Molecular Typing, Characterization and Pathogenicity of Clinical Multi-Drug Resistant *Staphylococcus aureus* isolates

By

Amir Afzal Khan

Department of Microbiology
Quaid-i-Azam University
Islamabad, Pakistan
2021

Molecular Typing, Characterization and Pathogenicity of Clinical Multi-Drug Resistant *Staphylococcus aureus* isolates

A thesis

Submitted in the Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

By

Amir Afzal Khan

Department of Microbiology
Quaid-i-Azam University
Islamabad, Pakistan
2021

Author's Declaration

I **Mr. Amir Afzal Khan** hereby state that my Ph.D. thesis titled **"Molecular Typing, Characterization and Pathogenicity of Clinical Multi-Drug Resistant *Staphylococcus aureus* Isolates."** is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

At any time if my statement is found to be incorrect even after I Graduate, the University has the right to withdraw my Ph.D. degree.

Mr. Amir Afzal Khan
Date: 01-11-2021

Plagiarism Undertaking

"Molecular Typing, Characterization and Pathogenicity of Clinical Multi-Drug Resistant *Staphylococcus aureus* Isolates." is solely my research work with no significant contribution from any other person. Small contribution / help wherever taken has been duly acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and Quaid-i-Azam University towards plagiarism. Therefore I as an Author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of Ph.D degree and that HEC and the University has the right to publish my name on the HEC/University Website on which names of students are placed who submitted plagiarized thesis.

Student / Author Signature: _____

Name: **Mr. Amir Afzal Khan**

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled titled **"Molecular Typing, Characterization and Pathogenicity of Clinical Multi-Drug Resistant *Staphylococcus aureus* Isolates."** was conducted by **Mr. Amir Afzal Khan** under the supervision of **Prof. Dr. Rabaab Zahra.** No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Microbiology, Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in field of **Microbiology**.

Student Name: **Mr. Amir Afzal Khan** Signature:_____

Examination Committee:

a) External Examiner 1: Signature:_____

Dr. Naeem Akhtar
Professor
Head of Department of Pathology
Rawalpindi Medical University, Rawalpindi

b) External Examiner 2: Signature:_____

Dr. Shahzad Hussain
National Institute of Health
Park Road, Islamabad

Supervisor Name: **Prof. Dr. Rabaab Zahra** Signature:_____

Name of HOD: **Prof. Dr. Aamer Ali Shah** Signature:_____

DEDICATED

TO

My Brother Professor Walayat khan, Haseeb Khan and Waqas khan, My wife, and all family members

CERTIFICATE

This thesis, submitted by **Mr. Amir Afzal khan** is accepted in its present form by the Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirement for the degree of Doctor of Philosophy (PhD) in Microbiology.

Examiner: _____

Dr.

Examiner: _____

Dr.

Supervisor: _____

Dr. Rabaab Zahra

Chairperson: _____

Dr. Aamer Ali Shah

Dated: 17-09-21

Contents

List of Abbreviations

%	Percentage
+ive	Positive
µg	microgram
α	Alpha
β	Beta
γ	Gamma
aadD	Adenyltransferase
agr	Accessory Gene Regulator
apoLp-III	Apolipophorin-III
ARGs	Antibiotic resistance genes
AST	Antibiotic Susceptibility Testing
ATCC	American Type Culture Collection
bp	Base Pair
C	Chloramphenicol
C^{o}	Degree Celsius/Centigrade
CA	Community Acquired
CDC	Centre of Disease Control and Prevention
CIP	Ciprofloxacin
CLSI	Clinical and laboratory Standard Institute
CN	Gentamicin
CSF	Cerebrospinal Fluid
CVP	Central Venous Pressure
DA	Clindamycin
DNase	Deoxyribonuclease
D-test	Double Disk Diffusion test
E	Erythromycin
EDTA	Ethylene Diamine Tetra-acetic Acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FOS	Fosfomycin
FOX	Cefoxitin
G	Gram
H_2O_2	Hydrogen Peroxide
HA	Hospital Acquired or Associated
HCl	Hydrochloric Acid
HVR	Hyper variable region
-ive	Negative
J	Joining or Junkyard
kDa	Kilo Dalton
l	Liter
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LZD	Linezolid
M	Molar
MDR	Multi-drug Resistant

MDR-MRSA	Multi-drug resistant methicillin resistant *S. aureus*
MDR-MSSA	Multi-drug resistant methicillin sensitive *S. aureus*
$MgCl_2$	Magnesium Chloride
MGE	Mobile Genetic Element
MHA	Mueller Hinton Agar
MICs	Minimum inhibitory concentrations
ml	Milliliter
MLS	Macrolide, Lincosamide and Streptogramin
MLST	Multilocus Sequence Typing
mM	Milli Molar
mm	Millimeter
M-PCR	Multiplex PCR
MRSA	Methicillin resistant *S. aureus*
MSA	Mannitol Salt Agar
MSSA	Methicillin sensitive *S. aureus*
N or n	Number
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NT	Non-Typeable
O 2	Oxygen
OPD	Out Patient Department
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PIMS	Pakistan Institute of Medical Sciences
Pmol	Pico-mole
PSM	Phenol-Soluble Modulin
PTC	Peptidyl transferase Centre
PVL	Panton-Valentine Leukocidin
QAU	Quaid-i-Azam University
QD	Quinupristin/Dalfopristin
RD	Rifampicin
ROS	Reactive Oxygen Species
Rpm	Revolutions Per Minute
S. aureus	*Staphylococcus aureus*
SCC	Staphylococcus Cassette Chromosome
SCC*mec*	Staphylococcal Cassette Chromosome *mec*
SCVs	Small colony variants
SDS	Sodium Dodecyl Sulphate
spa	Staphylococcal protein A
SSI	Skin and Soft Tissue Infection
SSSS	Staphylococcal scalded skin syndrome
SSTI	Soft Tissue Infections
ST	Sequence Type
SXT	Trimethoprim-Sulphamethoxazole
TBE	Tris Borate EDTA
TCS	Two-component signaling system

TE	Tetracycline
TE Buffer	Tris-EDTA Buffer
TSA	Tryptic soy agar
UK	United Kingdom
USA	United States of America
UTIs	Urinary Tract Infections
UV	Ultraviolet
V	Volt
VA	Vancomycin
VISA	Vancomycin Intermediate *S. aureus*
VRSA	Vancomycin resistant *S. aureus*
w/v	Weight by Volume
WBC	White Blood Cells

List of Tables

List of Figures

Acknowledgements

Praise to **ALMIGHTY ALLAH**, whose blessings enabled me to achieve my goals. Tremendous praise for the **Holy Prophet Hazrat Muhammad (Peace Be upon Him)**, who is forever a torch of guidance for the knowledge seekers and humanity as a whole.

I have great reverence and admiration for my research supervisor, **Dr. Rabaab Zahra,** Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan, for her scholastic guidance, continuous encouragement, sincere criticism and moral support throughout the study. Her guidance helped me in all the time of research and writing of this thesis, with her patience and vast knowledge.

I do not find enough words to express my heartfelt gratitude for **Prof. Dr. Eleftherious Melonakos,** chief**,** Infectious disease society, Rhode Island hospital, Brown *University, Alpert Medical School, at Brown*, USA. He supervised me during my studies in USA, during IRSIP fellowship. This experience would not have been as valuable without the guidance, support and inspiration provided by him. I am impressed by his scientific thinking, intellectual criticism and politeness. I am also deeply grateful to **Dr. Fouzia Naheed**, Post Doc fellow at QAU at EMBARK project, and impressed by her scientific thinking, intellectual criticism and politeness, and for valuable technical discussion during research work.

I am also deeply grateful to **Dr. Nagenderan Tharmalingam,** Post Doc fellow at Rhode Island hospital Associate for his mentorship, guidance, instruction and support at every step, during my research. I consider myself very fortunate for being able to work with considerate and encouraging professor like him. I would not have been able to finish my research work without his help and support.

I wish to express my most sincere appreciation to the current chairperson, **Dr. Aamer Ali Shah**, ex-chairpersons **Dr. Fariha Hasan** and **Dr. Safia Ahmad**, Department of Microbiology, Quaid-i-Azam University Islamabad Pakistan, for her valuable guidance, patience, support, criticism, encouragement and constructive suggestions throughout the course of this study.

I would also like to thank **Higher Education Commission, Pakistan**, for providing me research grant under the Project **"International Research Support Initiative Program (IRSIP)"** at USA.

I am extremely grateful to the entire faculty at the Department of Microbiology, Quaid-i-Azam University, Islamabad. I feel thankful to the non-teaching staff, Shabbir Sahib, Shafaqat sahib, Sharjeel, Tanveer, Arif and Shahid at Department of Microbiology, QAU, Islamabad, for their kind assistance. I am really thankful to Zia Bhai, Matiullah, Yousaf Amen, Saba Yasmeen, Amina Habib, Shahzeera Begam, Shahid Ali, Abdurauf Tareen, Mutuillah Khattak, Arshad, Zara, and all colleagues of Dr. Zahra lab, for their help and care throughout my study.

A non-payable debt to my loving **Ammi, Abbu**, my siblings and my wife **Pashmina Afridi** for bearing all the ups and downs of my research, motivating me for higher studies, sharing my burden and making sure that I sailed through smoothly. Completion of this work would not have been possible without the unconditional support and encouragement of my loving family members

Finally, I express my gratitude and apology to all those who provided me the opportunity to achieve my endeavors but I missed to mention them personally.

Amir Afzal khan

Abstract

Staphylococcus aureus (*S. aureus*) is one of the leading causes of community and hospital-acquired infections globally. These bacteria have adapted additional resistance mechanisms against various commonly used antibiotics. The increase in resistance to multiple antibiotics by *S. aureus* presents a major global problem. The objectives of the study were to assess the prevalence, study the carriage of antibiotic resistance genes and evaluate the molecular typing of *S. aureus* isolates from a tertiary care hospital in Islamabad.

