aimed at examining the infection caused by *S. aureus* where pus sample isolates were greater in number. A total of 332 isolates were tested 68 were iCR and after the antibiotic susceptibility test, it was confirmed that 100% of them were sensitive to vancomycin and linezolid. 51.6% were MRSA and 48.3% were MSSA. Among the MRSA strains, 36% were iMLSB and 6.33% were cMLSB phenotype. The overall result showed, that 24.9% of strains were resistant to all commonly used antibiotics, but MRSA were sensitive to vancomycin and linezolid. Avoiding the resistance might be harder and decreasing such rate can be done by adding the D-test to regular tests.

Mansouri S et al. (2014) evaluated a total of 162 *S. aureus* for inducible clindamycin resistance by D-test where 8.64% were iCR. Mostly, isolates cultured from urine and wound swabs were D-test positive as well as MRSA. The percentage of inducible clindamycin resistance was 11.95, and 4.18 in MRSA and MSSA separately. Similarly, 47.82% and 2.85% constitutive clindamycin resistance were found. Therefore, a D-test should be performed to prevent treatment failures of *S. aureus* infections caused by agents that are resistant to erythromycin and resistant to clindamycin.

Another study was aimed and done to assess different articles where a high number of MRSA were observed to have more inducible clindamycin resistance than MSSA. Through the years how the

resistance of clindamycin and MRSA has increased is quite alarming. At the same time, it varies from place to place. The highest peak prevalence of iCR was recorded in 2017 in Egypt which was 44%. In MRSA iCR is higher as it ranges from 3.6 to 77.8% than the MSSA and this varies from one country to another such as 25%-28% in India, 24.5% in Nepal, 46.7% in Malaysia, etc. The overall higher number of inducible clindamycin and MRSA from this study was 26.8%. (Muluneh Assefa, 2022)

1.3 OBJECTIVES

1.3.1 GENERAL OBJECTIVE

Detection of Inducible Clindamycin Resistance in Methicillin-Resistant and-Susceptible Staphylococcus aureus Isolated from Clinical Samples in a Tertiary Care Hospital of Dhaka City.

1.3.2 SPECIFIC OBJECTIVES

- to isolate *Staphylococcus aureus* from samples including wound swabs, pus, urine, and blood by culture.
- to detect and identify *Staphylococcus aureus* by Gram staining and biochemical tests.
- to confirm *Staphylococcus aureus* through the nuc gene by PCR method.
- to detect methicillin-resistant *Staphylococcus aureus* phenotypically by disc diffusion technique.
- to determine the mecA gene for molecular confirmation of MRSA.
- to determine antimicrobial susceptibility patterns of the isolated *s. aureus*.
- to detect inducible clindamycin resistance using D-test.
- To detect any correlation between MRSA and iCR (inducible clindamycin resistance)

Chapter II

Materials and Methods

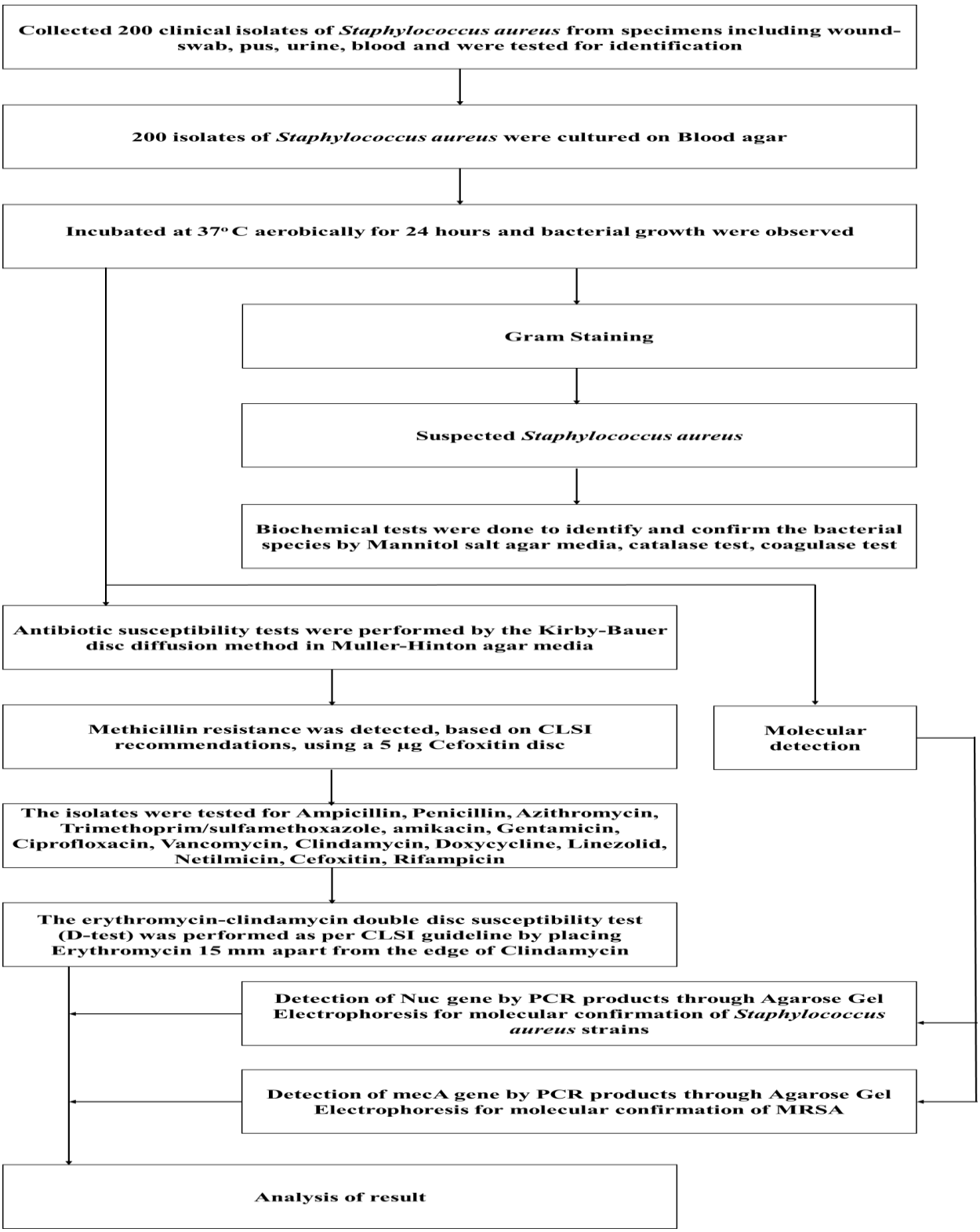
2.1 Materials and Methods

2.1.1 Methodology

Two hundred samples were received in the Microbiology Department of Bangladesh Specialized Hospital over 10 months (October 2022 to July 2023), where 83 were wound swab samples, 61 were pus samples, 32 were urine samples, and 24 were blood samples. Data regarding age, sex, indoor and outdoor patients were collected using a predesigned data collection sheet. **(Appendix-I)**

Among the two hundred samples 126 (63%) were female in number, whereas 74 (37%) were male as well as the ratio of indoor patients, and outdoor patients was 164:36 consecutively 82% and 18%. The *s. aureus* isolated strains were detected through subculture on blood agar media, then Gram staining and standard biochemical test were performed. Antibiotic susceptibility was done to detect methicillin resistance using cefoxitin and inducible clindamycin resistance by placing erythromycin and clindamycin 15mm apart among *S. aureus* isolates. Some commonly used antibiotics **(Appendix II)** were also used to observe the sensitivity pattern and correlation between methicillin resistance and inducible clindamycin resistance. By D-test inducible clindamycin was detected. However, the detection of the nuc gene by PCR was performed to confirm the s. aureus strains as well as detection of the mecA gene by PCR was done for the molecular confirmation of MRSA. The collected bacterial samples were sub-cultured in Blood agar media. (Ghosh et al., 2016**)** The samples were streaked for culture on the media and incubated at 37°C for 24-48 hours. Later than 24 hours the plates after incubation were observed for growth presence for the reidentification. The identification of the microorganisms was done as per standard techniques **(**Cheesebrough, 2006).

