Inducible Clindamycin Resistance Among Clinically Isolated Methicillin Resistant *Staphylococcus aureus* (MRSA) at Tertiary Care Hospitals in Bangladesh

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Masters of Science in Biotechnology

Department of Mathematics and Natural Sciences
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May, 2024

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2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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Ethics Statement

Ethical permission for the collection of isolates and using them for further research has been approved by the Institutional Review Board (IRB) of IEDCR; as a part of the AMR Surveillance conducted in the Microbiology Department. A written consent form had been signed by the patients before interviewing them for data collection. The samples and data collected are being used for research purpose only, and their names/contact information is kept confidential.

Abstract/ Executive Summary

Upon being virtually resistant to all β lactam antibiotics, Methicillin Resistant *Staphylococcus aureus* (MRSA) infections are being commonly treated by clindamycin due to various benefits. But due to the inducible Macrolide Lincosamide Streptogramin B (MLSB) phenotype, clindamycin is no longer effective. The research aimed to explore different methods to find the prevalence of MRSA and observe the inducible clindamycin resistance (iCR) in *S. aureus* isolated from clinical samples. By analyzing 45 isolates from wound and blood samples across various hospital departments, the research confirmed 31 (68.9%) MRSA strains via cefoxitin disk diffusion, including a notable prevalence of the *mecC* gene in 8 isolates. The study also reports iCR through D-tests, revealing that 64.4% of the *S. aureus* isolates exhibited inducible clindamycin resistance, with a higher occurrence in MRSA than MSSA strains. This highlights the urgency of continued surveillance and advanced molecular techniques to better understand and combat clindamycin resistance in MRSA.

Keywords: MRSA; Inducible Clindamycin Resistance; mecC; wound infections; septicemia; Bangladesh

Dedication (Optional)

I would like to dedicate my work to my parents, Dr. Prakash Saha and Anjoli Rani Das; who have supported me endlessly in pursuing the field of research.

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I am grateful to the honorable director of Institute of Epidemiology, Disease Control and Research (IEDCR), Prof. Dr. Tahmina Shirin for permitting me to conduct the research. I solemnly thank my research supervisor Prof. Dr. Zakir Hossain Habib (Chief Scientific Officer (Virology) and Departmental Head (Microbiology) for all the necessary guidance; and all the other employees of the microbiology department for assisting me to complete the work. A special appreciation to Akash Ahmed (Senior Lecturer, Brac University) for the counselling and advices on the laboratory work.

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List of Acronyms

Acronym	Full Meaning
CoNS	Coagulase Negative Staphylococci
CoPS	Coagulase Positive Staphylococci
MRSA	Methicillin-resistant Staphylococcus aureus
MSSA	Methicillin-sensitive Staphylococcus aureus
MLSBi	Inducible Macrolide Lincosamide Streptogramin B
MLSBc	Constitutive Macrolide Lincosamide Streptogramin B
iCR	Inducible Clindamycin Resistance
BORSA	Borderline Oxacillin-resistant Staphylococcus aureus
BlaZ	Beta-Lactamase gene
SCCmec	Staphylococcal Cassette Chromosome mec
PBP	Penicillin Binding Protein
CLSI	Clinical and Laboratory Standards Institute
PCR	Polymerase Chain Reaction
AMR	Antimicrobial Resistance
IEDCR	Institute of Epidemiology Disease Control and Research
IRB	Institutional Review Board
ATCC	American Type Culture Collection

MIC	Minimum Inhibitory Concentration
AST	Antimicrobial Susceptibility Testing
MS	Methicillin-sensitive
IPD	In-Patient Department
OPD	Out-Patient Department
KMCH	Khulna Medical College and Hospital
MMCH	Mymensingh Medical College and Hospital
RMCH	Rajshahi Medical College and Hospital
SOMCH	Sylhet Osmani Medical College and Hospital
UAMCH	Uttara Adhunik Medical College and Hospital

Chapter 1

Introduction

1.1 Literature Review

1.1.1 *Staphylococcus aureus* infections

Staphylococci, gram-positive cocci, are commensal bacteria present on the skin and mucosal membranes of mammals and animals [1]. Depending on the ability to produce coagulase enzyme, the genus is being divided into two major groups: Coagulase negative staphylococci (CoNS) and coagulase-positive staphylococci (CoPS) [1]. There are two types of coagulase enzymes produced by *Staphylococcus aureus* strains; bound and free. The bound coagulase or the clumping factor is attached to the cell wall and catalyze the conversion of fibrinogen to fibrin fibers but clumping of bacteria is not observed in this case. Free coagulase reacts with globulin plasma factor (coagulase-reacting factor) to form a thrombin like enzyme which converts fibrinogen to insoluble fibrin; and the clumping of cells can be observed in the tube [2]. A very familiar member of the CoPS is *Staphylococcus aureus*; a potent epidemic pathogen causing both nosocomial and community-acquired infections around the globe [3]. About 20-40% of the human population has *S. aureus* as commensal bacteria on their nasal mucosa [4]. However, it is also present on the skin as normal flora which can cause infections once the cutaneous immune defenses fail or by an open wound [5]. Infections caused by *S. aureus* can be classified into four groups: the carrier state, infections of the skin, infections of the deep tissues, and septicemia. The carrier state shows no morbidity but infections by the invasion of skin and deeper organs leads to increased cases of infection and deaths [6]. The bacteria invade the skin barrier is by producing epidermolytic toxins that damages the membrane, along with other extracellular components like toxic shock syndrome and

pyrogenic toxins. And exoenzymes such as coagulase and thermostable nuclease are widely used as identification markers for *S. aureus* [7].

Skin infections such as endocarditis, abscess, skin and soft tissue infection (SSTI) pleuropulmonary and device related infections are some of the most common infections caused by the opportunistic bacteria [4][5]. Staphylococcus aureus bacteremia (SAB) has been manifesting the industrialized world since the earliest 1950s which causes a wide range of complications causing increased morbidity of 20-40% [3]. Apart from a high mortality rate, SAB imposes a considerable strain on healthcare systems in terms of expenses and resources due to prolong hospital stays [8]. SAB can lead to a systematic inflammatory response, a development of sepsis which is a combination of inflammation and immunosuppression. The response sets off a series of mechanisms that damages the endothelium lining of blood vessels, which may lead to endocarditis [9].

1.1.2 Emergence of Methicillin Resistant *Staphylococcus aureus* (MRSA)

For the last two decades, the overall rate of SAB has become steady but the prevalence of Methicillin-resistant *S. aureus* (MRSA) has flourished all over the world [10-16]. MRSA is a highly infectious strain of *S. aureus* which is very difficult to treat with common curative drugs. Antimicrobial treatment for *S. aureus* includes β-lactams, including penicillin, methicillin, cloxacillin, oxacillin, flucloxacillin, and dicloxacillin [17]. Methicillin was first introduced in the late 1960s to treat β-lactamase producing staphylococcus and was widely used [18]. But later due to being physiologically toxic to human, its production was discontinued; however, methicillin resistant strains were discovered soon after its launch [19]. And more stable forms of penicillin were introduced for treatment such as oxacillin, flucloxacillin and dicloxacillin [19]. Although methicillin is no longer being produced or

used, the term Methicillin-resistant Staphylococcus aureus (MRSA) still persists and refers to being resistant to virtually all β-lactam antibiotics [19].