In this study, 1528 staphylococci isolates were collected from tertiary care hospital, Pakistan Institute of Medical Sciences (PIMS) Islamabad. Initial confirmation was done by Gram staining and observing growth characteristics on mannitol salt agar, while further conventional biochemical tests, such as catalase, DNase, and tube coagulase were performed to confirm *S. aureus*. Molecular identification of *S. aureus* isolates was done using 16S rRNA and nuclease (*nuc*) gene amplification through polymerase chain reaction (PCR). These isolates were from different specimens including pus, urine, blood, and tracheal secretions, etc. Patient demographic details such as specimen, age, gender, and ward were also recorded. Antibiotic susceptibility was evaluated using Kirby-Bauer disk diffusion method and minimum inhibitory concentration (MICs) by microbroth dilution method following Clinical and Laboratory Standards Institute (CLSI) guidelines. Cefoxitin disks of 30µg (Oxoid, UK) were used to differentiate between methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) isolates. The Double Disk Diffusion test (D-test) was performed to detect inducible clindamycin resistance. Screening of antibiotic resistance genes (ARGs) was performed using multiplex PCR. A molecular typing of study isolates was accomplished by accessory gene regulator (*agr*) typing, hypervariable (HVR) typing, staphylococcal cassette chromosome *mec* (SCC*mec*) typing, staphylococcal protein A (*spa*) typing, multi-locus sequence typing (MLST), and pulsed-field gel electrophoresis (PFGE). The hemolysin assay was carried out for selected isolates; other virulence and regulatory factors were evaluated through multiplex PCR. Representative isolates were selected

for expression analysis through Real-time quantitative PCR (RT-qPCR). Intracellular survival and pathogenicity of selected *S. aureus* strains were analyzed using *in vitro* Raw 264.7 cells and the in-vivo *Galleria mellonella* infection model. Chi-square test was applied to find correlation among phenotypic antibiotic resistance and their respective ARGs, and the association of Panton-Valentine leucocidin (PVL) toxin with SCC*mec* types.

From a total of 1528 staphylococci isolates, 485 were characterized as *S. aureus* based on standard microbiological procedures. Of total 485 isolates, based on cefoxitin resistance 65% of isolates were confirmed as MRSA and 35% were MSSA. For this study, 250 isolates (200 MRSA and 50 MSSA) were further analyzed. Antibiotic susceptibility results showed that *S. aureus* isolates were highly resistant to clindamycin (69%), ciprofloxacin (68%), and cefoxitin (65%), followed by gentamicin (46%), erythromycin (45%), tetracycline (31%), while with lower resistance to sulfamethoxazole/trimethoprim (21%), rifampicin (15%), chloramphenicol (7%) and for linezolid and quinupristin/dalfopristin (3% each). MRSA isolates were highly resistant to ciprofloxacin (85%), and gentamicin (64%). Resistance against erythromycin was 50%, tetracycline was 36%, sulfamethoxazole/trimethoprim and clindamycin resistance level were at 26% each while rifampicin was recorded as 20%. Among MRSA isolates, high levels of susceptibility were found against linezolid (96%), Quinupristin/dalfopristin (95%), and chloramphenicol (88%). Antibiotic susceptibility results showed that MSSA isolates were highly resistant to ciprofloxacin (80%), tetracycline (72%), and to erythromycin, sulfamethoxazole/trimethoprim, and gentamicin (32% each). Low resistance was noticed to clindamycin (12%) and rifampicin (20%). All MSSA isolates were sensitive to cefoxitin, chloramphenicol, quinupristin/dalfopristin, and linezolid. Clindamycin inducible resistance was observed in 4% of isolates. In all isolates, 85% were recorded as MDR, within MRSA 83% were confirmed as MDR-MRSA, while in MSSA 20% were MDR-MSSA. MICs against vancomycin confirmed that 5% were vancomycin-intermediate *S. aureus* (VISA), and 28% MRSA isolates were fosfomycin resistant. Molecular detection of ARGs showed

that in MRSA isolates, *mec*(C) gene had a low prevalence of 3%, and only 1 MRSA had both *mec*(A) and *mec*(C) genes in combination. Clinical MRSA carrying *mec*(C) alone and *mec*(A)+*mec*(C) in combination was also detected for the first time from Pakistan. Prevalence of ARGs in MRSA was recorded as 80% for *dfr*(B), 87% *tet*K, 81% *tet*M, 54% *mec*(A), 3% *mec*(C), 100% *bla*(Z), 95% *gyr*(A), 33% *gyr*(B), 9% *grl*(A), 6% *grl*(B), 80% *rpo*(B), 75% *aacph*D1, 13% *erm*(A), 81% *fos*(A), 56% *vat*(C), 29% *vat*(B), 4% *vat*(A), and 100% *erm*(C). While the prevalence of ARGs in MSSA were noticed as 75% *dfr*(B), 31% *tet*K, 52% *gyr*(A), 67% *rpo*(B), 75% *aacph*D1, 75% *erm*(A), 50% *vat*(C), 2% *vat*(B), and 100% *erm*(C).

In MRSA isolates a*gr*I was the most prevalent group (22%), while in MSSA *agr*III (16%) was the dominant group. Among SCC*mec* types, SCC*mec* III was the most prevalent (86%) one. Other prevalent *mec* types included type I (14%), II (23%), IV (40%), and VI (23%), while 7.1% were non-typeable (NT), whereas type V was not detected in any of the isolates. The study reports SCC*mec* type VI that has not been reported previously from Pakistan. The PVL toxin was observed in 24% MRSA and 10% in MSSA of the study isolates with distribution in all prevalent SCC*mec* types but was strongly associated with types VI, IV and II. In MRSA isolates, 42% were found to be positive for the HVR whereas 58% of isolates did not reveal the presence of HVR. In MRSA isolates 5 HVR types were observed, the most dominant HVR type was type 6 (60%), followed by type 5 (15%), type 7 (10%), type 3 (10%), and type 4 (5%).

In the present study, a total of 50 isolates (25 MRSA and 25 MSSA) were processed for *spa* typing, 10 different *spa* types were found in MRSA with t657 (24%) as the dominant *spa* type while 15 *spa* types were found in MSSA with t021 (24%) with the highest prevalence. Among these, four *spa* types (t657, t314, t632, and t5598) were common among MRSA and MSSA. One NEW *spa* type was identified in MSSA isolates. The current study advances information of *spa* types of local clinical *S. aureus* isolates as out of 21 *spa* types in this study, 17 are reported first time from Pakistan. A subset of 5 representative isolates (n=4 MRSA and n=1 MSSA) were further processed for MLST and PFGE analysis. Combining MLST and *spa* typing,

three genotypic patterns were identified; ST772-t657, ST1-t127 in MRSA, and ST1535-t3214 in MSSA. Among multi-locus sequence types, the MSSA with ST1535-t3214 has not been previously reported from Pakistan. Clonal analysis of representative isolates showed that two isolates were of same clone (100% similarity), 2 isolates showed 85% similarity and one isolate had 75% similarity index.

The alpha-hemolysin (45%, 60%) was more dominant as compared to beta-hemolysin (28%, 28%) and gamma-hemolysin (27%, 12%) in MRSA, and MSSA respectively. Among MRSA, alpha-hemolysin gene (*hla*) was detected in 80% of phenotypically positive isolates, followed by 65% for beta-hemolysin (*hlb*) and 58% were positive for gamma-hemolysin gene (*hlg*), and *hld* gene was found in 3.2% isolates. Prevalence of virulence and regulatory factors in MRSA isolates were recorded as 58%, 86%, 87%, 86%, 55%, 57%, 52%, 68%, 74%, 78%, 10%, 5%, 2%, 79% and 24%, for *sigB, codY, fnbA, fnbB, clfA, saeS, sarA, arlS, yycG, spoVG, sea, seb, sec, rot,* and PVL genes respectively. The percentages were 62%, 84%, 86%, 82%, 52%, 56%, 50%, 64%, 72%, 70%, 6%, 4%, 4%, 76% and 10%, for *sigB, codY, fnbA, fnbB, clfA, saeS, sarA, arlS, yycG, spoVG, sea, seb, sec, rot* and PVL genes respectively in MSSA isolates. Similar virulence factors were observed both in MRSA and MSSA. Macrophages induced intracellular killing effect was not observed after 20 hours of incubation for *agr* groups II, III, and *agr* mutant strain RN4220. Survival assay in *G. mellonella* infection model showed that HA-MRSA was more virulent than VISA, CA-MRSA, and MSSA. Hemocyte density experiment demonstrated that the CA-MRSA provoked a more pronounced immune response.

1. Introduction

1.1 *Staphylococcus aureus* (*S. aureus*) Discovery

A Scottish surgeon, Sir Alexander Ogston in 1880 demonstrated that a bunch shaped coccus was the reason for various pyogenic infections in humans; hence, he named the creatures as Staphylococcus. It is derived from two Greek words, "Staphyle" which means a bunch of grapes, and "coccus" that means a berry or grain (Ogston, 1882). The first person who possibly grew staphylococci in pure culture was Rosenbach. He considered that the organism was the same as that defined by Ogston so he embraced the name Staphylococcus for the genus to encompass these creatures. However, in a later aspect of the paper which contains the first description of this organism he used the name *S. aureus, aureus* means golden and is descriptive of the golden yellow colonies (Commission, 1958). At first, Staphylococcus was positioned in the family 'Micrococcaceae' (Paulin & Samson, 1973). In 1908, two species *S. epidermidis* and *S. aureus* were documented; these two species were moved to the genus Micrococcus in the 5th edition of the Bergey's Manual of Determinative Bacteriology (Winslow *et al.,* 1920). Hucker also concluded in the 6th edition that micrococci and staphylococci were not dissimilar (Hucker & Breed, 1948). However, the genus Staphylococcus was recorded in the 7th edition. Evans further indicated that staphylococci were physiologically different from genus micrococci as they can grow anaerobically, and they also ferment glucose (Evans, 1957).

1.2 *S. aureus*

S. aureus is a Gram-positive bacterium (Deurenberg & Stobberingh, 2008; Nulens *et al.,* 2008) that occurs in pairs, tetrads, and in the form of short-chains, and irregular "grape-like" clusters. They are non-spore forming and non-motile, facultative anaerobes, having a size of 1 μm diameter (Nulens *et al.,* 2008). The habitat in which *S. aureus* resides are the nasal passages, hair, warm-blooded animals and also with skin of humans. The main component of staphylococci cell wall contains both teichoic acid and peptidoglycan, which contains mainly glycine (Schleifer & Kandler, 1972). *S. aureus* can survive in harsh environments such as high salt

concentration of up to 15% Sodium Chloride (NaCl) and a pH ranging from 4.8 to 9.4 (Crossley*et al.,* 2009; Madigan, 2006). These characteristics allow it to survive and thrive in environments like host mucosal and epidermal layers as well as on the medical equipment surfaces (Vijayakumaran, 2013).