2.1.2 Flowchart of the study design



2.1.3 Culture:

For the isolation of organisms, they were cultured in Blood agar media **(Appendix-IV)**. In the beginning, the workplace was sterile using alcohol. Each plate was labeled with a marker and inside a Laminar air flow cabinet. Each sample was collected in an inoculating loop and a four-way streaking was performed on the blood agar. At first, the loop was burnt, the second and the fourth time as well, and had to skip the third time to get a discrete colony. To affect the dilution of the culture to get fewer organisms when streaked in each area at different points the loop was flamed as a result desired separated growth was observed. The plates were incubated for 24 hours at 37°C. after the 24-hour time frame, the growth was observed and a light golden yellow pigmented colony was visible with a small number of non-hemolytic which was 1-4 mm in diameter.

2.1.4 Gram Staining and Microscopy:

A crucial staining technique, Gram staining used to differentiate between gram-positive and gram-negative bacteria based on their cell wall's ability to retain the crystal violet dye. Gram-positive microorganisms' have high amount of peptidoglycan while gram-negative organisms have high lipid content. Due to having a thick layer of peptidoglycan gram-positive bacteria retain the primary color and stains in violet color. Whereas, the lipid layer of the gram-negative stains in red for their thinner peptidoglycan walls as a result they lose primary stain during the use of solvent. *S. aureus* is a gram-positive bacterium and shows violet staining under microscopes.

The smear was prepared from the culture plate and stained by Gram's stain as per standard procedure and was examined under a microscope for the gram-positive organisms. **(Appendix-III)**

2.1.5 Identification of staphylococcus aureus:

All organisms were identified using standard techniques including examination of colony morphology, hemolytic characteristics, staining properties, pigment production, and biochemical tests. **(Appendix V)** (Cheesebrough, 2006). The isolated organisms were inoculated in MSA, and tested for catalase, and coagulase test.

2.1.6 Antimicrobial Disc Diffusion test:

Antibiotic disk diffusion test is a procedure performed to identify antibiotic susceptibility, and sensitivity on organisms and to determine which combination of antibiotics works best for a given patient. The Kirby-Bauer antibiotic test was utilized to determine the antibiotic susceptibility of certain microorganisms. In the study, a total of fourteen commercially available antibiotics' potency were regulated. Susceptibility of antimicrobial agents of all isolates was done using Muller Hinton agar plates and the zone of inhibition was interpreted according to CLSI guidelines (CLSI, 2015). Antibiotic discs such as Ampicillin (10 µg), penicillin (10 µg), azithromycin (15 µg), trimethoprim/sulfamethoxazole (25 µg), amikacin (30µg), gentamicin (10 µg), ciprofloxacin (5µg), vancomycin (30 µg), doxycycline (30 µg), linezolid (30 µg), netilmicin (30 µg), and rifampicin (30µg) were used. Detection of MRSA was determined using 5 µg cefoxitin and the isolates that yielded a zone diameter <21. On the other hand, to determine the phenotype of strains against the MLS_B group of antibiotics the clindamycin (2 µg), and erythromycin (15 µg) were placed at a distance of 15 mm on the Muller Hinton agar.

2.1.7 Preparation of inoculum:

Using a sterile wire loop, isolated colonies of the test organisms were emulsified in 3 ml of sterile normal saline. The turbidity of the suspension was then compared with McFarland turbidity standard 0.5 **(refer to Appendix VI)** by adding normal saline and placing a printed card behind the test and standard inoculums under appropriate lighting conditions. This allowed for the preparation of the test inoculums.

2.1.8 Inoculation of the test:

A sterile swab stick was positioned at the bottom of the inoculum. The swab stick was pressed and rotated against the side of the tube above the suspension level to remove excess fluid. Subsequently, the swab was evenly streaked over the surface of the Muller Hinton agar **(refer to Appendix IV)** plate in three directions, rotating the plate approximately 60 degrees each time. The inoculated plate was then allowed to dry for 3-5 minutes.

2.1.9 Antibiotic Disc Placement:

Antibiotic discs were placed on the inoculating plate 15mm away from the edge of the plate and 25 mm apart from one disc to another from center to center. For the D-test, the clindamycin and erythromycin were placed at a distance of 15 mm on different MHA plates.

2.1.10 Inoculated plate incubation:

Within 30 minutes of placing the antibiotic discs, the inoculated plates were incubated aerobically overnight at 37°C. Both plates for the antibiotic sensitivity pattern of the *S. aureus* isolates and D-test were incubated.

2.1.11 Interpretation of zone of diameter:

After overnight incubation, the test plates were examined to confirm the confluent growth of organisms. The diameter of the zone of inhibition was measured in millimeters using a ruler on the underside of the plates and evaluated according to the standard chart of CLSI guidelines (CLSI, 2015). The presence or absence of a clear zone of inhibition around the disc on the tested organisms was interpreted as indicating resistance or sensitivity, respectively.

2.1.12 Detection of MRSA and inducible clindamycin in the antibiotic susceptibility test:

From the overnight incubation, through the zone of inhibition on the MHA plates, the Cefoxitin disc was utilized to detect methicillin resistance, and the isolates that yielded a zone diameter of <21 mm around the cefoxitin disc were considered as MRSA. Alternatively, test results were interpreted as three different phenotypes and they are:

1. (MLS_B sensitive) Moderate sensitive phenotype, if isolates were susceptible to clindamycin (zone diameter ≥ 21 mm) without a D-shaped zone and resistant to erythromycin (zone diameter ≤ 13 mm).
2. Inducible MLS_B phenotype, if isolates were susceptible to clindamycin (zone diameter ≥ 21 mm) with a D-shaped zone and were resistant to erythromycin (zone diameter ≤ 13 mm).
3. Constitute MLS_B phenotype, if isolates were resistant to both clindamycin (zone diameter ≤ 14 mm) and erythromycin (zone diameter ≤ 13 mm).

2.1.13 Preparation of stock sample:

Good strains of microorganisms when they grow should be preserved properly for any future usage. If the culture is preserved correctly their characteristic features will remain the same otherwise it might decline after a certain time.