The methicillin resistance is mediated by acquired mobile genetic element-staphylococcal cassette chromosome (SCC*mec*) carrying the *mecA* gene which encodes for PBP2A [20]. Penicillin binding proteins (PBP) is the targeted protein involved in cell wall biosynthesis and β-lactam antibiotics target this protein to inhibit peptidoglycan crosslink formation to cause cell lysis [20]. PBP2a/PBP2 is a structurally altered PBP which has lower affinity for β-lactam antibiotics, thus making the strain resistant [20]. The exogenous acquisition of the SCCmec is how methicillin-susceptible *S. aureus* becomes resistant to methicillin, and the mobile genetic element is transmissible among staphylococcal species [21]. The origin of mecA gene is still not clarified, but it is considered that *Staphylococcus sciur*i, a coagulase negative staphylococcus, is the evolutionary precursor of the *mecA* gene and have a ubiquitous presence among its species [22][23]. It is speculated that the SCCmec was formed by adopting *the S.fleurettii mecA* gene and its surrounding chromosomal region [22]. The SCCmec consists of two essential components: the *mec* gene complex which contains the *mecA* gene responsible for PBP2' and the *ccr* gene complex which encodes for site-specific recombinases [24]. Other components are characteristic nucleotide sequences of inverted repeats or direct repeats at both ends, and an integration site sequence (ISS) at the 3'-end of orfX [24]. There is a nucleotide diversity of *ccr* genes among various species of Staphylococci but *mecA* genes in SCCmec are near to identical regardless of the species carrying them [25]. Moreover, based on the combination of types of five classes of *mec* and eight classes of *ccr* gene complexes, the SCCmec is classified into 12 types till date [25][26].

Recently, a *mecA* gene homologue, *mecC* was identified which is 69% identical to *mecA* at DNA level and 63% identical at the amino acid level, and encoding for PBP2a/PBP2 [27]. It was initially called $mecA_{LGA251}$ and later was given the name *mecC* to distinguish from *mecA*

[28]. It has only been discovered recently, but it has been causing infections to human since the last 35 years [19]. Thus, only detecting *mecA* gene via PCR or doing a PBP2a/2′ slide agglutination is not enough to detect Methicillin resistance. Despite of being homologues, *mecC* MRSA produces a different antibiotic susceptibility result compared to *mecA* MRSA when assayed by Vitek 2 system [29]. When it comes to PCR, universal *mec* primers must be used which can amplify both *mecA* and *mecC* genes or *mecC* specific primers should be used. Otherwise, various MRSA strains will be misdiagnosed as methicillin sensitive if *mecC* gene is not detected.

1.1.3 Inducible Clindamycin Resistance (iCR)

The macrolide-lincosamide-streptogramin B (MLSB) group of antibiotics has been a common option to treat MRSA, among which clindamycin is mostly preferred for its good pharmacokinetic properties [30]. Clindamycin is also cost-effective, available for both oral and intravenous administrations. But clinicians have now started to feel discouraged to include clindamycin in the treatment regimen due to the emergence of inducible clindamycin resistance [31]. Macrolide (erythromycin) resistance occurs by two mechanisms which are using energy dependent efflux pumps and by modifying the drug binding [31]. The gene macrolide-streptogramin resistant, msr(A) gene in staphylococci encodes for the energy-dependent efflux pump that expels macrolides from the cell before it can bind to its targeted site [32]. But this mechanism is not responsible for creating resistance against lincosamides and group B streptogramins [32]. By the second mechanism, methylation of 23s rRNA-binding site occurs, a site which is shared by the other classes of antibiotics as well (lincosamides and streptogramin B) [33]. This is how erythromycin resistant staphylococcus develops resistance for clindamycin. Although this resistance mechanism is encoded by erythromycin resistance methylase *ermA* and *ermC* genes [34], the inducible resistance can be detected phenotypically only by D-test, following CLSI guidelines. However, clindamycin

resistance can be developed both constitutively (MLSBc) or be induced by other macrolides (MLSBi).

1.2 Research rationale

The aim of the study was to find the prevalence of Methicillin Resistant *Staphylococcus aureus* (MRSA) and test for inducible clindamycin resistance in *Staphylococcus aureus* isolates collected from patients with cases of wound infections and septicemia. Although there are several studies on MRSA but there is little data on the MLSB phenotype in Bangladesh. Thus, it is essential to investigate the occurrence of inducible clindamycin resistance in order to bring changes in the diagnostic testing procedures in this country. Because treatment failure may occur if only constitutive clindamycin resistance is assessed. Furthermore, molecular detection of MRSA by PCR must include testing for *mecC* gene of isolates showing phenotypically resistance to β-lactams but negative for *mecA* gene. This will help to cancel out the ambiguity make sure phenotypically tested MRSA strains are not being disregarded as sensitive or ambiguous.

These patients have received treatment from 2019 to 2021 from five different hospitals across Bangladesh. And the isolates had been collected as per the case definition appointed by the AMR Surveillance program of the microbiology department at the Institute of Epidemiology, Disease Control and Research (IEDCR). After confirming methicillin resistance among the *S. aureus* isolates by both Kirby Bauer Disk Diffusion and PCR, isolates were also tested for inducible clindamycin resistance by D-test. Then comparison has been made between constitutive clindamycin resistance and resistance induced by erythromycin. The results of the study shall encourage testing for inducible clindamycin resistance before the prescription of clindamycin for MRSA-infected patients. And the study will influence considering

detection of *mecC* gene apart from *mecA* resistant gene for the confirmation of MRSA strains.

Chapter 2

Methodology

2.1 Sample Collection

The study has been conducted using the isolates collected under AMR Surveillance of the Microbiology Department at IEDCR. Among the surveillance sites, five hospitals were selected and isolates of wound and septicemia cases dating from 2017 to 2022 were tested initially for the study. These sites were Khulna Medical College and Hospital (KMCH), Mymensingh Medical College and Hospital (MMCH), Rajshahi Medical College and Hospital (RMCH), Sylhet Osmani Medical College and Hospital (SOMCH) and Uttara Adhunik Medical College and Hospital (UAMCH). A total of 78 isolates were tested and only gram-positive organisms were further identified to be included in the study.

Sample Type	**Sample Size (Number of isolates tested)**	**Type of bacteria**	
		Gram Positive	**Gram Negative**
Wound Swab	60	47	13
Blood	18	8	10
Total	78	55	16

Table 1: Sample type and size.

2.1.1. Case Definitions

As per AMR Surveillance protocol, the case definitions for inclusion criteria are:

a) Wound infection & Abscess

Patients attending OPD or hospitalized with signs and symptoms of any infected wound including surgical wounds

i. wound not healing within last 3 days for gynecology to 10 days for general surgery and 20 days for orthopedic surgery
ii. pain and swelling surrounding the wound
iii. discharges of yellow/green-colored pus or any abscess.

b) Septicemia

Systemic inflammatory response syndrome (SIRS) caused by documented infection is known as septicemia.

SIRS is defined by the presence of two or more of followings:

- Respiratory rate >20/min
- Heart rate >90/min
- White blood count >12 $\times 10^9$/L or <4 $\times 10^9$/L
- Temperature >38.0°C or <36.0°C
- *Pa*CO2 <4.3 kPa (<32 mmHg) or ventilated

2.2 Identification and confirmation of *Staphylococcus aureus* species

2.2.1. Sample collection at sites

Wound Swab:

- The wound is cleansed by removing excess debris and surrounding base is wiped by alcohol pad to sterilize.
- A sterile culture swab damped with normal 0.9% sodium chloride is prepared.
- A small area of 1 cm of clean viable tissue is identified and the swab is taken from the clean tissue. The exudate, pus, or necrotic tissue needs to avoided as they contain microflora.
- The swab is inserted into a sterile container or transport media.

Blood Culture Sample:

- A site for venipuncture is selected.
- Two blood culture bottles are required for aerobic and anaerobic.
- A volume of 8-10 cc blood is drawn from the patient using butterfly Safety E-Z Collection Set.
- Put the blood in the bottles.
- The bottles are kept in the blood culture machine.
- The machine indicates if it is culture positive.
- Subculture is done to appropriate non-selective media.

2.2.2. Culture and Gram Staining

Nutrient media and blood agar were used to culture the isolates and then gram stained using the standard procedure. Staphylococci appears to be purple and cocci-shaped in clusters under the light microscope.