1.3 *S. aureus* infections

S. aureus is one of the most common naturally present cocci in the human host, with an expected 30-40 percent of the human beings demonstrated as carriers (Peacock *et al.,* 2001). *S. aureus* frequently exists on the nasal mucosa and is also isolated from other clammy surfaces; for example, the perineum and the axillae. It also acts as an opportunistic pathogen and can cause several diseases in humans that range from toxin-mediated to pyogenic and invasive infections, due to its ability to escape from immunity (Deurenberg & Stobberingh, 2008; Nizet, 2007). *S. aureus* has the ability to initiate infections in every tissue and organ of the human body (Lowy, 1998).

Due to the presence of specific toxins, a number of other infections can be associated with *S. aureus,* such as toxic shock syndrome (TSS), staphylococcal scalded skin syndrome (SSSS) and also causes food poisoning (Salyers & Whitt, 2002). Since the discovery of *S. aureus* during the 1880s, it has been known as a major virulent bacteria (Wertheim *et al.,* 2004), due to its extracellular virulence factors that promote pathogenesis and colonization of the host (Greenwood & Gentry, 2002). Since the mid-1990s, however, an increase in the number of MRSA infections in the community lacking risk factors for exposure to the health care system (Dantes *et al.,* 2013; DeLeo *et al.,* 2010). In the pre-antibiotic period, *S. aureus* has been commonly linked to lethal bacteremia, still most common in some location Such as Kansas City, Missouri (Abraham & Bamberger, 2020).

1.4 Treatment of *S. aureus* infections

In the pre-antibiotic era *S. aureus* mortality rate in blood infection exceeded 80%, and about 70% established metastatic infections (Skinner & Keefer, 1941). Use of penicillin in the early 1940s improved the prognosis of patients with staphylococcal infections. In early 1942, staphylococci that were penicillin-resistant documented in hospitals as well as in the community (Rammelkamp & Maxon, 1942). In 1944,

Kirby confirmed for the first time that penicillin-resistant *S. aureus* inactivated penicillin (Kirby, 1944). Later on, Bondi and Dietz recognized the role of penicillinase in hydrolyzing penicillin (Bondi & Dietz, 1945). In the late 1960s, over 80 percent of staphylococcal isolates involved both in hospital as well as in community-acquired infections were penicillin-resistant (Lowy, 2003). Penicillin-resistant *S. aureus* has disseminated quickly after its first presentation, followed by the further increase in penicillinase-producing multidrug-resistant (MDR) *S. aureus*, showing resistance to chloramphenicol, penicillin G, aminoglycosides (streptomycin and kanamycin), tetracyclines, and macrolides (e.g., erythromycin) (Lacey & Chopra, 1974; Lyon *et al.,* 1987). In 1960 semi-synthetic penicillins, such as methicillin (oxacillin and cloxacillin), were found to be active against penicillin-resistant staphylococcal infection (Bryskier, 2005). However, in 1961 from United Kingdom methicillin-resistant *S. aureus* (MRSA) was emerged in the form of MDR *S. aureus* (Lacey, 1975). In 1961, MRSA was reported for the first time from Europe (Jevons, 1961), later in 1968 from the United States at Boston city hospital (Barrett, McGehee Jr, & Finland, 1968) and then from other countries like Japan, Australia, and Europe (De & Wolf Miller, 2008). As more than 80% of Australian strains of methicillin-sensitive *S. aureus* (MSSA) produced a penicillinase, penicillinase-resistant penicillin was needed to be used in most patients (Nimmo *et al.,* 2003).

In treating complicated MRSA infections vancomycin is the drug of choice (Khatib *et al.,* 2009). Vancomycin derivatives such as oritavancin, dalbavancin, and telavancin have been shown to rapidly kill *S. aureus in vitro* in a concentration-dependent manner. Linezolid and daptomycin show good activity against resistant staphylococci (Fowler *et al.,* 2006). Cephalosporins such as ceftaroline and ceftobiprole have shown good activity against MRSA involved in skin and soft tissue infections (SSTI) (Anderson & Gums, 2008). The infections triggered by Gram-positive MDR bacteria represent health burden, mortality and morbidity, and increased expenditure to combat infections (Woodford & Livermore, 2009). MRSA is an important pathogen globally. Nasal passages of about 25%-30% of healthy persons are colonized by *S. aureus* while less than 2% are colonized with MRSA (Falagas *et al.,* 2013). Due to improper and misuse of antibiotics in developing

countries such as Pakistan, the infections triggered by resistant bacteria remedy become a persistent challenge (Hussain *et al.,* 2013).

1.5 Antibiotic resistance in staphylococci

In staphylococci, the main sites for antibiotics are nucleic acid, the cell wall, and the ribosome. Acquired resistance develops, when susceptible bacteria acquire either external genes or chromosomal mutations within the bacterial genome or more commonly, through horizontal gene transfer, involving the uptake of naked DNA, transduction, and/or conjugation (Neu, 1992; Normark & Normark, 2002). The main mechanism of acquiring the resistance determinants occurs through conjugation, whereas plasmids and mobile genetic elements act to transfer resistance determinants between the same species or diverse species (Lim & Webb, 2005; C. Walsh, 2003). Horizontal gene transfer can also help develop resistance through mobile genetic elements via transposons, plasmids, and through a staphylococcal cassette chromosome (Figure 1.1). Horizontally acquired resistance can happen via different mechanisms mentioned below;

1. Inactivation of drug
2. Modification of the target
3. Reduction in the permeability (Lim & Webb, 2005)
4. Efflux pumps (Lim & Webb, 2005).
5. By-pass mechanisms that involve acquiring a drug-resistant target.
6. Dislocation of the drug (antimicrobial compound) for the protection of the target.

   Acquiring resistance via mutation(s) can result in any of the following:

   i. Modification of the drug target that stops the inhibitor from binding
   ii. Depressing the chromosomally encoded multidrug resistance efflux pumps

Numerous step-by-step mutations change the conformation and structure of the cell wall and/or membrane to decrease drug access to its target.

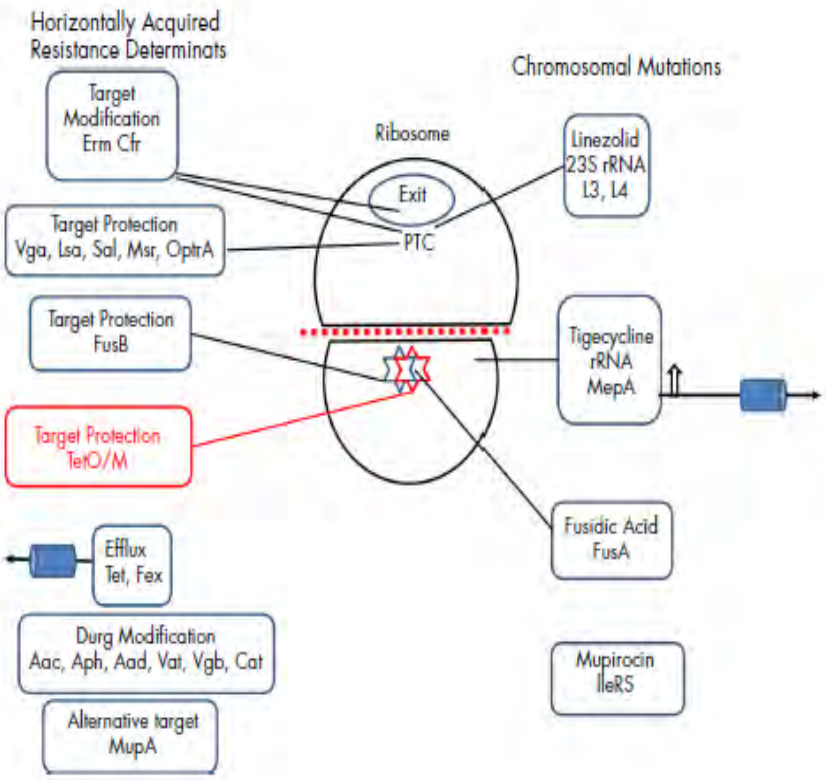


Figure 1.1: Mechanisms of antibiotic resistance in *S. aureus,* resistance may be either horizontal, acquired or due to mutational changes in the chromosome (T. J. Foster, 2017).

1.6 Minimum inhibitory concentrations (MICs) breakpoints

A breakpoint is a selected concentration (mg/L) of an antibiotic, which defines whether bacteria are resistant or susceptible to the antibiotic. Bacteria are considered sensitive if the MIC value is equal or less than the susceptibility breakpoint. According to the "British Society for Antimicrobial Chemotherapy Resistance Surveillance Project" bacteria will be considered as resistant or intermediate, if the MIC value is higher than the breakpoint value. The "European Committee on Antimicrobial Susceptibility Testing" (EUCAST) offers MIC breakpoints for almost all of the antibiotics which are in clinical use (Kahlmeter *et al.,* 2003).

Developing countries like Pakistan are facing adverse effects of the globally identified problem of antibiotic resistance. Due to extensive usage of broad-

spectrum antibiotics, various MDR microorganisms such as staphylococci have emerged as a major threat in our hospital setups. Several sources can cause the spread of staphylococcal infections from one person to others, for example, contact with pus or wound of an infected person, and through the belongings of an infected person like towels, sheets, clothes, etc. Staff working in healthcare settings is also the major source of transfer of resistant organisms from one person to another (Ehsan *et al.,* 2013).