2.1.14 Preservation of isolated organisms:

With all the aseptic precautions in a laminar air flow cabinet with the help of a straight wire loop few colonies of each organism were stabbed into the media Trypticase Soy broth and incubated overnight at 37^{o} C then the cryovials were immersed with sterile liquid 300µl glycerin to exclude air and reduce dehydration and were preserved in the refrigerator at -20^{o}C. subcultures were done every month into a fresh slant and were kept at -20^{o}C (Chesbrough, 2006).

2.2.1 Molecular method:

Polymerase Chain reaction or PCR for the detection of the nuc gene for molecular confirmation of *Staphylococcus aureus* strains, and detection of the mecA gene for the molecular confirmation of MRSA.

2.2.2 Procedure of PCR:

2.2.3 Bacterial pellet formation and DNA extraction:

The colonies of the specific gram-positive bacteria were sub-cultured into Muller Hinton agar media at 37^{o}C for 24 hours. Colonies each of the bacterial isolates were inoculated by a loop into an Eppendorf tube filled with sterile trypticase soy broth and was incubated at 37°C overnight, following the incubation centrifuged at 4000 rpm for 10 minutes to pellet the cells. The supernatant was discarded as much as possible while keeping the cell pellets. Again, a small amount of

trypticase soy broth was added and mixed evenly, later centrifuged at 4000 rpm for 10 minutes at room temperature. The supernatant was discarded carefully and the pellet was kept at -20^{o}C. The pellet in the Eppendorf was filled with 300µl sterile distilled water, vortexed until mixed and the mixture was heated at 100^{o}C water bath for 10 minutes. After heating, immediately the tubes were placed on ice for 5 minutes for cold shock. Instantly after cold shock, the suspension was centrifuged at 14000 rpm at 4^{o}C for about 6 minutes. The supernatant was then transferred by micropipette into a new sterile Eppendorf tube and was stored at -20^{o}C to be used for a PCR run later.

2.2.4 Mixing of master mix and primer with DNA template:

Primers were mixed with Tris-EDTA (TE) Buffer according to the manufacturer's instructions. For each sample, a total of 13 µl of the mixture was prepared by mixing 6 µl of master mix (mixture of dNTP, taq polymerase $MgCl_2$, and PCR buffer), 1 µl of forward primer, 1µl of reverse primer, 2 µl of DNA template, and 3 µl of nuclease-free water in the PCR tube. After a brief vortex, they were prepared for PCR.

2.2.5 PCR Thermal cycle parameters:

Table 1: PCR Thermal Cycling parameters for primer *Nuc, MecA*

Gene	**Temperature (ºC)/time cycle**					**Cycle no.**
	Initial denaturation				**Final extension**	
		Denaturation	**Annealing**	**Extension**		
Nuc	95/10 min	94/1 min	55/30 sec	72/1.30 sec	72/3.30 sec	37
MecA	94/4 min	94/30 sec	53/30 sec	72/1 min	72/1 min	35

By using the reference cycling parameters on the mentioned chart were followed for the PCR (Parvez et al., 2018)

2.2.6 Gel electrophoresis:

For the preparation of 100ml of a 1.6% agarose solution, 1.6g agarose was measured on an electric balance, and 100 ml 1X buffer was added. The agarose solution was heated until the agarose properly dissolved and till the solution turned transparent, it was heated in a microwave oven. After a while when the temperature of the solution reduced, 1.52 µl of ethidium bromide dye (EtBr) was added, it is generally added as its DNA bands are quite visible under the ultraviolet (UV) light. After the gel turned solid the comb was removed carefully and the gel was placed in the electrophoresis chamber where the chambers were then loaded with TBE Buffer keeping the wells deluged under the buffer. On the first well 4µl of 1000bp DNA ladder, then 5µl of DNA solution

was added sequentially where the well was loaded in a segment as follows- ladder, negative control (water), positive reference strain, and other DNA PCR products. The gel was run at 90 volts for about 45 minutes until the run was complete. After the gel run, it was sighted under the short-wave UV light for desired DNA band detection.

Table 2: Selected primers were used in the study:

Primer pairs	**Target gene**	**Sequence (5'-3')**	**Product size, bp**
mecA P4 2821 mecA P7 2822	mecA	TCCAGATTACAACTTCACCAGG CCACTTCATATCTTGTAACG	162
nuc F nuc R	Nuc	GCGATTGATGGTGATACGGTI AGCCAAGCCTTGACGAACTAAAGC	270

2.2.7 Visualization of agarose gel:

The gel was removed from the tray and visualized by UV illumination and gel was photographed for documentation and it was done using a digital camera. The size of the amplified DNA was assessed by comparing it with the bands of the DNA ladder.

Chapter III

Result

3.1.1 Results

In the study, a total of 200 *S. aureus* isolates were obtained of which 83 were wound swab samples, 61 were pus samples, 32 were urine samples, and 24 were blood samples. The data found demonstrates the distribution of various strains of *Staphylococcus aureus* isolated from different samples. Among the isolated bacteria, most of the MRSA (53.1%), MSSA (30.7%), and iCR (51.5%) isolates were from wound swabs. Out of the 200 samples, more than 90% were gram-positive *S. aureus* bacteria which showed purple cocci shape under the microscope. All the isolates were tested against standard biochemical tests and they gave respective results in the tests: In MSA (Mannitol Salt Agar) *S. aureus* turned yellow from pink through Mannitol fermentation representing MSA positive. Whereas *S. aureus* resulted positive by forming bubbles in the catalase test and the appearance of clumps indicated coagulase positive.

48% (96/200) strains were screened as cefoxitin resistant out of the 200 *S. aureus* strains. The DNA of these isolates was extracted, and amplicons were detected for the mecA gene. Before that, all *S. aureus* strains were detected with the nuc gene by PCR molecular identification of actual strains of *S. aureus.* In the findings, all cefoxitin-resistant strains came out bearing the mecA gene, conveying that among all the collected samples, 48% were MRSA strains and the rest were MSSA (52%). In between the 96 MRSA (96/200) strains, 53.1% were from the wound swab samples, 69.7% were from female and 83.3% were hospitalized patient that given in the table 4, 5, 6 respectively. In the MSSA 30.7% were from wound swabs, 29.8% were from pus, 18.27% from blood, and 20.1% from urine samples. Later when the D-test was done, iCR was found mostly from wound swabs (51.5%) and from blood samples, it was less in number which was 6.25%. all these data are demonstrated in Table 3.

Table 3: Distribution of *Staphylococcus aureus* in various samples:

Clinical Specimen	***S. aureus*** **N= 200**	**MSSA (%)** **N= 104**	**MRSA (%)** **N= 96**	**iCR (%)** **N=64**
Pus	61	31(29.8%)	30(31.2%)	21(32.8%)
Wound swab	83	32 (30.7%)	51(53.1%)	33(51.5%)
Blood	24	19 (18.27%)	5(5.2%)	4(6.25%)
Urine	32	22 (20.1%)	10(10.4%)	6(9.37%)

While outlining **Table 4**, it can be found that, among the 200 collected samples, 37% were male (n=74) and 63% were female (n=126). Among which 30.2% of the samples from males, 69.7% were from females was found in MRSA strains, 43.2% samples from males, and 56.7% were from females in MSSA strains. Finally, 17.1% of samples were males and 82.8% were females found in iCR strains.