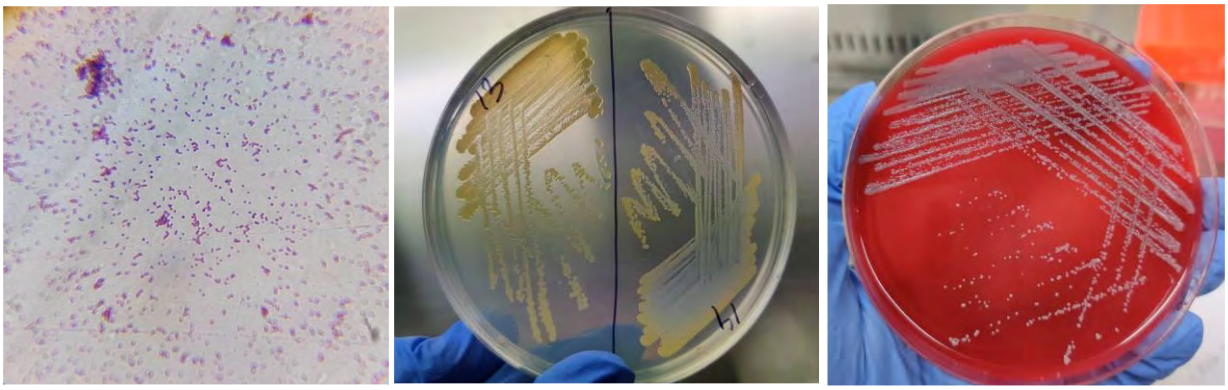

Figure 1: Gram staining showing purple cocci in clusters (left), golden yellow colonies on nutrient agar indicating S. aureus (middle), and CoNS on blood agar.

2.2.3. Subculture on Mannitol Salt Agar (MSA)

Suspected colonies were inoculated on Mannitol Salt Agar (MSA) which is a selective media for *S. aureus*. A 7.5% NaCl in the media encourages the growth of halophilic bacteria and inhibits the growth of bacteria other than staphylococci. So, *S. aureus* colonies appear to be yellow forming yellow zones due to the fermentation of mannitol.

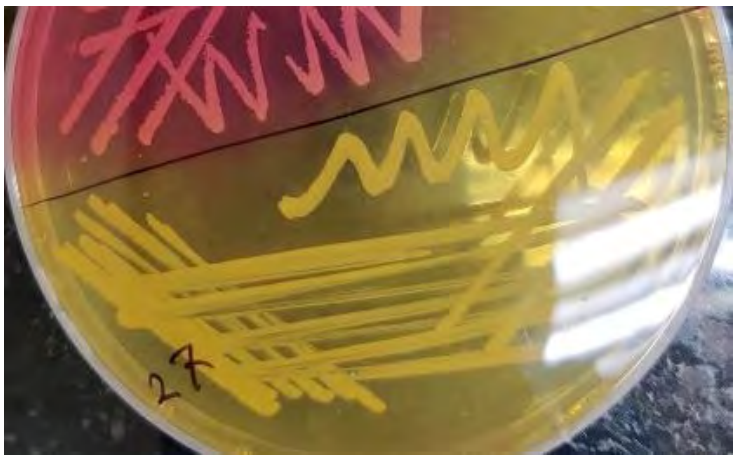

Figure 2: Yellow colonies in contrast to red colonies.

2.2.4. Catalase Test

Using a sterile cotton swab, the colonies were picked up and dipped in 3% hydrogen peroxide solution on a slide to observe effervescence. Being a facultative aerobe, *S. aureus* produces catalase enzyme.

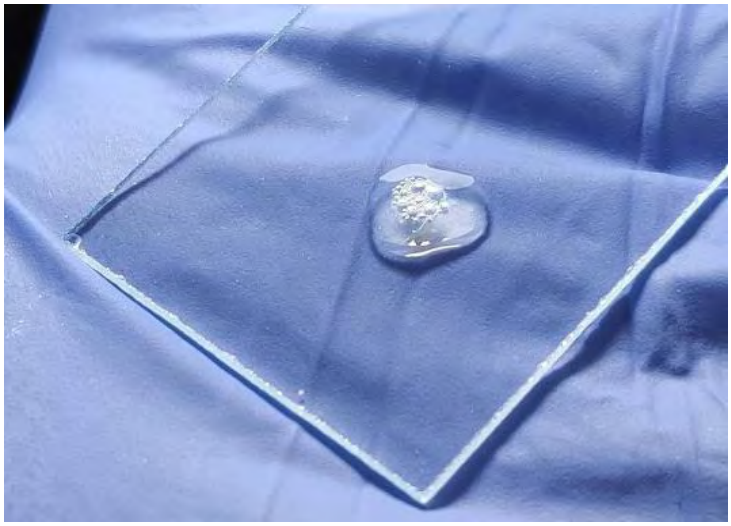

Figure 3: Effervescence during catalase test.

2.2.5. Coagulase Test

Tube coagulase method was followed using EDTA treated human plasma. Two or three isolated colonies were selected and emulsified in 0.5 ml of plasma. The tubes are observed for clot formation after 4-hour incubation at 37ºC. If there was no clot formation within 4 hours, the tubes were kept at room temperature for 24 hours. For negative control, plasma without inoculation was used. And for positive control, a reference strain of *S. aureus* (ATCC 25923) was used. Isolates that showed clot of the plasma were confirmed as *S. aureus* and those without clot were defined as CoNS.

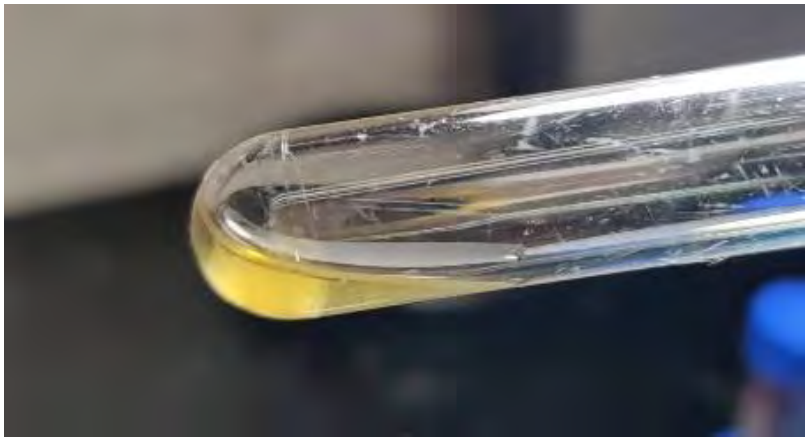

Figure 4: Clot formation during coagulase test.

2.2.6. Using VITEK 2 Automated System for Further Confirmation of the Identification of *Staphylococcus aureus*

The VITEK 2 microbial testing system is a high-throughput platform that can identify the organism and provide its antibiotic susceptibility chart. It is an effective method of detecting multi-drug resistant bacteria such as MRSA.

Colonies from a fresh culture on nutrient medium was inoculated in a 0.45% NaCl solution and adjusted to a 0.5 McFarland standard concentration. As all the isolates were gram positive, ID-Gram Positive (bioMérieux) cards were used according to the manufacturer's recommendations. The inoculated solutions were then loaded with the GP cards into the VITEK 2 system. The plastic card is manufactured to have 64 in-built microwells, containing dehydrated substrates for the biochemical reactions. As the cards are inserted into the filling chamber, the negative pressure causes the bacterial suspension to be sucked into the wells and the substrates are rehydrated. Then the cards are put into the incubator chamber to be analyzed, the pH change or the color change is detected by an optical sensor and the combination of each reaction dictates the identification of the organism accordingly.

bioMérieux Customer: IEDCR

Microbiology Dept.
Microbiology Chart Report

Printed October 10, 2023 12:34:48 PM BDT

Patient Name: MMCH, WW
Location:
Lab ID: 910202324

Patient ID: WW03210020
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Staphylococcus aureus
BP Infection Site:

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:** 3.95 hours	**Status:** Final
Selected Organism	99% Probability **Staphylococcus aureus**	
	Bionumber: 030402066763231	
ID Analysis Messages		

Biochemical Details																	
2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	+	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	-	39	lLATk	+	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	-	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Figure 5: Chart report generated by VITEK 2 showing the detection of S. aureus with 99% probability.