1.7 Antibiotic resistance in *S. aureus*

Antibiotic resistance is an emerging issue globally. Several factors are involved in the emergence of antibiotic resistance such as; animal growth enhancers, improper usage of antibiotics, and traveling to other geographical regions (Cohen, 1992; Tomasz, 1994). In MSSA, cell wall synthesis inhibited by β-lactam drugs, by binding the intrinsic penicillin-binding proteins (PBPs) eventually killing the bacterium, while in the case of MRSA the existence of modifiedPBP2a rather than PBP due to which MRSA gain resistance to penicillin and methicillin and thus reduces the binding affinity for a β-lactam antibiotic (Rohrer & Berger-Bachi, 2003). *mec*A gene in *S. aureus* isolates encodes PBP2a is responsible for methicillin resistance. On the other hand, in the case of MRSA, PBP2a existence reduces the binding capacity of β-lactam antibiotics cannot bind PBPs, as a result peptidoglycan formation is not affected hence rendering the strain resistant to the effects of methicillin (Berger-Bachi & Rohrer, 2002). MRSA was restricted to hospitalized patients till the 1990s, but later its community transmission was also observed. Besides β-lactam resistance, *S. aureus* has also adapted other resistance mechanisms (Herold *et al.,* 1998).

The first report of the community-associated MRSA (CA-MRSA) was noticed in individual who injected intravenous drugs from Detroit (Levine *et al.,*1986; Saravolatz *et al.,* 1982). According to the Centre for Disease Control and Prevention (CDC), an MRSA is isolated within 48 hours of admission from a specimen in a hospital. (Deurenberg *et al.,* 2008). CA-MRSA strain USA 300 outbreak was reported from California involved in SSTIs amongst collegiate football team

(Nguyen *et al.,* 2005). In 2000, a substantial increase in MRSA was observed and this MRSA was labeled as CA-MRSA. Classically, CA-MRSA infections emerge on the other hand in healthy people who do not have exposure to the healthcare settings hence no risk factors including indwelling medical devices as well as surgery are involved (DeLeo *et al.,* 2010). Risk factors associated with CA-MRSA are repeated crowding, skin-to-skin contact, and soft tissue infections. Generally, the CA-MRSA involved in SSTIs that are necrotizing fasciitis and impetigo as well as necrotizing pneumonia is also one of the infections (Rubinstein *et al.,* 2008). Risk factors for a person to be prone to colonization with hospital-acquired MRSA (HA-MRSA) are related to those for nosocomial colonization with MSSA. Patients that undergo operative procedures, or who are treated in the trauma or burns, and intensive care facilities are usually exposed to the HA-MRSA. The immunocompromised individuals or patients with foreign material in them are more at risk for HA-MRSA infections. Wounds, damaged skin like eczema, and catheters that are introduced as intravascular materials increase the risk for MRSA colonization (Coia *et al.,* 2006).

1.8 Penicillin resistance in *S. aureus*

In 1942, *S. aureus* resistance to penicillin was reported due to the acquisition of a plasmid that spread in the following years (Lowy, 2003). Resistance to penicillin is due to the β-lactamases enzymes encoded by *bla*(Z) gene. (Lowy, 2003). In *S. aureus* the native PBPs (1-4) are the target for β-lactam. (Pinho *et al.,* 2013; Reed, 2015). On the other hand, MRSA exhibits the modified PBP2a that differ from, the native PBPs of *S. aureus*, thus reducing the binding capacity for the β-lactams (Fishovitz *et al.,* 2014). *mec*(A) gene carried by the Staphylococcal cassette chromosome *mec* (SCC*mec*) which is a large mobile genetic element (MGEs) and is integrated into the genome of MRSA (Kwan *et al.,* 2005).

The transposable elements like Tn552 are responsible for the β-lactamase structural gene *bla*(Z) (Jensen & Lyon, 2009). Besides this, develop resistance to other β-lactam antimicrobials like cephalosporins, monobactams, carbapenems, and cephamycin antibiotics, leading to the progress of a new drug "Methicillin" in 1960, a semisynthetic penicillinase-resistant antibiotic of the β-lactam class of antibiotics (Ito *et al.,* 2003; Wisplinghoff *et al.,* 2004). Generally, MRSA showed resistance

against all β-lactam antibiotics. We know that in the case of MRSA the presence of a novel PBP2a reduces the binding capacity of β-lactam antibiotics. One study from Peshawar city of Pakistan reported high resistance in MRSA isolates, exhibiting 100% non-susceptibility to penicillin (Naeem *et al.,* 2013). Another study from Peshawar reported highest resistance against cefotaxime and penicillin with 75% and 100% isolates being resistant, respectively (Khattak *et al.,* 2015).

1.9 Methicillin Resistant *Staphylococcus aureus* (MRSA)

1.9.1 Mechanism of methicillin Resistance

Resistance against methicillin and oxacillin is regulated by the *mec*(A) gene expression which encodes for acquired PBP2a and thus reduces the binding capacity of β-lactam drugs (Chambers, 1988). MGEs such as, SCC*mec* harbors the *mec*(A) gene, which serves as a vehicle for horizontal transfer of resistance genes between different Staphylococcus species. It remains unclear which species of staphylococcal contributed the 5 SCC*mec* types present in MRSA isolates. (Enright *et al.,* 2000). The expression of the *mec*(A) gene is regulated by 2 genes, such as *mecR1and mecI.* (Kim *et al.,* 2012; Mirkarimi *et al.,* 2016). The *mecI* gene codes for a repressor protein MecI while the *mecR1* encodes MecR1 that is a protein that acts as a signal transducer (Mirkarimi *et al.,* 2016). MecI attaches with the *mec*(A) gene promoter region and represses the production of PBP2a in the absence of β-lactam antibiotics (Kim *et al.,* 2012; Mirkarimi *et al.,* 2016).

1.9.2 MRSA carrying *mec*(C) gene

Phenotypically oxacillin resistant strains, negative for *mec*(A) gene, led to the proposition that the presence of another mechanism for oxacillin resistance, for example, hyperproduction of β-lactamase or alteration of the PBP binding target site (Geha *et al.,* 1994). Only a few MRSA negative for also reported (Ba *et al.,* 2013). In 2011, homologue of a *mec*(A) gene carried by the SCC*mec* type XI elements was first found in bovine and human isolates in Denmark, UK, and Eire (García-Álvarez *et al.,* 2011; Shore *et al.,* 2011). Originally, it was termed *mec*ALGA251 and afterward named as *mec*(C) (Ito *et al.,* 2012), which shares 69% sequence homology with *mec*(A), produces a negative PCR result for *mec*(A) and negative slide

agglutination test for PBP2a. MRSA isolates harboring the *mec*(C) gene have also been reported from other European countries, including France (Laurent *et al.,* 2012), Finland (Gindonis *et al.,* 2013), Sweden (Monecke *et al.,* 2013), Germany (Christiane *et al.,* 2011; Schaumburg *et al.,* 2012b), Austria (Loncaric *et al.,* 2013), Switzerland (Basset *et al.,*2013), Norway (Medhus *et al.,* 2012) and Belgium (Paterson *et al.,* 2012; Vandendriessche *et al.,* 2013) from various host species, comprise livestock, companion animal and wildlife, including sheep (Kriegeskorte *et al.,* 2012; G. K. Paterson *et al.,* 2012), domestic cat (Medhus *et al.,* 2012; Walther *et al.,* 2012), domestic dog (Paterson *et al.,* 2012), brown rat (Paterson *et al.,* 2012) and hare (Loncaric *et al.,* 2013). In the literature, MRSA with *mec*(C) has also been identified in human clinical samples from Sweden (Swedish Institute for Communicable Disease Control 2012). MRSA carrying a *mec*(C) gene especially the community-acquired isolates are involved in a variety of infections (Petersen *et al.,* 2013). Generally, there is a low occurrence of *mec*(C) positive human isolates, less than 1% in clinical MRSA from Belgium (Deplano *et al.,* 2014).

1.10 Global burden of MRSA

The reported frequency of MRSA in the USA ranging from 7% to 60% (Khan *et al.,* 2018; Sabbagh *et al.,* 2019). Prevalence of MRSA from Latin American countries has been accounted for 45% in Colombia, 28% from Ecuador, 62% from Peru, and 26% from Venezuela, respectively (Reyes *et al.,* 2009). MRSA prevalence in various European countries has been reported as 10.6% from Spain (Garcia *et al.,* 2011) 0.3% from Netherlands (Greenland *et al.,* 2011), 0% from Sweden (Andersson, 2012), 12.2% from Belgium (Jans *et al.,* 2013), 7.2% from Luxembourg (Mossong *et al.,* 2013), 0.9% in the Netherlands to 56% in Romania (ECDC, 2013), 54.3% from Portugal (Conceicao *et al.,* 2013), and 9.2 % from Germany (Heudorf *et al.,* 2014). From various Asian countries prevalence of MRSA accounted for 56.8% in Hong Kong, 77.6% in South Korea, 57% in Thailand, 74.1% in Vietnam, 86.5% in Sri Lanka, and 65% in Taiwan, and 38.1% in Philippines (Song *et al.,* 2011), 50.4% from China (Shang *et al.,* 2016) 38-56% reported in different studies from India (Chavadi *et al.,* 2018), and 75% from Nepal (Gurung *et al.,* 2020). MRSA prevalence in the Middle East accounted for 32-55%, reported in different studies

from Saudi Arabia (Sirkhazi *et al.,* 2014), 16% and 35% from Iran (Havaei *et al.,* 2014; Mirkarimi *et al.,* 2016) 10% from Jordan (Aqel *et al.,* 2015), and 22-50%, reported in different studies from Iraq (Abdulrahman & Taher, 2018; Hussein *et al.,* 2017; Pirko *et al.,* 2019).

MRSA prevalence in East Africa has been reported as 32% (Ojulong *et al.,* 2008) and 42% from Uganda (Kateete *et al.,* 2011), 71% from Sudan (Kheder *et al.,* 2012), 82% and 31% in Rwanda (Masaisa *et al.,* 2018; Ntirenganya *et al.,* 2015), 10 to 50% from Tanzania (Kumburu *et al.,* 2017; Kumburu *et al.,* 2018; Mawalla *et al.,* 2011; Moremi *et al.,* 2012), and 72% from Asmara (Garoy *et al.,* 2019). The first MRSA in Pakistan was identified in 1989 (Ashiq & Tareen, 1989). In last decade, the prevalence of MRSA in different cities of Pakistan has been reported as 42% from Lahore (Bukhari *et al.,* 2011), 60% from Rawalpindi (Perveen *et al.,* 2013), 32% from Peshawar (Naeem *et al.,* 2013), 36% from Peshawar (Ullah *et al.,* 2016), 52% from Karachi (Siddiqui *et al.,* 2017), 87% from Hyderabad (Brohi & Noor, 2017) and 67% from Rahim Yar Khan (Hussain *et al.,* 2019).