Table 4: Distribution of methicillin resistance *Staphylococcus aureus,* methicillin-susceptible *Staphylococcus aureus,* and Inducible clindamycin resistance (iCR) in different sex of patients:

Sex of patients			
	MRSA **N=96(%)**	**MSSA** **N=104 (%)**	**iCR** **N=64 (%)**
Male (N=74) 37%	29 (30.2%)	45 (43.2%)	11 (17.1%)
Female (N=126) 63%	67 (69.7%)	59 (56.7%)	53 (82.8%)

Form **table 5**, the idea can be taken that,

82% were inpatient (n=164), 18% were outpatient (n=36). Of which, 83.3% were inpatient, 16.6% were outpatient among MRSA (n=96) strains. 80.7% were inpatient, 19.2% were outpatient among MSSA (n=104) strains, 87% were inpatient and 12.5% were outpatient among iCR (n=64) strains.

Table 5: Distribution of methicillin-resistant *Staphylococcus aureus,* methicillin-susceptible *Staphylococcus aureus,* and Inducible clindamycin resistance (iCR) among two different types of hospital care patients:

Hospital care of patients			
	MRSA **N=96 (%)**	**MSSA** **N=104 (%)**	**iCR** **N=64 (%)**
Outpatient care (N=36) 18%	16 (16.6%)	20 (19.2%)	8 (12.5%)
Inpatient care (N=164) 82%	80 (83.3%)	84 (80.7%)	56 (87%)

Afterward, antibiotic sensitivity testing for the MRSA strains was performed, and the phenotype was examined and recorded. From **Table 6**, resistance in different classes of antibiotics was higher among the MRSA isolates than the MSSA isolates. In between that, all MRSA isolates were cefoxitin, ampicillin, penicillin-resistant. The isolates of inducible clindamycin showed no resistance to vancomycin and high resistance to azithromycin (79.6%).

Table 6: Isolates displaying both resistance and sensitivity to various antibiotics were tested:

Antibiotics	**MRSA (N=96)**		**MSSA (N=104)**		**iCR (N=64)**		**Total (N=200)**	
	R	**S**	**R**	**S**	**R**	**S**	**R**	**S**
1. Cefoxitin	96	0	0	104	47	17	96	104
2. Ampicillin	96	0	76	28	45	19	172	28
3. Penicillin	96	0	84	20	49	15	180	20
4. Azithromycin	86	10	78	26	51	13	164	36
5. Trimethoprim/ Sulfamethoxazole	80	16	61	43	38	26	141	59
6. Amikacin	48	48	14	90	31	33	62	138
7. Gentamicin	71	25	16	88	29	35	87	113
8. Ciprofloxacin	74	22	63	41	32	32	137	63
9. Vancomycin	8	88	0	104	0	64	8	192
10. Doxycycline	18	78	4	100	4	60	22	178
11. Linezolid	12	84	5	99	2	62	17	183
12. Clindamycin	81	15	68	36	0	64	149	51
13. Rifampicin	13	83	5	99	3	61	18	182
14. Netilmicin	38	58	11	93	11	53	49	151
15. Erythromycin	48	48	16	88	64	0	64	136

R= Resistant. S=Sensitive

*values of inducible clindamycin resistance alone are provided.

Then, MRSA and MSSA strains were examined to exhibit resistance towards erythromycin and clindamycin by performing the D-test. By summarizing **Table 7,** it was found among MRSA strains (n=96) 11.4% were sensitive to both erythromycin and clindamycin, 31.2% were resistant to both which was constitutive MLSB, 50% were D-test positive whereas 7.2% were D-test negative. Among the MSSA strains, 42.32% were both sensitive to erythromycin and clindamycin, 22.1% were both resistant, 15.3% were D-test positive, and lastly, 20.1% were D-test negative.

Table 7: Susceptibility pattern of clindamycin and erythromycin among the isolates:

Susceptibility pattern (Phenotype)	**Number (percentage) MRSA (N=96)**	**Number (percentage) MSSA (N=104)**	**Number (percentage) All *S. aureus* (N=200)**
E= S, C=S (Susceptible)	11 (11.4%)	44 (42.32%)	55 (27.5%)
E= R, C= S (D-test +ve) iMLSB	48 (50%)	16 (15.3%)	64 (32%)
E= R, C= R (cMLSB)	30 (31.2%)	23 (22.1%)	53 (26.5%)
E=R, C= S (D-test negative) MS	7 (7.2%)	21 (20.1%)	28 (14%)

R= Resistant, S=Sensitive, E= Erythromycin, C=Clindamycin,

iMLSB= Inducible Macrolide-lincosamide-Streptogramin B

cMLSB= Constitutive Macrolide-lincosamide-Streptogramin B

MS= Macrolide-streptogramin B phenotype

From the figure 1, it describes the iMLSB, cMLSb, MLSB phenotype.

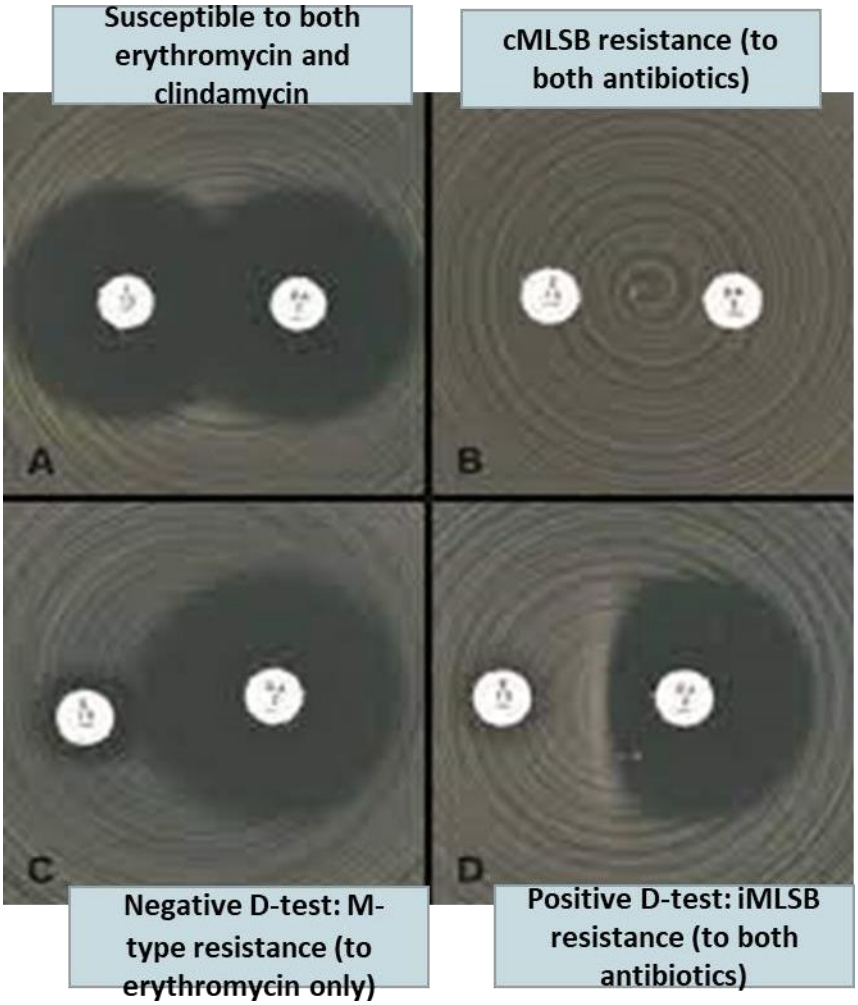


Fig 1: Different types of antibiotic disc results of MLSB group antibiotics.