2.3 Detecting Methicillin Resistant *Staphylococcus aureus* (MRSA) by Kirby Bauer Disk Diffusion method

Fresh culture was used to form a bacterial suspension equivalent to 0.5 McFarland standard. Using a sterile cotton swab, a bacterial lawn was created on Mueller Hinton agar and a 30µg cefoxitin disk was placed. The plates were incubated for 24 hours at 37°C. According to Clinical & Laboratory Standard Institute (CLSI 2021) guidelines, the zone diameter for resistance is ≤ 21 mm and for susceptible is ≥ 22 mm. The reference strain *S. aureus* ATCC 25923 was used as a negative control.

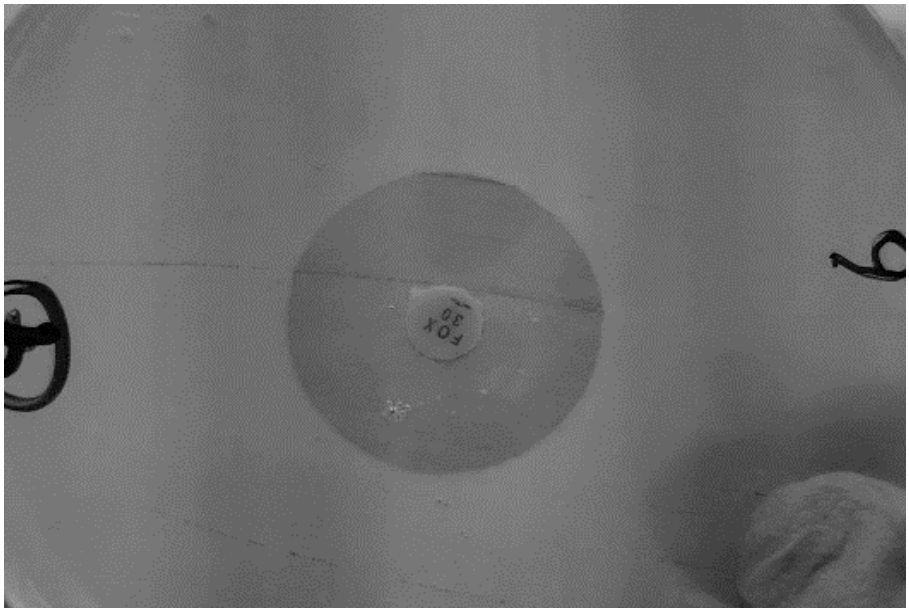

Figure 6: Disk diffusion method using cefoxitin.

2.4 Detection of inducible clindamycin resistance (iCR) by double disk diffusion method.

The isolates were also tested with erythromycin and clindamycin separately to determine their constitutive resistance. And for the D-test, erythromycin and clindamycin disks were placed in close proximity (15mm to 26mm) on MHA, known as a double disk diffusion method. Formation of a D-zone around the clindamycin disk indicates inducible clindamycin resistance. Isolates which were susceptible to clindamycin but is D-test positive are considered to show the inducible MLSB phenotype. All the susceptibility tests were done according to the guidelines mentioned in the 31st Edition of CLSI M100 supplement booklet.

Antibiotic Disk Name	Zone Diameter Interpretation (mm)		
	Sensitive	Intermediate	Resistant
Erythromycin	≥ 23	14-22	≤13
Clindamycin	≥21	15-20	≤14
Cefoxitin	≥ 22 mm		≤ 21 mm

Table 2: Interpretation of cefoxitin, erythromycin, and clindamycin zone diameters in S. aureus

D test Positive **D test Negative**

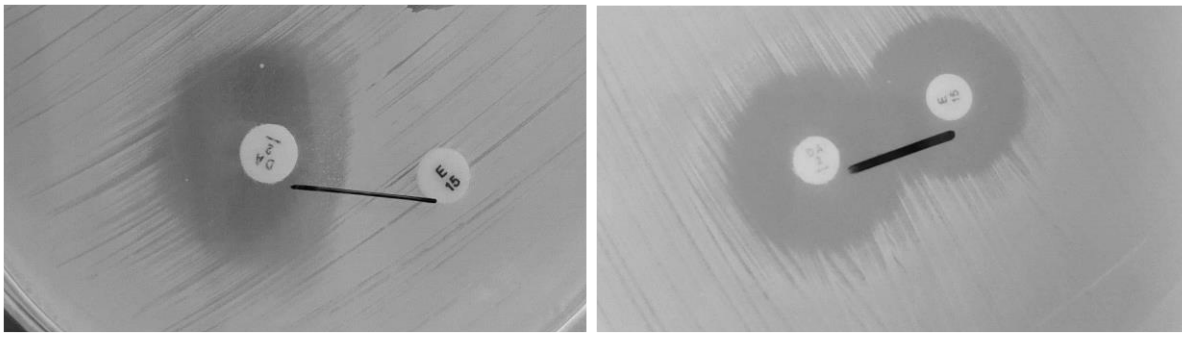

Figure 7: A positive Double Disk Test (D test) shows flattening of the zone of inhibition of clindamycin in close proximity to erythromycin while a negative test shows a regular zone.

2.5 Molecular Identification by PCR

Conventional PCR was done to further confirm the identity and detect resistant genes of the cefoxitin resistant or phenotypically confirmed MRSA strains.

2.5.1. Chromosomal DNA extraction by Boiling Method

The DNA was extracted using 2 mL nuclease free water to dissolve 2-3 isolated colonies from a non-selective media. Then the solutions were boiled at 95°C for 15-20 mins and

centrifuged at 5000 rpm for 10 minutes. After centrifugation, the supernatant was collected carefully and transferred to a sterile Eppendorf.

2.5.2. PCR for the identification of *Staphylococcus aureus* and resistant genes.

The identification of the phenotypically confirmed MRSA isolates as *S. aureus* was done by the PCR of *S. aureus* specific *nuc1* gene that encodes for the thermonuclease enzyme. And to confirm the phenotypic methicillin resistance, *mecA* was targeted initially. And for isolates in which *mecA* was not detected, a homologue of *mecA* known as *mecC* gene was targeted. All the information about the primers' sequence and the cycling conditions are given in Table 2.

A 25.0µl PCR mixture was prepared using 2.5 µl of nuclease free water, 2.5 µl of forward primers, 2.5 µl of reverse primers, 12.5 µl of PCR mastermix solution and 5 µl of genomic DNA. The PCR was conducted using an AB Applied Biosystem-2720 Thermal Cycler PCR machine.

Gene	Forward Primer	Reverse Primer	Amplicon size (bp)	Conditions	Number of Cycles	Reference
Nuc1	5'-GCGATTGATGGTGGATACGGT-3'	5'-AGCCAAGCCTTGACGAACTAAAGC-3'	279	94°C for 5 mins,94°C for 30s, 55°C for 30s,72°C for 60s, 72°C for 5 min	30	35
mecA	5' TCCAGATTACAACYYCACCAGG-3'	5' CCACTTCATATCTTGTAACG-3'	162	94°C for 15 mins,94°C for 30s, 55°C for 30s,72°C for 60s, 72°C for 5 mins	40	36
mecC	5'-GAAAAAAAGGCTTAGAACGCCTC	5'-GAAGATCTTTTCCGTTTTCAGC-3'	138	94°C for 15 min,94°C for 30s, 59°C for 1	40	37

	-3’			minute,72°C for 10 mins, 72°C for 10 mins		

Table 3:Description of oligonucleotide primers used in the amplification of nuc1, mecA and mecC genes.