1.11 Vancomycin resistance in *S. aureus*

The drug of choice for HA-MRSA infection treatment is vancomycin (Loomba *et al.,* 2010). About 60 years ago, glycopeptide antibiotics were introduced for clinical use. Initially, vancomycin was the only glycopeptide antibiotic, then in the mid-1980s, teicoplanin was introduced in Europe (Bambeke *et al.,* 2004). Vancomycin and teicoplanin are only glycopeptide molecules available for clinical use. Vancomycin inhibits peptidoglycan chain synthesis, by binding with the C-terminus of a dipeptide (Reynolds, 1989). . The replacement of D-Ala-D-Ala residue with D-lactate (D-Lac) or D-serine (D-Ser) in enzyme encoded by operons, such alterations can prompt the variable expression of glycopeptide resistance, altered D-Ala-D-Lac and D-Ala-D-Ser lead to poor binding affinity of drug, and results in vancomycin resistance. (Courvalin, 2006). First time in *S. aureus* isolates vancomycin resistance was reported in 2003 from Hershey Medical Center, USA (Bozdogan *et al.,* 2004).

The isolates are characterized as vancomycin-intermediate *S. aureus* (VISA) if the minimum inhibitory concentrations (MICs) for vancomycin is 4-8 mg/L. Some

studies from Pakistan have reported the occurrence of VISA strains with prevalence of 13% (Hakim *et al.,* 2007), 0.22% (Taj *et al.,* 2010), 3% (Khan *et al.,* 2014), and 8.97% (Ullah *et al.,* 2016). One study from Lahore observed that 22 isolates were VISA and 5 isolates showed resistance to vancomycin having a MICs value of ≥16mg/L and were considered as vancomycin-resistant *S. aureus* (VRSA) (Ghias *et al.,* 2016). Another study from Pakistan, noticed the vancomycin resistance in 14% MRSA while recorded low resistance in MSSA (3%) using the E-test method. Low prevalence of VRSA were reported from Asia (5%), 1% from Europe, 4% from America and 3% from South America (Wu *et al.,* 2021) and 14% from Pakistan (Saeed *et al.,* 2020). Vancomycin resistance was reported first time in clinical Enterococci confer by *VanA* gene cluster (Leclercq *et al.,*1988), and is found on transposons similar to Tn1546, located either on chromosome or plasmids, basically encodes D-Ala-D-Lac ligases (Cong *et al.,* 2020). *VanA* gene was found in 74% vancomycin-resistant isolates (Azhar *et al.,* 2017). One study from India reported 6 VRSA isolates, using MICs evaluation (Tiwari & Sen, 2006). Another study reported that only 8 isolates were resistant to vancomycin, six with a MIC of 32 mg/L and two with a MIC of 64 mg/L (Hasan *et al.,* 2016). However, vancomycin resistance is commonly found in Enterococci (Kristich *et al.,* 2014) (Dahl & Sundsfjord, 2003) (Kelesidis *et al.,* 2011), but rare in *S. aureus* may be attributed to a fitness cost associated with acquisition of *vanA*-mediated vancomycin resistance (Foucault *et al.,* 2009; Périchon & Courvalin, 2009).

1.12 Tetracycline resistance in *S. aureus*

Tetracycline resistance can either be due to ribosome modification encoded by *tet*M gene or *tet*K gene which encodes efflux protein. Among the range of diverse *tet* genes encoding efflux pumps, *tet*K is mainly identified in *S. aureus* (Trzcinski *et al.,* 2000). *Tet*K is encoded by pT181 (Jensen & Lyon, 2009) plasmid which is a multi-copy plasmid integrated into chromosomal SCC*mec*III cassette (Jensen & Lyon, 2009) while the *tet*O/M are naturally encoded on chromosomally positioned at integrative and conjugative elements (ICE) such as Tn1545 and Tn916. Tn916 and Tn1545 are mobile genetic elements, belong to ICE family, in several bacterial species *tet*M gene is harbored by Tn916 (Lunde *et al.,* 2021). One study from

Pakistan reported 100% tetracycline resistance in MRSA isolates (Romeeza *et al.,* 2010). A previous study from Pakistan reported tetracycline resistance in 74 isolates and the *tet*K gene was found in 58 isolates (Ullah *et al.,* 2012). Other studies from Pakistan have reported tetracycline resistance in *S. aureus* isolates as 65% (Mirza, 2007), 62.5% (Rafiq *et al.,* 2015), and 33% and 42% resistance to minocycline and doxycycline, respectively (Ullah *et al.,* 2016)

1.13 Aminoglycoside resistance in *S. aureus*

In the 1970s, gentamicin was introduced to treat hospital acquired infections mainly due to *S. aureus*, but resistance was soon developed against gentamicin encoded by transposons. In clinical *S. aureus,* aminoglycoside modifying enzymes encoded by MGEs are responsible for aminoglycoside resistance (Jensen & Lyon, 2009). Neomycin and gentamicin resistance is due to the presence of acetyltransferase-phosphotransferase (aacA-aphD) encoded by Tn4001 transposon (Culebras & Martínez, 1999). Transposon Tn5405 carries the genes Phosphotransferase (aphA) and adenyltransferase (aadD) which encodes for aminoglycoside resistance (Takano *et al.,* 2008).

A variable rate of gentamicin resistance over the years has been reported from different cities of Pakistan in MRSA isolates, such as 100% in Islamabad (Hussain *et al.,* 2005), 80% from Karachi (Naqvi *et al.,* 2007) 96% from Karachi (Taj *et al.,* 2010), 76% from Rawalpindi (Perveen *et al.,* 2013), 67% from Kohat (Hussain *et al.,* 2013) and 87% from Lahore (Muhamad Sohail & Zakia Latif, 2018). One study from Jordan in 2012 documented that *aac(6')/aph(2")* were the most prevalent resistance mechanism, identified in 45% *S. aureus* isolates, including 34% MSSA and 52% MRSA, respectively, and *ant* (aminoglycoside-4'-O–nucleotidyltransferase) (4',4") gene was identified in 31% *S. aureus* isolates. Twenty one percent of *S. aureus* isolates carried the *aph(3')III* gene, while 22% MRSA and 16% MSSA carried the *aac(6')/aph(2")* and *ant(4',4")* in combination. The second leading gene in combination was identified as *aac (6')/aph (2")* with *aph(3')III* in 17% and 7% in MRSA and MSSA, respectively (Bdour, 2012). Few studies from

India reported gentamicin resistance in *S. aureus* isolates as 60% (Rajaduraipandi *et al.,* 2006) and 100% (Sarma & Ahmed, 2010).

1.14 Linezolid resistance in *S. aureus*

Peptidyl transferase Centre (PTC) is the catalytic center of the ribosome, the main binding site for linezolid. It overlays the binding site for clindamycin and chloramphenicol and hinders aminoacyl moiety of aa-tRNA blocking peptidyl transferase and prevent protein synthesis. (Long & Vester, 2012). The US Food and Drug Administration (FDA) approved linezolid for treating MRSA infection and its use as vancomycin substitute (Micek *et al.,* 2007). Another mechanism for linezolid resistance is observed by the phenicol and oxazolidinone transferable resistance marker *OptrA*, which has rapidly emerged in animal sources and human in enterococci from China (Huang *et al.,* 2016). Previous study conducted in Pakistan reported *cfr* gene in 78% of linezolid resistant *S. aureus* isolates (Azhar *et al.,* 2017). Other reports from Turkey and India documented all *S. aureus* as susceptible to linezolid (Sipahi *et al.,* 2013; Vijayamohan & Nair, 2014).

1.15 Resistance towards chloramphenicol

Presumably, the same mechanism of action was shared by florfenicol and chloramphenicol inhibiting peptidyl transferase by interfering with the aminoacyl end of aa-tRNA. In *S. aureus*, resistance towards chloramphenicol is due to enzymatic modification of chloramphenicol acetyltransferase (Foster, 1983). Previous studies from Pakistan reported chloramphenicol resistance in *S. aureus* isolates as 10% (Idrees *et al.,* 2009), 93% (Taj *et al.,* 2010), and 21.84% with MICs of 32mg/L (Fayyaz *et al.,* 2013). One study from Kuwait reported annual variation in the case of antibiotic resistance. Resistance in the case of rifampicin and chloramphenicol increased from 0.1% and 2.6% to 1.6% and 9.6%, respectively (Udo & Boswihi, 2017).

1.16 Erythromycin resistance in *S. aureus*

Erythromycin belongs to class macrolides which block the ribosomal polypeptide exit tunnel next to PTC. Macrolides are not widely used in the developed world to treat the staphylococcal infections (Rayner & Munckhof, 2005). Previous studies

from Pakistan have reported erythromycin resistance from Islamabad as 82% (Hussain *et al.,* 2005), 95% from Karachi (Perwaiz *et al.,* 2007), 86% from Rawalpindi (Perveen *et al.,* 2013) and 68% MRSA isolates from Kohat (Hussain *et al.,* 2013). Globally 93% erythromycin resistant hospital-acquired strains harbored the *erm*(B) gene (Huang *et al.,* 2006; Schmitz *et al.,* 2000). In southwestern Alaska, 80% of MRSA isolates showed resistance to erythromycin (Baggett *et al.,* 2003). Studies from India reported erythromycin resistance in *S. aureus* isolates as 60.5% (Anupurba *et al.,* 2003) and 33.3% (Abbas *et al.,* 2015).

1.17 Streptogramin resistance in *S. aureus*

Since 1970 in Europe, an antibiotic named pristinamycin which is a streptogramin was used to combat serious *S. aureus* infections. It was introduced in 1999 for treating MRSA infections (Allington & Rivey, 2001). Dalfopristin A binds with PTC, inhibiting peptide bond formation by interfering with the binding of aa-tRNA, in a similar fashion as clindamycin (Walsh *et al.,* 2016; Wilson, 2014).