3.1.2 Molecular detection of the Isolates by PCR:

To amplify the desired genes, DNA from the isolates were extracted by using the PCR method. After the biochemical identification of the template DNA, 2µl of template DNA was utilized in PCR to enhance the likelihood of gene detection including the Nuc (R/F), the mecA P4, and mecA P7. All the samples resulted nuc positive by confirming the stains as *Staphylococcus aureus* as it

identifies the microorganism. And 96 isolates, confirmed as MRSA based on phenotype, exhibited positive results for the detection of the mecA gene through PCR.

The PCR findings are depicted in the figure below:

Fig 2: Gel Electrophoresis of *Staphylococcus aureus* samples for the Nuc gene for the identification

Here, lane 1 contains a **1000 bp DNA ladder,** lane 2 contains **negative control- Water, and at the end of lane 18 there's a positive control-reference strain.** And in between there are 15 isolates. In the PRC result, the last 10 results show positive, which confirms the presence of the Nuc gene, and the first four isolates show negative indicating the absence of the Nuc gene. The positive ones confirm that these are strains of ***Staphylococcus aureus.***

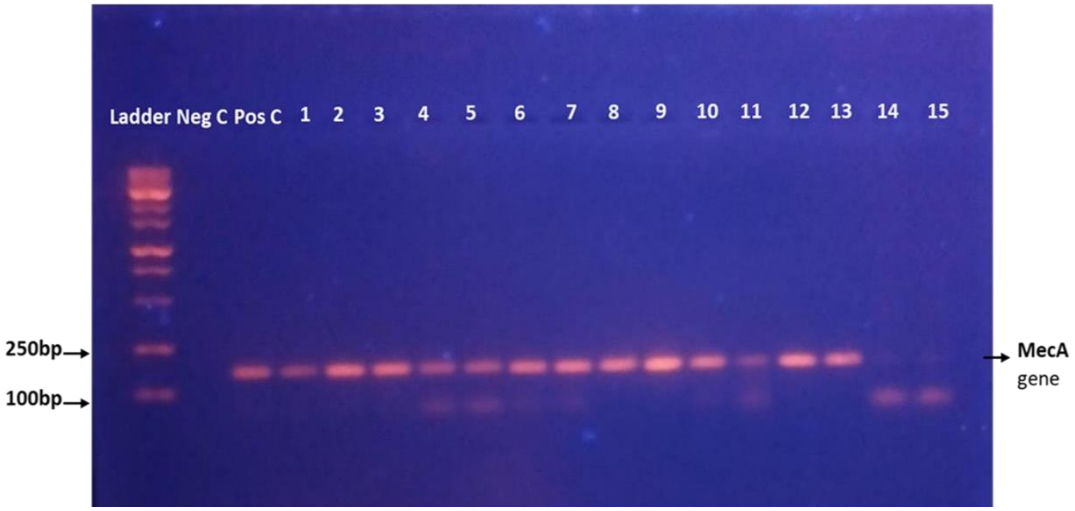


Fig 3: Gel Electrophoresis of *Staphylococcus aureus* samples for the mecA gene for the confirmation of MRSA

Here, lane 1 contains **1000 bp DNA ladder,** lane 2 contains **negative control- Water, and on lane 3 there's positive control-reference strain after 15 isolates are loaded on the wells.** In the PRC result, the first 13 results show positive, which confirms of the mecA gene is present, and the last two isolates show negative indicating the absence of the mecA gene. The positive ones confirm that these are strains of ***Staphylococcus aureus*** MRSA positive.

Chapter IV

Discussion

4.1.1 Discussion

In the current study, *S. aureus* was frequently isolated from pus, wound swabs, blood, and urine samples, whereas a higher number of isolates came from wound swabs. Besides, methicillin-resistant *Staphylococcus aureus* (53.1%), as well as resistant inducible clindamycin (51.5%) isolates also were mostly from the wound swab. Other studies, show, most of the MRSA and iCR were isolated from wound swab-blood (58.82%) (Ghosh S et al., 2016), urine-pus samples (Devi Thapa et al., 2021), pus (53.5%), and wound swab (46.5%) (Shameem et al., 2012). The study has resulted in 69.7% of female patients having MRSA and 82.8% having iCR whereas the percentage of inpatients was sky-high including 83.8% MRSA and 87% iCR. In one study in India, it shares, 38% of MRSA were outpatient and most of the MRSA was found in male patients (50%) (Devi Thapa et al., 2021).

Cefoxitin, ampicillin, and penicillin were observed 100% resistant among the total 200, and 100% resistant in MRSA isolates. MSSA were 100% sensitive to cefoxitin as well as vancomycin whereas iCR isolates showed 0% resistance in vancomycin. A study done in Bangladesh a few years ago also adds to the 100% resistance of cefoxitin (MRSA) (Shameem et al., 2012). Again, is shared Vancomycin and linezolid exhibited 100% sensitivity. In addition, a study shares All isolates included in the study demonstrated sensitivity to both vancomycin and linezolid. (Ghosh S et al., 2016).

In my study, cefoxitin was significantly used to observe the MRSA identification was conducted using the disc diffusion method, revealing that 48% of the isolates were Methicillin-resistant Staphylococcus aureus (MRSA). At the same time, molecular detection of MRSA was done using

the mecA gene for confirmation. Likewise, other studies such were investigated in India showed that they also confirmed the MRSA by PCR method using the mecA gene. (Bala et al., 2021)

Clindamycin is considered one of the effective drugs for treating infections caused by resistant staphylococci, including Methicillin-resistant Staphylococcus aureus (MRSA). In the study, phenomenon of resistant inducible clindamycin is also known as the iMLSB phenotype and was observed in 50% and 15.3% of MRSA and MSSA, respectively. Similarly, a study a few years back also indicated that 48.3% of MRSA and 0% of MSSA had inducible clindamycin resistance (Shameem et al., 2012).in the same study conducted reported Coagulase-negative *staphylococci.*

This study observed a notable correlation between MRSA and inducible clindamycin resistance, as well as the presence of the MS phenotype in both MRSA and MSSA isolates. Out of different studies (Shameem et al., 2012), (Devi Thapa et al., 2021) has detected MS phenotypes in MRSA and MSSA as well.

The current study identified constitutive clindamycin resistance in MRSA isolates (31.2%) and MSSA (22.1%). This trend contrasts with findings from other studies and from (Bala et al., 2021) that shows constitutive clindamycin-resistant (51.6%) MRSA, and MSSA (13.3%). And form (Devi Thapa et al., 2021) MRSA (20%) and MSSA (17.4%). These findings suggest that the occurrence of both inducible and constitutive resistance in staphylococcal isolates fluctuates across different regions. Furthermore, the study observed a lower prevalence of constitutive clindamycin resistance compared to inducible clindamycin resistance. The emergence of antibiotic resistance is closely linked to the extensive utilization of the specific antibiotic (Devi Thapa et al., 2021). To the best of my knowledge, this study represents the initial documentation of clindamycin inducible resistance among both MRSA and MSSA strains in India.