2.5.3. Gel Electrophoresis for visualization of amplified genes

A 1.5% agarose gel was prepared using 1xTBE buffer and ethidium bromide (EtBr) dye was added for staining. 5 µl of the PCR product and 100bp ladder were loaded into the wells and run for 1 hour at 100V. The gel was then visualized under an UV transilluminator.

Chapter 3

Data Interpretation and Presentation.

Data was analyzed using software such as SPSS and DATATab, by operating chi-square test of independence to assess the association between variables. If p is less than or equal to 0.05, their association is considered statistically significant. And the charts and tables were generated by MS Excel and MS word.

3.1. Isolation and identification of Staphylococci from clinical samples.

Organism Isolated	**Wound swab**	**Blood**	**Total**
Staphylococcus aureus	42	3	45
Staphylococcus sciuri	4	0	4
Staphylococcus saprophyticus	1	0	1
Staphylococcus xylosus	0	5	0
Total	47	8	55

Table 4:VITEK02 results of Staphylococci organisms from blood and wound swab samples

A total of 45 out of 55 gram-positive isolates were confirmed as *S. aureus* by biochemical tests, VITEK02 and molecular methods. While 42 isolates from wound infections were identified as *S. aureus*, 3 cases of septicemia have been confirmed. The rest of the isolates were identified as coagulase negative *Staphylococci* (CoNS): *S. sciuri*, *S. saprophyticus* and *S. xylosus*. As these members of CoNS are common part of the normal skin microflora of humans and animals, they have been dismissed as contaminants.

3.2. Screening of MRSA by Cefoxitin Disk Diffusion method.

Out of 45 isolates confirmed as *S. aureus*, 33 (71.7%) isolates were methicillin resistant and 13(28.3%) were sensitive. Overall, the percentage of methicillin resistant strains is greater compared to methicillin sensitive and all the 3 isolates from blood samples are methicillin resistant.

Sample Type	**No. (%) of MRSA isolates**	**No. (%) of MSSA isolates**	**Total No. (%) of Isolates**
Wound Swab	28 (66.7)	14 (33.3)	42 (93.3)
Blood	3 (100)	-	3 (6.67)
Total	31 (68.9)	14 (31.1)	45

Table 5: MRSA screening of S. aureus from clinical samples.

3.3. Age wise distribution of patients infected with MRSA and MSSA.

Age Group	MRSA (n=31)	MSSA (n=14)	Total (n=45)	P value
0-9	4	1	5	0.0256
10-19	4	1	5	
20-29	4	1	5	
30-39	10	1	11	
40-49	3	6	9	
50-59	1	3	4	
60-69	5	0	5	
70+	1	0	1	

Table 6: Age distribution of patients infected with MRSA and MSSA.

The average age of patients included in the study is approximately 34.68 years, or 35 years. According to the results of this study, patients aging from 30-39 years were mostly affected with MRSA. The p-value has been calculated to be 0.0256 by chi-square test suggests a correlation between age and being infected with MRSA.

3.4. Gender wise distribution of patients infected with MRSA and MSSA.

Sex	MRSA (%) n=31	MSSA (%) n=14	Total No. (%) n=45
Female	19 (76)	6 (24)	25 (54.3)
Male	13 (65)	7 (35)	20 (43.5)

Table 7: Gender distribution of patients with MRSA and MSSA infections.

Among 45 cases, 25 (54.3%) were female and 20 (43.5%) were male patients from whom samples were collected. The findings also show that more female patients were infected with MRSA than male.

3.5. MRSA and MSSA infections in relation to the type of patients.

Patient Type	MRSA (%) n=31	MSSA (%) n=14	Total No. (%) n=45
In-Patient Department	25 (69.4)	11 (30.6)	36 (80)
Out-Patient Department	7 (77.8)	2 (22.2)	9 (20)

Table 7: Number of MRSA and MSSA isolates collected from in and out patients.

From a total of the 45 isolates of *S. aureus*, most isolates were methicillin resistant and collected from the in-patient rather than out-patient department.

3.6. Distribution of MRSA and MSSA strains according to the types of departments in the hospitals.

Department	MRSA n=31	Non-MRSA n=14	Total n=45
ICU	1	0	1
Medicine	6	3	9
Surgery	25	10	35

Table 8: MRSA and MSSA isolates collected from patients belonging in different hospital departments.

The graph below portrays the number of MRSA and MSSA cases within the three departments and the type of treatment received. The findings of the study show that there is an excessive number of MRSA cases in the surgical ward and among in-patients with wound infections.

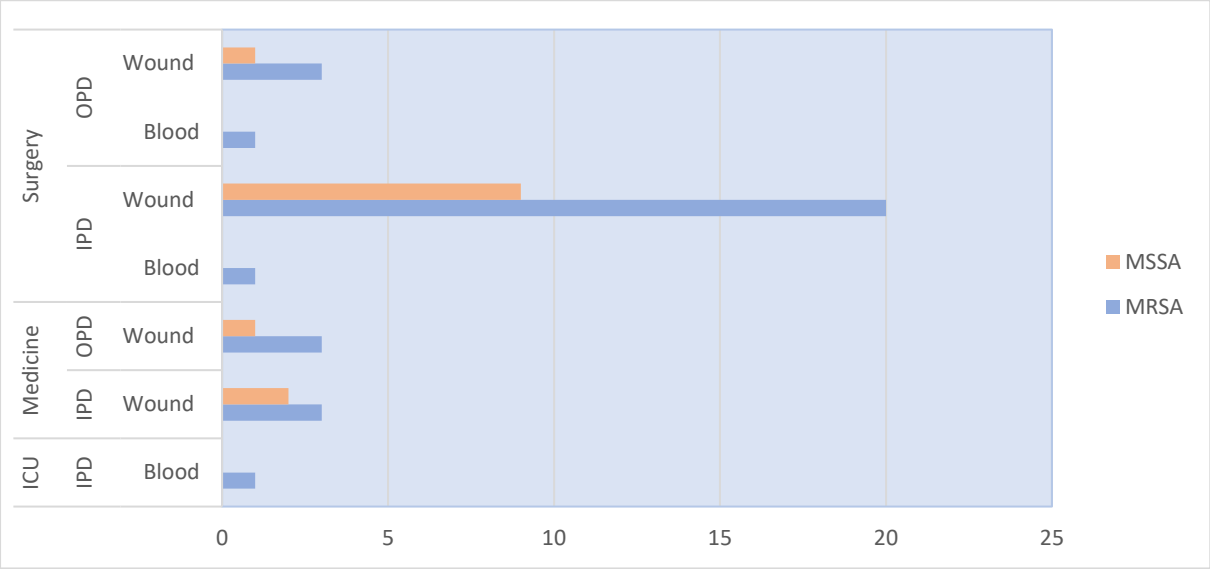

Figure 8: Graph showing MRSA strains isolated from wound and blood samples of patients in different departments.

3.7. Molecular detection of *S. aureus* specific *nuc1* gene, *mecA* and *mecC* in MRSA strains.

Cefoxitin resistant *S. aureus* strains were further tested to detect *nuc1* and resistant genes by conventional PCR. Among 32 MRSA isolates, there were one isolate from blood with neither *mecA* nor *mecC*. Majority of the resistant strains have the *mecA* gene rather than *mecC*.

Sample Type	*Nuc 1*	*mecA*	*mecC*	*mecA* and *mecC* negative
Wound Swab	28	22	6	-
Blood	3	-	2	1
Total	31	22	8	1
		31		

Table 9: Detection of nuc1, mecA, and mecC genes among MRSA strains.