Quinupristin B binds with the starting point of ribosomal polypeptide exit tunnel blocking the formation of peptidyl tRNA short chains, inflowing the tunnel at the opening of polypeptide translation, causing the release of peptidyl tRNA (Allignet *et al.,* 1998). Streptogramins antibiotics are of 2 types, A and B, made of macrocyclic lactone rings. Type B act on the 50S ribosomal subunit, while. Type A streptogramins inhibits protein synthesis by hindering peptide bond formation (Harms *et al.,* 2004). One study reported the pristinamycin resistance in 10 *S. aureus* isolates carrying the *vat* (B) gene. These isolates showed resistance towards quinupristin-dalfopristin and pristinamycin and harbored the *erm*(A) gene (Allignet *et al.,* 1996).

1.18 Fusidic acid resistance in *S. aureus*

Fusidic acid is a topical antibacterial agent usually used to treat skin infections as well as colonization in eczema patients caused by *S. aureus*. However, resistance quickly arises if used as monotherapy due to single-step mutation. , and was effective in combination with rifampicin against MRSA infections and also as an efficient linezolid alternative. Widespread usage of this topical drug may

compromise this activity soon which is alarming (Williamson *et al.,* 2017). The elongation factor G is the target of fusidic acid, that attaches with the ribosome, and as a result inhibits protein synthesis. (Bodley *et al.,*1969).

Studies from Pakistan have reported fusidic acid resistance in 2% MRSA isolates from Karachi (Perwaiz *et al.,* 2007), and later 18% from the same city (Zafar *et al.,* 2011), 20% from Islamabad (Hussain *et al.,* 2005), and 17% from Peshawar (Ahmad *et al.,* 2014). One study reported that increase in fusidic acid resistance was mainly due to the FusC and FusB mechanisms which are plasmid-encoded (O'Neill & Chopra, 2006). FusC is positioned inside the SCC*mec* elements (SCC*mec* and SCC*fus*) while the *fus*B gene is plasmid-mediated, which is associated with the *tir*S gene, encode the Tirs protein, which contributes to virulence (Askarian *et al.,* 2014; Patot *et al.,* 2017). Resistance to FusA is because of impulsive chromosomal mutants that affect EF-G, prevent the drug attachment to its target (Nagaev *et al.,* 2001). FusB is a small protein of ~25 kDa in size, attached with the EF-G fusidic acid complex. One study from Saudi Arabia reported fusidic acid resistance in 96% *S. aureus* isolates (Baddour *et al.,* 2006). Many countries such as Russia, South Africa and UK use fusidic acid as a drug of choice (Loeffler *et al.,* 2005; Marais *et al.,* 2009; Stratchounski *et al.,* 2005).

1.19 Ciprofloxacin resistance in *S. aureus*

Topoisomerse and DNA gyrase are essential genes for bacterial survival, involved in bacterial DNA replication and the main target for quinolones (Saiki *et al.,* 1999). Fluoroquinolones acts by inhibiting either topoisomerase IV and/or DNA gyrase (Sugino *et al.,* 2000). In clinical *S. aureus* isolates fluoroquinolone resistance comprises of both mutational changes to the topoisomerase IV and DNA gyrase, resulting in a decrease in drug binding capacity (Hooper, 2002). Amino acid substitutions due to mutation result in compromising the drug binding site, commonly named as quinolone resistance-determining region (Hooper & Jacoby, 2015).

Various reports from Pakistan have documented ciprofloxacin resistance in *S. aureus* isolates with prevalence of 86% (November 2002 to February 2003) (Naqvi

et al., 2007), 64% (January to December 2009) (Taj *et al.,* 2010) and 97% (April 2012 to April 2016) (Muhammad Sohail & Zakia Latif, 2018). Other studies from neighboring country India reported ciprofloxacin resistance in *S. aureus* isolates as 100% (August 2001) (Sarma & Ahmed, 2010), 81% (Kumar *et al.,* 2012), and 87% collected during September 2009 to December 2010 (Pandya *et al.,* 2014).

1.20 Rifampicin resistance in *S. aureus*

A broad-spectrum antibiotic named rifampicin has bactericidal activity that specifically targets DNA-dependent RNA polymerase, which is essential for bacterial growth and survival. Rifampicin interacts specifically with β-subunits of the RNA polymerase encoded by the *rpo*(B) gene (Pandya *et al.,* 2014). In *S. aureus* and other bacteria rifampicin resistance is associated with a mutation in a particular region (cluster II and I) in the *rpo*(B) gene (Aubry-Damon *et al.,* 1998). Rifampicin monotherapy is questionable, due to the quick emergence of resistant mutants in a single-step reaction. Amino acid substitutions on all sides of the binding site of the drug lower the affinity for the target (Aubry-Damon *et al.,* 1998). It is commonly prescribed in tuberculosis treatment and is used in prolonged combination therapy as a front-line drug. Rifampicin cannot be used as a monotherapy to treat MRSA infections due to the rapid emergence of mutant phenotypes (Aboshkiwa *et al.,* 1995). It can be used with fusidic acid in combination (Drancourt *et al.,* 1997), and quinolones (Zimmerli *et al.,* 1998) to avoid the rapid emergence of rifampicin resistance (Moellering, 2008). A study from Pakistan reported rifampicin resistance in 81.2% of MRSA isolates collected during September 2012 to September 2013 in Peshawar (Ullah *et al.,* 2016).

Fosfomycin has broad-spectrum bactericidal activity against bacteria by hindering the synthesis of the bacterial cell wall (Michalopoulos *et al.,* 2011). In the treatment of MRSA infection fosfomycin is used alone or in combination with other drugs. (Falagas *et al.,* 2009). However, resistance to fosfomycin in clinical *S. aureus* has emerged and been increasing continuously (Lee, *et al.,* 2020). Fosfomycin neutralizes the enzyme UDP-*N*-acetylglucosamine-3-enolpyruvyltransferase, also identified as MurA (encoded by the *murA* gene), interferes with peptidoglycan synthesis (Michalopoulos *et al.,* 2011). Bacterial resistance towards fosfomycin is

either due to the chromosomal mutation or fosfomycin modifying plasmid-encoded enzymes. *MurA* gene mutations lead to reduced affinity of fosfomycin for MurA (Jiang *et al.,* 2011), reduced intake of fosfomycin due to mutation in *uhp*T and *glp*T (Takahata *et al.,* 2010), and the catalytic activity of *Fos*B, *Fos*X, *Fos*A, *Fos*C genes (Garcia *et al.,* 1995; Lee *et al.,* 2012). Fosfomycin resistance genes such as *fos*B observed in Staphylococcus species are located mainly on the plasmid (Fu *et al.,* 2016). There is limited information available in the literature about fosfomycin usage for *S. aureus.* One study reported the presence of *fos*B gene in 46% of *S. aureus* isolates which were phenotypically fosfomycin resistant (Etienne *et al.,* 1991). One study from Pakistan reported the fosfomycin resistance in 31% isolates (Taj *et al.,* 2010). In a previous study, fosfomycin was found active against MRSA strains (Lu *et al.,* 2011). One study from China reported fosfomycin resistance in 30% MRSA isolates (Guo *et al.,* 2013). Another study from China reported 9 *fos*B positive MRSA strains among 67 fosfomycin resistant strains with elevated MIC value of 128mg/L (Fu *et al.,* 2016). Three other subtypes of *fos*B, designated as *fos*B6, *fos*B5, and *fos*B4 were also identified. *Fos*B6, *fos*B4 and *fos*B1 genes were carried by small plasmids of 2.5kb and flanked by an analogous replication gene (Fu *et al.,* 2016).

1.22 Clindamycin inducible resistance

Erythromycin can induce clindamycin resistance in clindamycin sensitive strains by activating the *erm*(C) gene (Aktas *et al.,* 2007). Only those strains of Staphylococcus show inducible resistance which are erythromycin-resistant and clindamycin sensitive. Inducible resistance is detected by performing the disk diffusion method, called double disk diffusion test (D-test) by placing erythromycin and clindamycin disks 15-20mm apart. Clindamycin is usually used for treating CA-MRSA infections (Prabhu *et al.,* 2011). One study from Pakistan noticed inducible clindamycin resistance in 16% isolates (Ullah *et al.,* 2016). Another study from India reported constitutive and inducible clindamycin resistance in MRSA as 16% and 20%, respectively (Prabhu *et al.,* 2011).

1.23 Treatment of MRSA Infections

MRSA is usually MDR and has very limited therapeutic options. Clindamycin is usually used for treating CA-MRSA infections (Prabhu *et al.,* 2011). The drug of choice for HA-MRSA infection treatment is vancomycin (Loomba *et al.,* 2010). Different drugs have also been approved to treat infections caused by MRSA and these include daptomycin, linezolid, tigecycline, and quinupristin/dalfopristin (Llarrull *et al.,* 2009; Loomba *et al.,* 2010). In 2011, one study reported fosfomycin activity against MRSA strains (Lu *et al.,* 2011). Some new β-lactam groups of antibiotics targeting the PBP2a include cephalosporins like ceftobiprole and ceftaroline that show good activity against MRSA (Morosini *et al.,* 2019).

1.24 Hospital-acquired (HA) MRSA and community-acquired (CA) MRSA

With the discovery of MRSA, initially, it was only limited to hospitals and known as hospital-acquired (HA) or hospital-associated MRSA. However, in the 1990s, another type of MRSA emerged that was referred to as community-acquired (CA) MRSA, which brought about a big change in the overall epidemiology of MRSA (Dumitrescu & Lina 2012; Udo *et al.,* 1993). Both types of MRSA can be differentiated and are defined by the CDC (Deurenberg & Stobberingh, 2008).

CA-MRSA is defined as an MRSA strain that is cultured from a clinical specimen within 48 hours of the patient's admission to the hospital who already had infection at the time of hospitalization or from the patient who is not associated with any HA-MRSA infection risk factors (CDC, 2017; McClure *et al.,* 2006). HA-MRSA risk factors for include long-term hospitalization, drug usage, recent surgery, and recent hospitalization (CDC, 2017; Herold *et al.,* 1998). HA-MRSA causes several infections ranging from surgical site infections to invasive infections. HA-MRSA harbor SSC*mec* types I, II, and III, whereas CA-MRSA isolates carry the type IV or type V (David & Daum, 2010) and rarely have Panton-Valentine Leucocidin (PVL) (Udo *et al.,* 2006).