The findings suggest that most MRSA isolates displayed resistance to azithromycin, trimethoprim/sulfamethoxazole, gentamicin, and ciprofloxacin. This pattern closely resembles observations from a study conducted in South Africa (Adebayo et al., 2006).

In the study, approximately half of the MRSA isolates and over half of the MSSA isolates exhibited sensitivity to both erythromycin and clindamycin. Therefore, these drugs remain viable options for treating less severe staphylococcal infections in the study area. Nevertheless, the routine disc diffusion test along with the D-test must be done.

4.1.2 Conclusion

In conclusion, while many strains of *Staphylococcus aureus* primarily cause skin and soft tissue infections, some have the potential to lead to more severe infections such as bloodstream infections, pneumonia, or infections of the joints and bones. However, such diseases are cured using certain antibiotics and these leads to incidence of MRSA and MLSB resistance, and different studies give a broad view of the current situation as well as in this study it is quite clear that, resistant pattern of antibiotics are increasing in number. The prevalence of inducible resistance in *S. aureus*, including MRSA strains, is on the rise, potentially leading to the development of multiple-drug resistance patterns in the future.

From all assessments it was determined that the incidence of MLSB resistance exhibits geographical variability, influenced by infection patterns and drug utilization practices. Consequently, continuous surveillance for MLSB resistance in S. aureus is imperative, necessitating the implementation of D-tests on erythromycin-resistant isolates in all healthcare settings to prevent therapeutic shortcomings. Routine screening of these strains, appropriate

administration of clindamycin, and molecular identification and genotyping of resistant genes are critical. Clinicians should also be vigilant regarding in vitro inducible clindamycin resistance.

Chapter V

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Appendix

APPENDIX-I

Predesigned data collection sheet.

Serial No.	**Sample Receiving Date**	**Sample Id No.**	**Gender**	**Age**	**Specimen**	**Indoor/outdoor patient**
1.						
2.						
3.						
4.						
5.						
6.						
7.						
8.						
9.						
10.						

APPENDIX-II

Table 1: List of antibiotic discs, their amount and their ranges of inhibition zone [23]:

Serial no.	Antimicrobial agent	Disc code	Disc potency (µg)	Range		
				Resistance (mm)	Intermediate (mm)	Susceptibility (mm)
1.	Ampicillin	Amp	10	≤16	-	≥17
2.	Penicillin	P	10	≤28	-	≥29
3.	Azithromycin	Azi	15	≤13	14-17	≥18
4.	Trimethoprim/ Sulfamethoxazole	Sxt	25	≤13	14-18	≥19
5.	Amikacin	Ak	30	≤16	-	≥18
6.	Gentamicin	Gen	10	≤18	-	≥18
7.	Clindamycin	CD	2	≤14	15-20	≥21
8.	Ciprofloxacin	Cip	5	≤16	16-20	≥21
9.	Vancomycin	Va	30	≤17	-	≥17
10.	Doxycycline	Do	30	≤12	13-15	≥16
11.	Linezolid	Lzd	30	≤20	-	≥21
12.	Netilmicin	Net	30	≤18	-	≥18
13.	Cefoxitin	Fox	5	≤21	-	≥22
14.	Rifampicin	Rif	30	≤16	17-19	≥20
15.	Erythromycin	E	15	≤ 13	14-22	≥23

APPENDIX-III

Gram staining technique:

The technique was used for the observation of the morphological characteristics of the bacterial strains. The slide was carefully cleaned for having good staining.

The reagents required are as follows:

- Crystal violet
- Lugol's iodine
- Acetone-alcohol decolorizer
- Neutral red
- Carbol fuchsin

Methodology:

Smear preparation:

A portion of bacterial culture was extracted using a sterile needle and suspended in sterile Ringer's solution. The suspension was adequately diluted. A drop of the suspension was placed on a slide, and a thin film was created, allowing it to dry in the air.

Fixating of the smear:

The smear was fixed by gently heating the slide over a burner. After fixation, the slide was allowed to cool down before proceeding with re-staining.

1. **Primary staining:**

The dried and fixed smear was covered with crystal violet stain for 1 minute. Subsequently, it was washed with tap water.

2. **Mordanting:**

Following the washing step, the smear was covered with Lugol's iodine and left for 1 minute.

3. **Decolorization:**

Decolorization was performed using acetone-alcohol until the blue color was washed out.

4. **Counter staining:**

Following decolorization, the smear was covered with neutral red stain and left for 1 minute. Then, the slide was washed with tap water. Finally, the smear was covered with carbol fuchsin stain and kept for 30 seconds before being washed with tap water.

The slide was examined under the microscope by using oil immersion lens. Gram positive *s. aureus* was observed under microscope which appeared purple in color with cluster in shape.

APPENDIX-IV

The compositions and methods of different media used is given below:

Blood Agar Media components:

Ingredients	**Amount (g/L)**
peptone	0.5
Beef extract	0.3
Sodium chloride	0.5
Sheep blood	5
Agar	15

pH was measured 6.8.

The mixture of ingredients and distilled water was autoclaved at 121°C for 15 minutes under 15 lbs pressure. After cooling down to 50°C, 5% sheep blood was added and thoroughly mixed. The resulting mixture was then poured into sterile Petri dishes.

Samples were inoculated into 20 ml of Blood agar taken on a medium-sized Petri plate and incubated at 37°C for 24 hours. Following the 24-hour incubation period, the morphology and cultural characteristics of the colonies on the media were observed to identify and confirm the presence of *S. aureus*. The presence of a clear zone around the colonies, indicating beta hemolysis, was considered a positive result for the presence of *S. aureus.*

Mannitol Salt Agar (MSA):

Ingredients	**Amount (g/L)**
peptone	10
Mannitol	10
Lab-lemco powder	1.0
Sodium chloride	75.0
Phenol red	0.025
agar	15

Mannitol Salt Agar (MSA) is a selective and differential medium. *Staphylococcus aureus* produces yellow colonies with yellow zones due to mannitol fermentation. In contrast, other Staphylococci do not ferment mannitol and produce small pink or red colonies with no color change to the medium.

To test the sample, it was inoculated into 20 ml of MSA on a medium-sized petri plate and incubated at 37°C for 24 hours. After the incubation period, the morphology and cultural characteristics of the colonies on the media were observed to identify and confirm the presence of *S. aureus.* The appearance of a yellow color on the media was considered a positive result for the presence of *S. aureus*.

Muller Hinton Agar media:

To prepare the MHA (Mueller-Hinton Agar) base, 38 grams of the dehydrated MHA base powder were dissolved in 1000 ml of distilled water. The pH of the solution was measured and found to

be 7.4. Media was delivered and calculated at 121^{O} C for 15 minutes under 15lbs/ sq. inch pressure. After cooling to about 55^{O}C, media was poured into sterile petri dish.