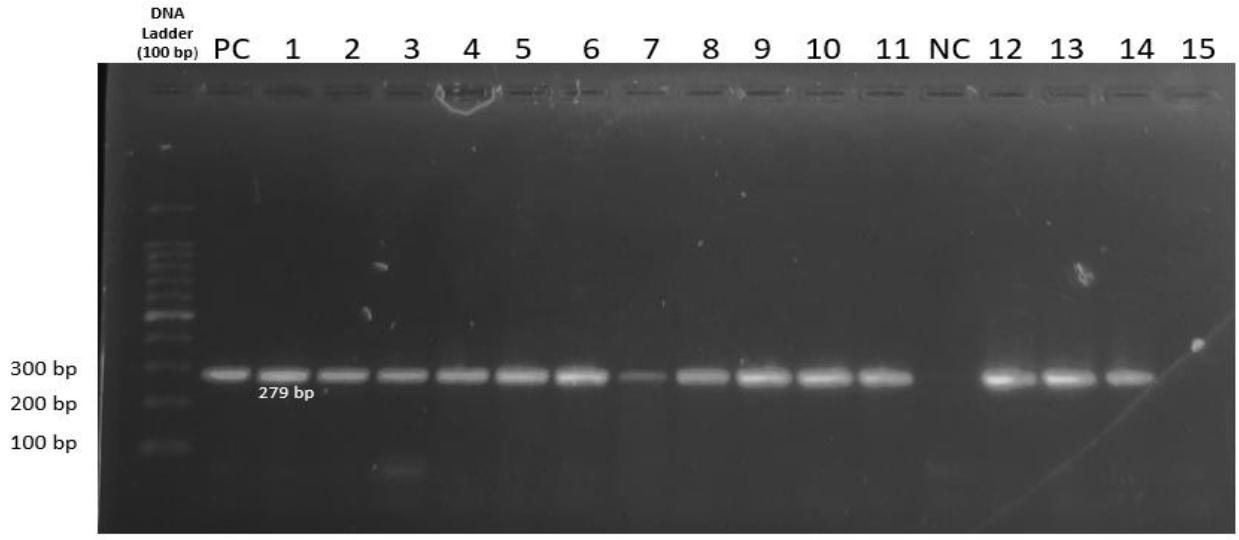

Figure 9: Visualization of nuc1 gene amplification by gel electrophoresis.

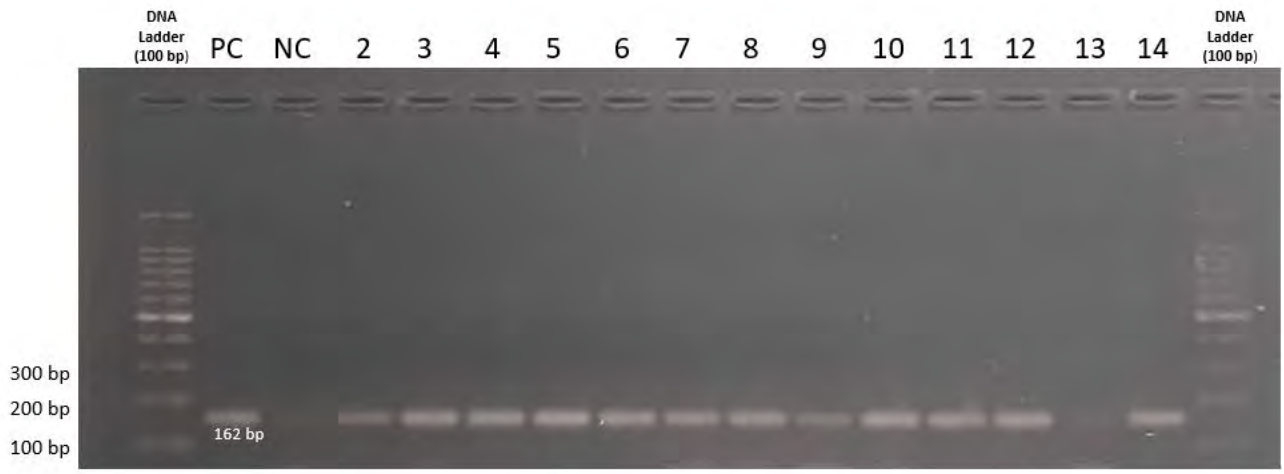

Figure 10: Visualization of mecA gene amplification by gel electrophoresis.

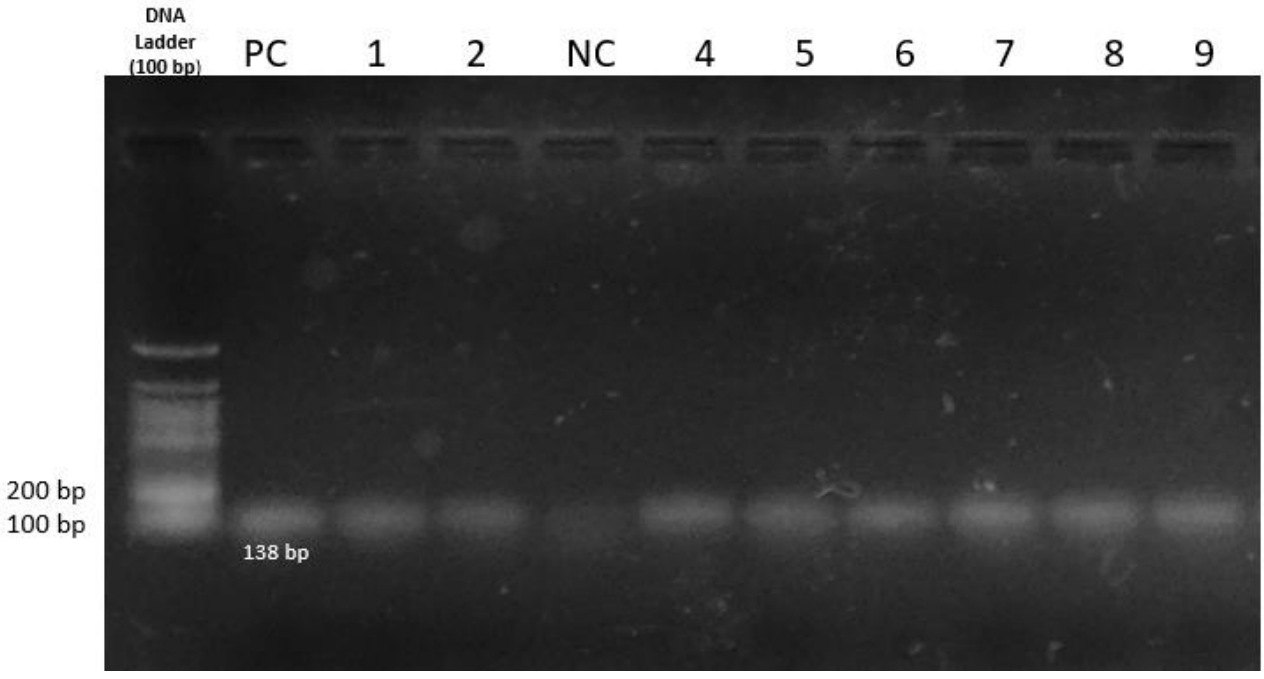

Figure 10: Visualization of mecC gene amplification by gel electrophoresis.