One study from India revealed that 21% and 33.3% resistance was shown against erythromycin in CA-MRSA and HA-MRSA, respectively (Bhutia *et al.,* 2015). In another study, a clone of CA-MRSA was isolated from a football player in the US

having skin abscesses, susceptible to most of the antibiotics except macrolides and β-lactam drug, carried SCC*mec* type IV and PVL toxin, associated with severe necrotizing infections (Kazakova *et al.,* 2005).

Phenotypically, CA-MRSA are probably more susceptible to non-β-lactam antibiotics than hospital-acquired strains, harbor PVL toxin, and SCC*mec* type IV, V, or VII (Grundmann *et al.,* 2006). Clinically, CA-MRSA isolates are frequently related to more antibiotic susceptibility, the occurrence of extra virulence factors probably induces severe infection in immune-compromised individuals (Elston, 2007). Classically, CA-MRSA cause diseases in healthy young people that have no interaction with healthcare nor they were identified at a risk for CA-MRSA (DeLeo *et al.,* 2010).

CA-MRSA is mostly involved in SSTI such as necrotizing fasciitis and impetigo. CA-MRSA show high virulent capacity in some hosts, especially in the case of young children (Healy *et al.,* 2004). HA-MRSA and CA-MRSA are often considered as different epidemiological entities, and it should be noted that MRSA move from the community to hospitals, from hospitals to the community. The resistance rate for gentamicin, linezolid, chloramphenicol, fusidic acid, and rifampicin has been reported from India as 13%, 10%, 3%, 3%, and 0%, respectively in CA-MRSA while it was 20%, 20%, 13%, 3%, and 0% respectively for HA-MRSA (Bhutia *et al.,* 2015).

1.25 Transmission of MRSA

Humans can be affected by MRSA by skin-to-skin contact with an already infected human. Although, one study reported that MRSA can spread from humans to animals and vice versa (Lowder *et al.,* 2009). Contaminated fomites can be a source of transmission, for example, clothing, towels, or sports equipment used by an infected individual or contact with pus from an infected wound. For many years, MRSA was thought to be a human pathogen, later on, the concept was changed when a dairy cow was infected by MRSA (Devriese *et al.,* 1972).

1.26 Techniques Used for MRSA Typing

Several molecular typing methods are used in epidemiological studies of MRSA. Such techniques define the relationship among different clones of MRSA circulating in a particular area or during an outbreak (Crisóstomo *et al.,* 2001; Enright *et al.,* 2000). The two main categories of typing techniques are sequence-based typing and band-based typing. The former is the most preferred technique because it generates reliable data. For MRSA, several molecular typing methods are being commonly used including pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), SCC*mec* typing, Staphylococcal protein A (*spa*) typing (de Sousa, 2004). All these techniques have one or more disadvantages including being costly, difficulty in interpretation, and time consuming (Stefani *et al.,* 2012). The cost-effective and easily interpretable is the SCC*mec* typing that determines the MRSA molecular types based on the differences in the genetic architecture of the SCC*mec* elements (IWG-SCC, 2009; Li *et al.,* 2013).

1.27 Staphylococcal Cassette Chromosome *mec* (SCC*mec*)

1.27.1 History of SCC*mec*

In 1961, the first strain of MRSA (NCTC 10442) was reported from the United Kingdom, harbored SCC*mec* type I and in the 1960s it spread globally. SCC*mec* is transferred between staphylococci (Hanssen *et al.,* 2006). SCC*mec* is mobile that is MSSA clone turn out to be the MRSA clone due to the integration of SCC*mec* elements. These MRSA clones disseminated in each country and are more divergent in Asian hospital than previously reported (Enright *et al.,* 2002; Ko *et al.,* 2005). It is now called the Archaic clone. Then in 1982, SCC*mec* type II carried by N315 MRSA strain was isolated from Japan. This strain was known as the New York or Japan clone and it also disseminated worldwide. In 1985, another MRSA strain (85/2082) was isolated from New Zealand that harbored SCC*mec* type III (Ito *et al.,* 2001). In the 1990s, type IV SCC*mec* was detected in several clones of MRSA all over the world. In 2004, a strain of MRSA (WIS) was described in Australia that harbored the SCC*mec* type V while type VI was observed in MRSA from Portugal in the strain HDE288 (Ito *et al.,* 2001; Ito *et al.,* 2004; Oliveira, Milheiriço, & de

Lencastre, 2006; Takano *et al.,* 2008; Van Duijkeren *et al.,* 2004; Vandenesch *et al.,* 2003).

Up till now, eleven diverse types of SCC*mec* elements, designated as SCC*mec* type I-XI, have been recognized (Havaei *et al.,* 2014; IWG-SCC, 2009). The size of the element ranges from 20.9-66.9 kb, type IV, and VI being the smallest and type III the largest one. It is seen that the types I, IV, V, VI, and VII are resistant to β-lactam antibiotics only, while types II and III show resistance towards several antibiotics belonging to different antimicrobial classes (Deurenberg & Stobberingh, 2008). The markers for multiple antibiotics are located on plasmids (e.g. pUB110, pT181, and pI258), or transposons (e.g. Tn554) integrated into the SCC*mec* element (Deurenberg *et al.,* 2005).

1.27.2 SCC*mec* Typing

SCC*mec* typing classifies MRSA into 11 distinct types based on *ccr* and *mec* genes differences of the SCC*mec* element. These molecular types are designated as SCC*mec* type I-XI. This technique is based on the PCR method (Havaei *et al.,* 2014; IWG-SCC, 2009). The techniques for SCC*mec* typing varied and developed gradually from time to time. The old techniques were based on traditional PCR methods of using multiple primer sets e.g. 20-30, that identified the individual *mec* and *ccr* gene complexes. One such example is of Okuma *et al*, that employed 21 primer sets and multiple single PCR experiments for identifying SCC*mec* types I-IV (Okuma *et al.,* 2002). This technique was further simplified by Kondo *et al*, to identify *ccr* and *mec* gene complexes and in the J-region major differences (Kondo *et al.,* 2007), but the same problem of requiring many primer sets and multiple single-target PCR reactions persisted (Kondo *et al.,* 2007).

Then, a better advancement in simplifying SCC*mec* typing was made when multiplex-PCR was developed by De-Lencastre and his colleagues for identifying SCC*mec* types I-V (Milheiriço, Oliveira, & de Lencastre, 2007; Oliveira & de Lencastre, 2002), but this technique was in the initial stages of development, had a problem of limitlessness in typeability and difficulty in interpretation. Later on, this technique was developed and simplified by many researchers (Milheiriço *et al.,*

2007; Oliveira & de Lencastre, 2002; Zhang *et al.,* 2012; Zhang *et al.,* 2005). Previous studies from Pakistan have reported SCC*mec* type III as the most prevalent one with prevalence of 47% (Asghar, 2014), 71% (Zafar *et al.,* 2011), and 77.27% (Arshad *et al.,* 2015). The spread of SCC*mec* types in MRSA strains has also been observed worldwide, SCC*mec* III, II were most predominant in Asia IV, II in America and Europe while IV and V were mostly distributed in Africa (Asadollahi *et al.,* 2018).

1.27.3 SCC*mec* Types in CA- and HA-MRSA

CA-MRSA and HA-MRSA can be differentiated by two important genetic markers, the PVL gene carriage and the structure of the SCC*mec* element (Boyle-Vavra & Daum, 2007). CA-MRSA strains are more contagious and communicable than traditional HA-MRSA strains (DeLeo *et al.,* 2010; Elie-Turenne *et al.,* 2010; Miller *et al.,* 2012). According to SCC*mec* types, HA-MRSA carries the large (34-67kb) SCC*mec* types I, II, or III, while CA-MRSA have the smaller SCC*mec* type IV, or occasionally type V or its variant, V_T (Boyle-Vavra *et al.,* 2005; Ito *et al.,* 2001; Ma *et al.,* 2002). The PVL gene is commonly associated with SCC*mec* type IV, and possibly with type V or V_T, but not with type I, II, and III. SCC*mec* type IV is small in size and most mobile among all SCC*mec* types (Boyle-Vavra & Daum, 2007). SCC*mec* typing revealed that *mec* type IV or V in combination with the PVL gene are the two important genotype markers for identifying CA-MRSA strains (Tristan *et al.,* 2007). Due to small size and functional recombinases, SCC*mec* type IV is the most mobile, and for this reason, it rapidly spreads in the community (Liu *et al.,* 2010). The type V SCC*mec* also resembles type IV as it also harbors the *mec*(A) gene and lacks other resistance determinants (Berglund *et al.,* 2008). Small SCC*mec* types are replacing the larger SCC*mec* types i.e. I, II, and III in the hospital settings (Strandén *et al.,* 2009). In literature, PVL correlation with SCC*mec* types is reported as 90% and 62% in type IV and V SCC*mec* types respectively (D'Souza *et al.,* 2010; Hannan *et al.,* 2015)

1.27.4 Significance of SCC*mec* Typing

Molecular typing is important for a particular pathogen to determine its origin, dissemination, and evolution that will further help in developing a strategy to control its spread (de Sousa, 2004). SCC*mec* typing is not only important to identify MRSA but also for assigning proper definition to the MRSA clones (Milheiriço *et al.,* 2007). The importance of such typing is well described in the literature for proper identification of MRSA clones. A proposal was put forward by Enright and his colleagues which was agreed, in 2002, by an international sub-committee of International Union of Microbiology Societies, Japan that the MRSA clones supposed to be named by both the MLST and SCC*mec* typing information, e.g. ST5-MRSA-II (Enright *et al.,* 2002).