For 200 samples, Several Muller-Hinton Agar (MHA) plates were prepared, each properly labeled with the corresponding samples. Autoclaved swabs were dipped into bacterial suspensions and rotated to ensure complete saturation with the suspension. Before dipping the cotton swab, the test tubes containing the bacterial suspension were vortexed. The swab was then streaked multiple times on the dried surface of the MHA plate to create a pure lawn, ensuring contact of the swab's cotton with all edges of the plate. The agar plate was rotated 90 degrees each time it was streaked to ensure even distribution of the inoculum. Finally, the plates were allowed to dry out.

Trypticase Soya broth:

Ingredients	**Amount (g/L)**
Pancreatic digest of casein	17
Papaic digest of soybean meal	3
Sodium chloride	5
Di-basic potassium phosphate	2.5
Glucose	2.5

pH was measured 7.3.

After dissolving the media, then dispensed 50ml in each sterile bottle with rubber cap and wrapped with brown paper. Autoclaving at 121^{O} C for 15 minutes under 15lbs/ sq. inch pressure was done and finally stored at 4^{O} C.

APPENDIX-V

Different biochemical tests given below:

Catalase test:

Catalase test was done in order to ensure whether the bacteria was able degrade hydrogen peroxide. As certain bacteria have the ability to produce catalase enzyme which eventually results in release of oxygen from hydrogen peroxide. To differentiate between the bacteria that produce catalase enzyme which includes *Staphylococci* from the other *Streptococci* that doesn't produce catalase.

$$2H_2O_2 \xrightarrow[\text{catalase}]{} 2H_2O + O_2 \text{ (gas bubbles)}$$

On a sterile glass slide placed inside a Petri plate a loop full of bacterial colony was placed. 1 drop of 3% H_2O_2 was added onto the microorganism by a dropper and rapid oxygen bubble formation was observed immediately. **[12] [16]**

Coagulase test:

Slide coagulase: One drop of normal saline (0.9% NaCl) was placed on a clean glass slide, with a minimum spreading one or two colonies of the test organism were taken on the glass slide and emulsified with the drop of saline from a smooth milky suspension. One drop of undiluted human plasma was added to bacterial suspension with help of a straight inoculating wire loop. Appearance of coarse clumping visible to the naked eye within 10 seconds indicates positive *Staphylococcus aureus.*

Mannitol salt agar:

Mannitol Salt agar, one of the selective medias, on which coagulase positive *S. aureus* as a result of mannitol fermentation produces yellow zones along with yellow colonies. On the other hand, other type of Staphylococci, coagulase negative ones, produces small pink or red colonies where the color of media remains same as they do not ferment mannitol. An acidic byproduct is formed when an organism is able to ferment mannitol and it causes the agar to turn yellow from phenol red present in the agar media**.**

APPENDIX-VI

MacFarlane Turbidity standard

Composition and preparation:

1% (V/V) solution of chemically pure (0.36N) H_2SO_4 and 1% (W/V) solution chemically pure (0.048M) barium chloride was prepared in nutrient broth in separate sterile flasks. Then 9.9ml of H_2SO_4 and 0.1 m; of barium chloride were added to the clear screw cap test tube and sealed. The barium sulphate suspension corresponds approximately to MacFarland standard tube no.1 with corresponding cell density of $30x10^8$ organisms/ml.

To make the turbidity standard of cell density to one half of the Macfarlane Standard tube no.1 which corresponds to the density of $1.5x10^8$ organisms/ml for determination of antibiotic sensitivity by Kirby-Bauer technique, 0.5ml of 1% (W/V) barium chloride was added to 99.5ml of 1% (V/V) H_2SO_4 (0.36N) mixed well and 5-10ml distributed in sterile screw capped teste tubes and sealed.

APPENDIX-VII

Reagents and buffers

Crystal Violet (100 ml)

To prepare the stain, 2 g of crystal violet was dissolved in 29 ml of 95% ethyl alcohol. Separately, **0.8** g of ammonium oxalate was dissolved in 80 ml of distilled water. The two solutions were then mixed together to form the stain, which was stored in a reagent bottle at room temperature.

Safranin (100ml)

To prepare the solution, 2.5 g of safranin was dissolved in 10 ml of 95% ethanol. Distilled water was then added to the solution to reach a final volume of 100 ml. The resulting solution was stored in a reagent bottle at room temperature. **Gram's iodine (300 ml)**

To prepare the solution, 1 g of iodine and 2 g of potassium iodide were added to 300 ml of distilled water. The mixture was stirred on a magnetic stirrer overnight. Subsequently, the solution was transferred to a reagent bottle and stored at room temperature.

Phosphate buffered saline (PBS)

To prepare PBS, the following steps were taken:

1. Dissolve 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4, and 2.0 g of KH2PO4 in 800 ml of distilled water.
2. Adjust the pH to 7.4 using HCl.
3. Adjust the final volume to 1 liter by adding distilled water.
4. Sterilize the solution by autoclaving.
5. Store the solution at room temperature.

10 x TBE (pH 8.3)

To prepare the buffer, 54.0g of Tris-base, 27.5g of boric acid, and 20ml of 0.5 M EDTA (pH 8.0) were combined. Distilled water was added to the mixture to bring the total volume to 500 ml. The buffer was then stored at room temperature.

Gel loading buffer

A 10x concentrated loading buffer was prepared by combining 800µl of 20% Ficoll 400, 400µl of 0.1 M EDTA (pH 8.0), 10µl of 0.25% bromophenol blue, and 200µl of 1% SDS in 590µl of distilled water. The resulting solution was stored at 4°C in 1ml aliquots.

Ethidium bromide solution

A solution of 2.5mg of ethidium bromide (Sigma, USA) was dissolved in 5 ml of distilled water, resulting in a concentration of 0.5mg/ml. The solution was then covered with aluminum foil and stored at room temperature.

MacFarlane turbidity standard

Sulfuric acid 99.5 ml, Barium chloride 0.5 ml of 1% Distilled water.

APPENDIX-VIII

Result Pictures given below:

Gram-Staining:

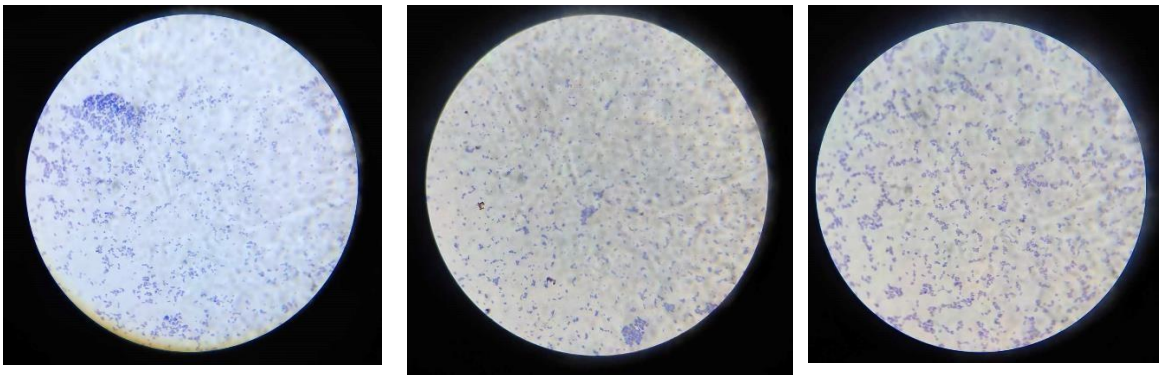

Fig: The Gram staining test yielded a positive result, indicating the presence of *Staphylococcus aureus.*