3.8. Inducible Clindamycin Resistance (iCR) in *S. aureus*

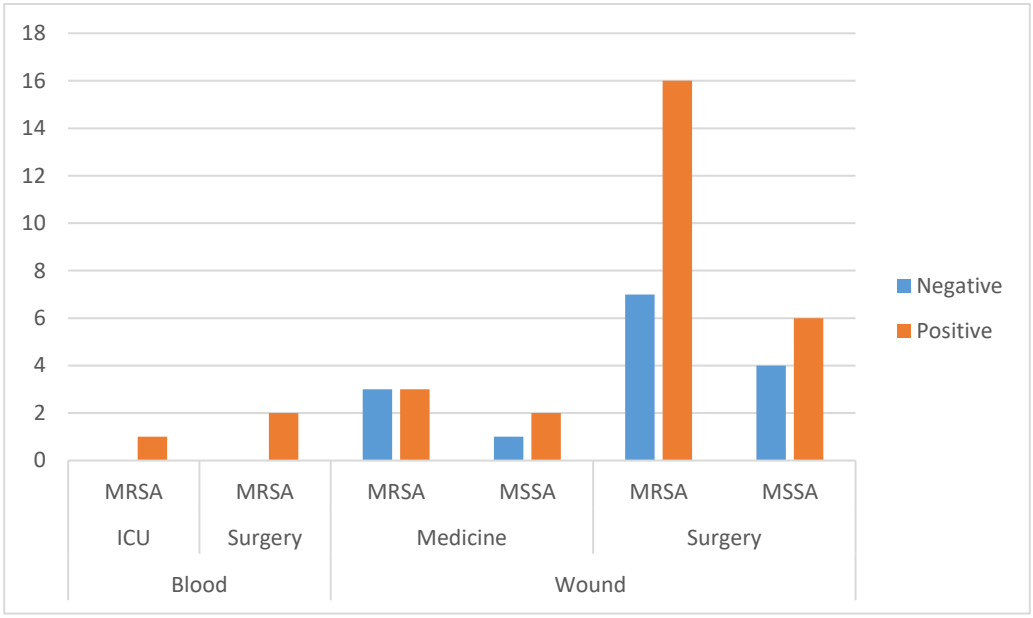

Figure 11: Observation of inducible clindamycin resistance in MRSA and MSSA isolates in the three departments.

About 29 (64.4 %) out of 45 isolates show inducible clindamycin resistance (iCR) after performing the D-test. The prevalence of iCR was the highest among the patients with post-surgical wound infections.

Phenotype	**No. (%) of MRSA isolates**	**No. (%) of MSSA isolates**	**Total (%)**
No resistance ER-S, CL-S	9 (29.0)	5 (35.7)	14 (31.1)
Constitutive resistance ER-R, CL-R	1 (3.23)	1 (7.14)	2 (4.44)
MLSBi/iCR ER-R, CL-S D+	21 (67.7%)	8 (57.1)	29 (64.4)
MS ER-R, CL-S D-	0	0	0
Total	31	14	45

Table 10: CL, clindamycin; ER, erythromycin; D+, D-test positive; D–, D-test negative; S, Sensitive; R, Resistant.

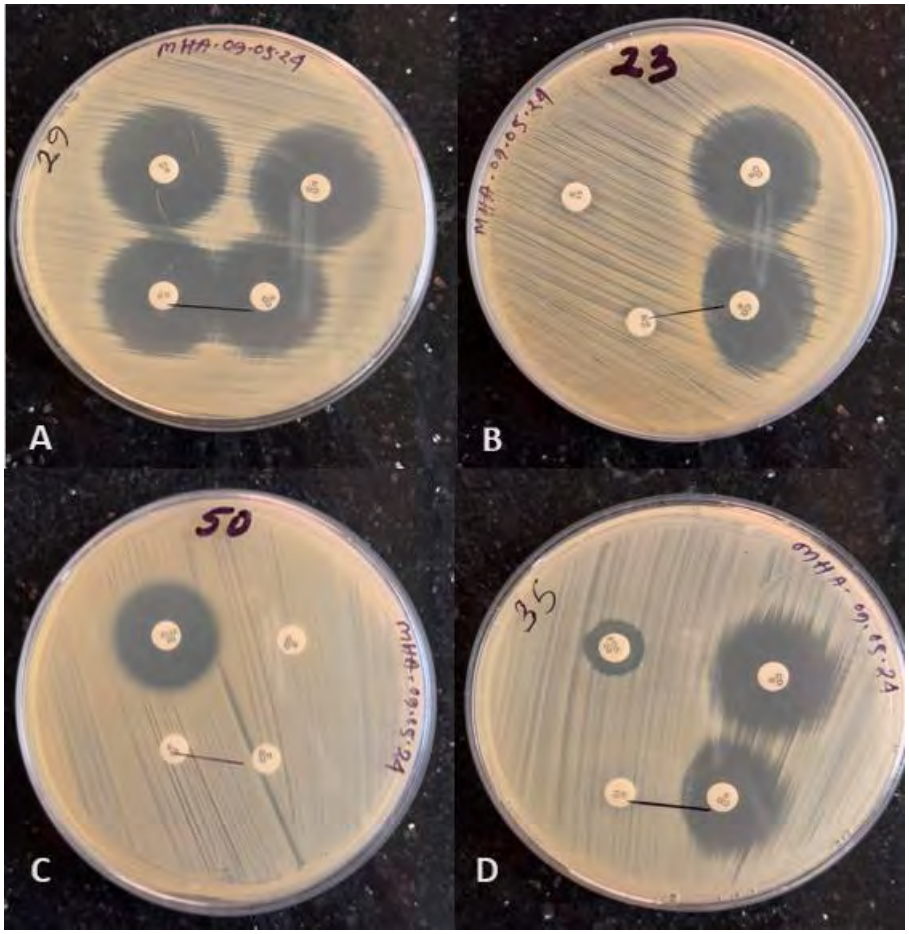

Figure 12: Results of D-test showing different phenotypes of clindamycin resistance.

E, Erythromycin; CD, Clindamycin; FOX, Cefoxitin. In picture A, absence of iCR is detected by a regular zone around the clindamycin disk. Picture B shows a positive D-test indicated by complete resistance around E and flattening of zone around clindamycin. The same disks, when placed apart more than 26mm, showed regular circular zones. Picture C depicts constitutive resistance of both erythromycin and clindamycin with a 6mm zone diameter. Lastly, picture D is an example of the orientation of the disks which can be used to detect MRSA strains with iCR. It consists of a D-test, single clindamycin disk for constitutive resistance, and a cefoxitin disk for MRSA screening.

Chapter 4

Discussion

While there are numerous studies on the prevalence of *S. aureus* and MRSA infections in both hospital and community settings in Bangladesh, the phenomenon of inducible clindamycin resistance (iCR) is relatively less explored. Keeping the graveness of this knowledge gap in mind, the study aimed to demonstrate the occurrence and percentage of iCR in MRSA strains isolated from wound swab and blood samples. These isolates were collected from both in-patients and out-patients in the surgery, ICU, and medicine departments of five different tertiary care hospitals all over Bangladesh. A combination of conventional, automated, and molecular methods was applied to confirm *S. aureus* and MRSA identification. In the next step, their consecutive erythromycin and clindamycin resistance was assessed by disk-diffusion methods, and their MLSB phenotype was assessed by a D-test as mentioned in the CLSI 2021 guidelines.

A total of 45 isolates were identified as *S. aureus* by coagulase testing and VITEK02, among which 31 (68.9%) were determined to be methicillin-resistant strains by the cefoxitin disk diffusion method. Isolates from wound samples showed 66.7% methicillin resistance, while all three blood isolates were MRSA. Overall, the study has revealed an overall higher number of cases of MRSA than MSSA. Most patients infected with MRSA belonged to the age group of 30-39 years, and females were more infected than men. In terms of the type of treatment received, patients infected with MRSA were greater than MSSA among inpatients compared to outpatients who were not admitted to the hospital.

The percentage of MRSA in this study is similar to the results found by previous studies in the country. A multicenter study in 2005 reported an overall 55.7% MRSA incidence from clinical samples in different regions [38], but recent studies show the rate has increased over

the years. In 2018, a study investigating the different sources of MRSA found that 72% of the *S. aureus* isolated from different clinical samples in Dhaka were methicillin-resistant by both phenotypical and molecular methods [39]. Other studies have revealed the prevalence of MRSA to be 53.1% in wound-infected patients and 72% in burn-unit patients [40, 41]. Most studies use disk diffusion or MIC tests to detect MRSA and then use PCR to detect the mecA gene for molecular characterization.

Cefoxitin disk diffusion is a standard procedure that is widely used to detect mecA-mediated MRSA strains for both diagnosis and research purposes due to its availability and ease of reading. As per CLSI guidelines, the cefoxitin disc is used as a surrogate test for detecting methicillin resistance and is equivalent to oxacillin broth microdilution or agar dilution methods. A study comparing the different methods has found oxacillin disks to frequently produce hazy zones that may be misinterpreted as susceptible [42]. Thus, cefoxitin disks are used as a substitute for oxacillin disks. However, these tests are only directed towards detecting resistance encoded by the mecA gene and not its homologue, the mecC gene. In our study, we used conventional PCR for molecular characterization of these resistant isolates by using nuc1, mecA, and mecC primers. All 31 of the resistant isolates were positive for the nuc1 gene, and only 22 (71%) isolates carried the mecA gene. The rest of the mecA-deficient isolates were tested with mecC primers, and 8 (25.8%) isolates were positive. A homologue of the mecA gene, the mecC gene, was initially detected in a dairy herd, a zoonotic source. The mecC-MRSA strains have been commonly isolated from human sources as well, and they also encode for the PBP2A protein. A review of the prevalence of the mecC gene in European countries showed that it was skin and wound infection cases that had the most mecC-MRSA and also included 2 bacteremia cases [43]. To relate, our results show that MRSA in six wounds and two blood samples hold the mecC gene. In Bangladesh, studies investigating the methicillin-resistant bacteria in houseflies included PCR of the mecC gene

for mecA-negative strains, but it was not detected [44] [45]. Similarly, none of the mecA-negative *S. aureus* isolates from food-related samples have tested positive for mecC, as mentioned in the studies [46] [47]. To our knowledge, the mecC gene has not been reported in Bangladesh till today, but PCR results in our study have shown these 8 isolates to be *S. aureus*-specific nuc1 and mecC gene positive. However, more advanced molecular techniques, such as sequencing of the gene or whole genome sequencing, can confirm its detection.

In the case of one isolate from a blood sample, neither the mecA nor the mecC were amplified, even after repeating several times. But this isolate was considered to be methicillin-resistant based on VITEK02 AST results showing resistance against cefoxitin, oxacillin, and benzylpenicillin. The phenotypic resistance can be explained by the reports that *S. aureus* beta lactamase, encoded by the blaZ gene, is a penicillinase that can be present in mecA-negative *S. aureus* strains [48]. This particular category of *S. aureus*, which is not truly methicillin-resistant or sensitive, has been named borderline oxacillin-resistant *S. aureus* (BORSA) [48]. Beta-lactamase (BlaZ) is a class A β-lactamase and has a low hydrolytic effect on penicillinase-resistant penicillin (PRP), such as oxacillin. This is due to a hyperproduction of beta-lactamases, which causes effective degradation of penicillin but also inactivates PRPs. Other reported mechanisms include the synthesis of a new oxacillin hydrolyzing β-lactamase or spontaneous point mutations in PBP genes [49]-[51]. The blaZ gene encoding for class A β-lactamase is present on the plasmid, so the absence of the mecA or mecC gene on chromosomal DNA can lead to the misidentification of methicillin resistance [52]. A study on the characterization of the blaZ gene in BORSA and MSSA strains revealed that the gene in BORSA was associated with increased oxacillin hydrolysis, whereas it had no effect on oxacillin susceptibility in MSSA stains [53]. However, our thesis

requires further amino acid sequencing and oxacillin MIC tests to confirm the methicillin-resistant gene-negative isolate as BORSA.

In the next step of the thesis, a D-test was performed to observe the inducible clindamycin resistance phenotype in 29 (64.4%) out of the total 45 *S. aureus* isolates, with a greater percentage in MRSA strains than MSSA strains (67.7% vs. 57.1%). Clindamycin, a drug of the lincosamide class, has gained popularity for the treatment of both methicillin-resistant and methicillin-sensitive *S. aureus* infections due to its low cost, good tissue penetration, accumulation in the abscesses, and satisfactory bioavailability [54]. The drug is available in both oral and intravenous forms and can even prevent certain virulence properties of staphylococci [55]. Unfortunately, cases of treatment failure by clindamycin due to iCR are being reported, but Bangladesh has limited case studies and awareness on this matter [56]. The only information and data available on iCR are from a study held at the Bangladesh Institute of Health Sciences (BIHS) Hospital in 2011, which investigated the prevalence of iCR in staphylococci [57]. This study also reported a high percentage (48% of iCR among MRSA strains and all negative for MSSA cases), but overall, only 22% iCR was observed in the total samples [57]. To compare, our results show a significant rise in the prevalence of iCR, both overall and in MRSA strains, since the time of that study. But both studies show a similar percentage of constitutive clindamycin resistance, which is 5% and 4.44%, respectively. One notable result of our study is that there weren't any strains showing the MS phenotype, which is described as sensible to clindamycin but resistant to erythromycin and is D-test negative. It indicates that all the erythromycin resistance in the strains is mediated by the *erm* genes, which are inducing resistance to clindamycin, rather than the efflux pump mechanism. This outcome is the same as a study in Turkey that also determined the rate of iCR in staphylococci isolated from clinical samples and found zero MRSA isolates with the MS phenotype, indicating all MRSA strains were inducible clindamycin resistant [58].

However, this was not the case for a study in India, which showed about almost half of the total isolates to have the MS phenotype, but compared to our study, a much higher percentage (84%) of D-test-positive MRSA strains were identified [59]. Induced resistance to clindamycin leads to a cross-resistance to Streptogramin B-group antibiotics, making treatment for MRSA infections more complicated. Lastly, while iCR can be detected phenotypically by the D-test and is a standard procedure, gene amplification of the erm genes in our study can confirm the methylase activity towards erythromycin as the inducer macrolide.

Conclusion and Recommendations

MRSA has become one of the most prevalent multi-drug resistant organisms, leaving only a few effective treatment options. The objective of the thesis was to exhibit the prevalence of methicillin-resistant *S. aureus* (MRSA) strains from wound and blood samples at tertiary care hospitals and test for inducible clindamycin resistance (iCR). The research mainly focused on the detection or screening of MRSA. And the outcomes of our studies show alarming rates of 68.9% MRSA and 64.4% iCR, mostly among the in-patients in the surgical department with wound infections. Our research has also detected the homologue of the mecA gene, the mecC gene, which hasn't been reported in Bangladesh yet. This suggests that amplification of only the mecA gene by PCR for only molecular-based detection may lead to the misidentification of MRSA as methicillin-sensitive *S. aureus* (MSSA). Research studies or diagnosis methods that solely use molecular detection should target both the *S. aureus*-specific nuc1 gene and the two methicillin-resistant genes to avoid misidentifications. The finding of an isolate with no methicillin-resistant gene also suggests including assessment of beta-lactamase activity or detection of blaZ genes in cases of mecA and mecC-deficient MRSA. This is due to the emergence of BORSA, which doesn't contain the PBP2A-encoded resistant genes but shows a lower level of resistance against oxacillin. Cefoxitin disk diffusion and oxacillin MIC tests

are acceptable and reliable methods to screen MRSA phenotypically. Clindamycin is prescribed frequently for *S. aureus*-related infections because it is cheaper and more effective against both MRSA and MSSA infections [60]. But the prescription of clindamycin based on only the constitutive susceptibility test is not sufficient due to concerns over inducible clindamycin resistance. Luckily, a D-test, which includes placing a clindamycin disk in close proximity to erythromycin, is a simplistic and cost-effective procedure to avoid treatment failure or relapse in patients. It is advisable that diagnostic labs become aware of this phenomenon and introduce the D-test to their routine AST for *S. aureus* infections. Finally, the study also encourages further investigation of clindamycin treatment failures among patients, as case reports on this matter are not yet available in Bangladesh. A few of these steps can prevent prolonged hospital stays, medical costs, and, most importantly, death.

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