Biochemical results:

<u>Catalase test:</u>

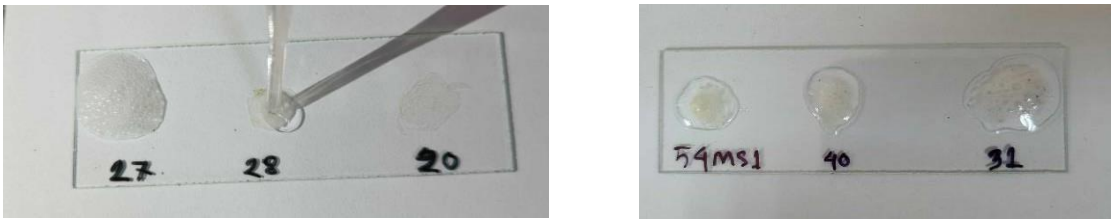

Fig: The catalase test produced a positive result characterized by the formation of bubbles, indicating the presence of catalase activity in the sample. Conversely, a negative result was observed when no bubbles were formed, indicating the absence of catalase activity.

Coagulase test:

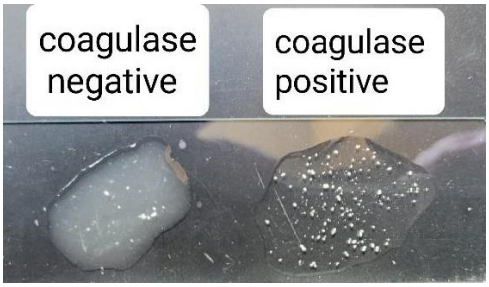

Fig: *S. aureus* positive result showing forming clumps.

Mannitol salt Agar:

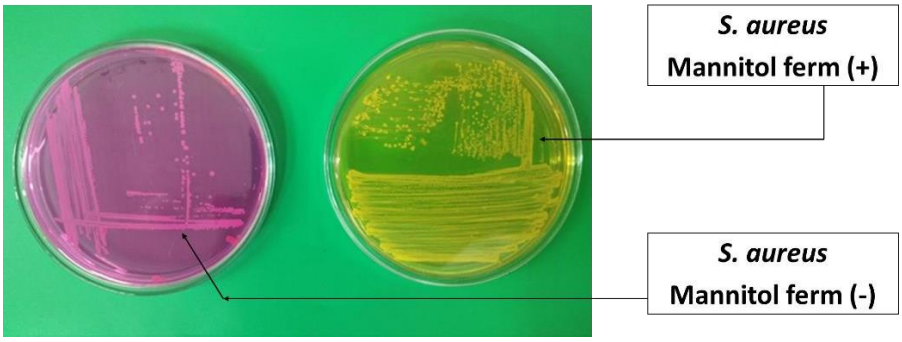

Fig 1: *S. aureus* on mannitol salt agar, showing both negative and positive results, yellow representing *S. aureus*.

Antibiotic susceptibility test:

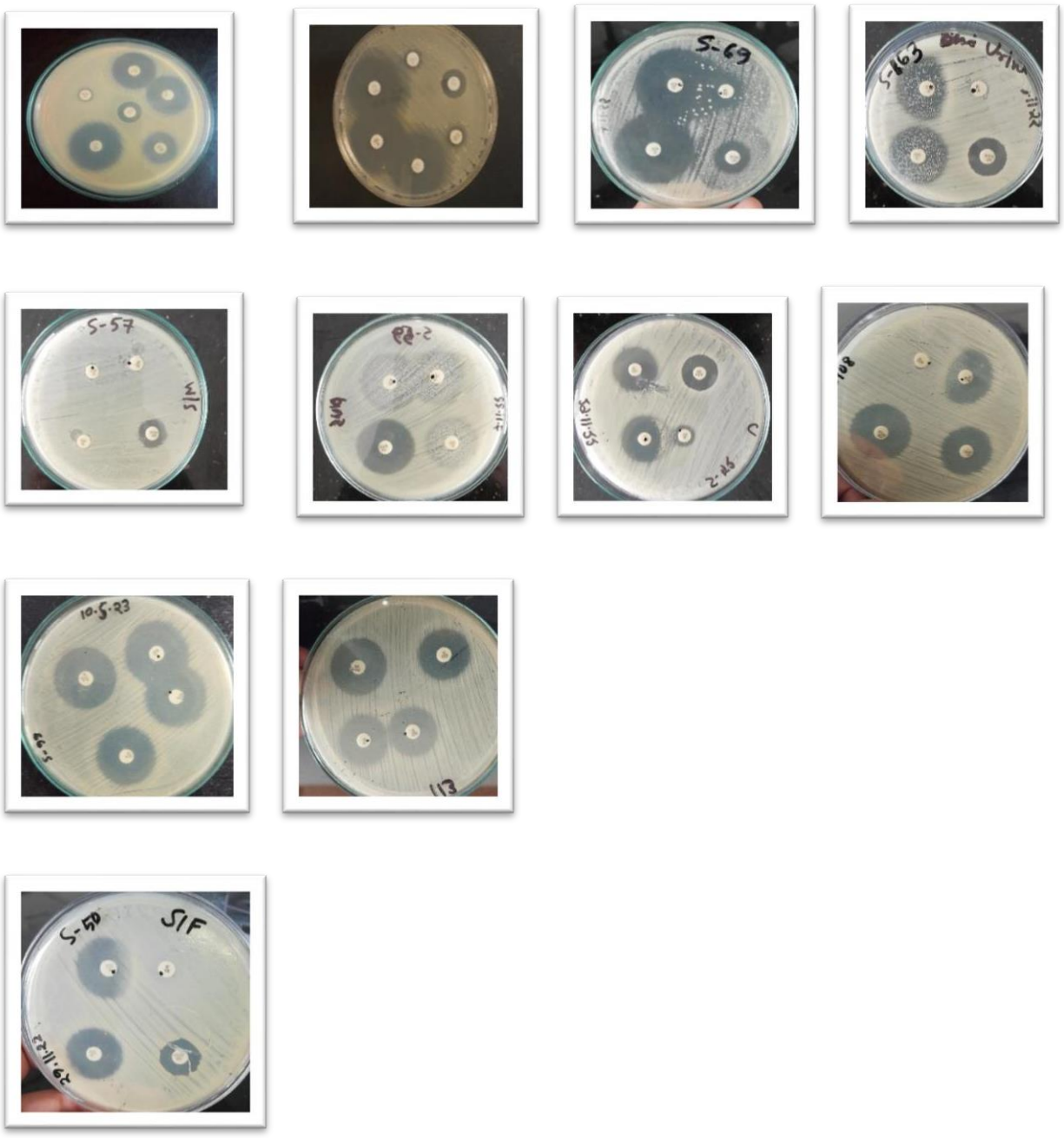

Fig: Antibiotic susceptibility test and D-test