1.28 Staphylococcal protein A (*spa*) typing

Spa typing is characterized as an essential virulence factor and has been revealed to be associated with *S. aureus* pneumonia pathogenesis and is expressed in invasive diseases helping in proteinaceous biofilm formation. *Spa* (42 kDa) protein contains several sections each with different functions: such as N-terminal known as signal sequence (S region) with 4-5 extremely homologous immunoglobulin G (IgG)-binding domains in tandem (A, B, C, D, and E regions) (Merino *et al.,* 2009). The C-terminal region also known as the X region has 2 domains: first, an octapeptide structure, the repeat region XR, used for *spa* typing, and the XC involved in fixing of the cell wall. The function of *spa* protein is to interact with Fc Portion of human IgG resulting in compromising the host immune system. *Spa* typing was developed which required only one locus sequencing, specifically repeated short sequence region of Protein A gene and having a better discriminatory power than MLST (Khandavilli *et al.,* 2009). *Spa* typing uses a variable number of tandem repeats of 24bp sequence of the polymorphic X region, as a genetic marker. Certain *spa* types are more prevalent in certain regions of the world. In Denmark, an increase in numbers of t437 isolates was observed between 2006 and 2008 (Song *et al.,* 2011). In Europe and Asia, an increase in *S. aureus* having *spa* type t437 isolates was observed (Brauner *et al.,* 2013). t030 is more prevalent in hospitals from Turkey while t044 and t042 in the US and North Africa, while in Kenya all MRSA have

ST239 (t037) which is a globally distributed hybrid of ST30 and ST8, and is responsible for numerous outbreaks in many continents (Omuse *et al.,* 2016).

One study reported a total of 45 dissimilar *spa* types, with type t002 as the most frequent one (6%), followed by *spa* types t127, t318 and t008 (3% each), besides that t002 has been most frequently reported *spa* type among MSSA in 26 European countries (Satta *et al.,* 2013). Another study from Iran categorized the molecular types into five *S. aureus spa* types; t044 (7.7%), t969 (11.1%), t790 (16.7%), t030 (22.2%) and t037 (33.3%) while 8.9% isolates were NT (Goudarzi *et al.,* 2016). Previous study from Bosnia and Herzegovina reported the predominant *spa* types t030 and t037 in *S. aureus* isolates collected during December 2009 to May 2010 (Uzunović-Kamberović *et al.,* 2013). Another study reported 18 MRSA and 22 MSSA having diverse *spa* types, where predominant *spa* type in MRSA was t037 (40.6%), while in case of MSSA they were quite diverse, only 2/23 MSSA shared identical *spa* types, 2 new *spa* types in MRSA (t13149, t13150) and 3 in MSSA (t13194, t13193 and t13182) were also identified (Omuse *et al.,* 2016).

1.29 Accessory gene regulator (*Agr*) typing

1.29.1 Quorum sensing in *S. aureus*

In the case of staphylococci and other facultative pathogens, primary interactions between the bacterium and host occur through secreted proteins or proteins bound to the bacterial outer cell surface. Such protein products help the organism in escaping from host defenses, in sticking to tissue matrix and cells, in colonizing the host and in degrading tissues and cells, for both nutrition and protection and are encoded by accessory genes, together known as the virulon. The virulon genes are regulated in a highly synchronized manner coupled with the biological requirements of the organism. Staphylococci demonstrate this principle as they have developed a major quorum sensing system (QS) accessory gene regulatory system (*agr*), representing both central regulatory element controlling virulon expression and determinant of evolutionary diversification (Wright *et al.,* 2005).

1.29.2 The *agr* locus

Expression of *agr* locus controlled by two divergent promoters such asP2 and P3 . The 4-gene operon having *agr* B, D, C, and A form the P2 transcript. *Agr* A and C comprise a classical two-component signaling system (TCS) and *Agr* B and D join to generate the activating ligand which contains a 5 amino acid thiolactone (or, in one case, a lactone) ring and itself is a 7-9 amino acid peptide. *Agr* C is bound and activated by AIP, which in turn activates *Agr* A and then active *Agr* A up-regulates its own promoter (P2) (Novick *et al.,* 1995). *Agr* A is also transcribed by an independent weak promoter, P1 whose role is unclear in the regulatory circuit. Gram-positive bacteria use a similar mechanism of auto-activation systems (Kleerebezem *et al.,* 1997). *Agr* belongs to an extremely complex regulatory network in which *agr* expression is regulated by numerous regulatory genes. These regulatory genes directly or indirectly up or down regulate *agr*. These include *sarU*↑, *sarX*↑, *sarA*↑, *srrAB*↓, *mgrA*↑, σβ↓ and *arlRS*↓ (Bénédicte *et al.,* 2001). Other researchers suggested that studying the overall control of the accessory gene regulon and mechanisms by which these regulatory elements affect *agr* regulation could help locate sites where interference would be clinically beneficial (Novick & Geisinger, 2008). One study from Pakistan reported that among *agr* groups *agr* I (45.8%) was most prevalent, followed by *agr* III (29.1%) and *agr* II (4.1%), while no *agr* IV was found in MRSA isolates (Khan *et al.,* 2014). Other studies have also reported *agr* I as the most dominant in MRSA isolates with prevalence of 54.8% from Iran (Mohsenzadeh *et al.,* 2015) 64.3% from Iran (Goudarzi *et al.,* 2016) and 86% from India (Jain *et al.,* 2019).

1.29.3 Correlation of *agr* group with specific clinical features

In *S. aureus*, the *agr* group correlates with the clinical features of the infection. Most exfoliatin-producing strains are from *agr* group IV (Gilot *et al.,* 2002), while VISA isolates belong to *agr* group II. (Sakoulas *et al.,* 2002). According to Dufour *et al*, as MGEs encode PVL, TSST-1, and Enterotoxin so it suggests either *agr* group-specific selective factors or some kind of linkage disequilibrium in MGEs play a role in their spread (Dufour *et al.,* 2002).

1.29.4 *Agr* function in *vivo*

Agr controls a variety of virulence factors in *S. aureus*. The *agr* mutant strain is reported to have attenuated virulence in mammalian models, exhibiting reduced virulence in *Galleria mellonella* (Peleg *et al.,* 2009). A study was conducted regarding ventilator Staphylococcal pneumonia on large scale which showed that only 20% of the infection causing organisms were *agr*-positive. Although this revealed that diseases may have been caused by *agr*-negative organisms but the patients infected with *agr*-negative organisms showed significantly better outcome than those infected with *agr*-positive organisms (Novick & Geisinger, 2008; Wright *et al.,* 2005). A few other studies reported that *S. aureus* human clinical isolates are *agr*-positive and expressed the *agr* poorly (Fowler *et al.,* 2004; Sakoulas *et al.,* 2002).

1.30 Hypervariable region (HVR)

In 1991, a new region between IS431-*mec* and the *mec*(A) gene in SCC*mec* was described (Ryffel *et al.,* 1991) which was termed as the HVR because of DNA length polymorphism. One MRSA strain's HVR region was sequenced and ten repeat units of 40bp each were identified (Ryffel *et al.,* 1991). Most of the studies either have analyzed the hybridization patterns of these direct repeat units PCR products or have compared the gel band sizes (Nishi *et al.,* 1995) while direct repeat unit (dru) region was sequenced in one study (Nahvi *et al.,* 2001). Since the number of these repeat units may be different among isolates, the amplification of HVR region can be used to type and classify MRSA strains. One study reported eight types of HVR which were HVR types 3, 5, 7, 8, 9, 10, 11, and 12 direct repeat units (dru) (Senna *et al.,* 2002). Another study from Iran reported HVR types 1-11 (Nia *et al.,* 2013).

1.31 Multilocus Sequence Typing (MLST)

MLST is the process in which microorganisms are characterized via the identification of alleles using DNA sequences of internal fragments of housekeeping genes. Seven housekeeping genes are selected because of their availability in specific species and show enough distinction within the species (Sabat *et al.,* 2006). The seven housekeeping genes dispersed all overthe genome, augmentingthe

discriminatory power of the technique (Cooper & Feil, 2004). The sequencing of the PCR products and the number characterize the allele is determined to each locus on the basis of its sequence. Allele sequence for *S. aureus* is 645,163 which was last updated on 04-06-2021 (https://pubmlst.org/organisms/staphylococcus-aureus). Sequences in MLST highly conserved and evolve slowly. MLST is not preferably used for short epidemiological studies (Pourcel *et al.,* 2009).

According to the literature, ST80 is a leading CA-MRSA strain of Europe, while in the USA it was ST8, in Australia ST93 is the most prevalent one, ST30, and ST59 (t437) were reported from Oceania and Asia, respectively (Tristan *et al.,* 2007). A related study identified a major clone known as, ST59-MRSA-t437 in Asia and Western Australia, CC59 clones were incidentally reported from Europe as well as from USA (Coombs *et al.,* 2010; Ho *et al.,*2012; Ho *et al.,* 2008; Ko *et al.,* 2005; Wu *et al.,* 2010). One study from Belgium reported that four isolates had *spa* type t437, and the dominant sequence type in CA-MRSA were ST338 (Brauner *et al.,* 2013). A collective research showed in 16 European countries identified 22 (6%) *S. aureus* isolate Clonal Complex (CC) 59 had *spa* type t437, out of which one isolate was MSSA. Interestingly, another group reported the most dominant clone ST59 with increased frequency since 2007 in Northern Europe (Poland, Sweden, and Finland) (Glasner *et al.,* 2015). In the United States, MRSA population encompasses two main MRSA lineages USA100 and USA300 (both are PFGE types rather than ST type); USA100 is assigned to ST8 and *spa* type t008 while USA300 also belongs to ST8 with t008 harboring the PVL toxin; both are ST8 but they have different PFGE banding responsible for causing soft tissue and skin infection (STSI) in children. The USA100 MRSA lineage having ST5 (CC5), t002 (CC002) were PVL-negative and a common reason for *S. aureus* infection in inpatient settings (O'Hara *et al.,* 2016).

The local predominant strain in India and Pakistan among hospital MRSA strains was ST239, the foremost sequence type observed (Shabir *et al.,* 2010). The ST772 a single locus variant (SLV) of ST1 (MW2) (USA400) was first time reported from Malaysia and Bangladesh (Afroz *et al.,* 2008). SLV are bacterial sequence type that

differ at only one test loci from the ancestral genotype (Yu *et al.,* 2012). One study from India reported the occurrence of ST772, ST22, and ST239 in *S. aureus* isolates (Goering *et al.,* 2008).

1.32 Virulence factors

Various virulence factors such as the production of several enzymes like hydrolytic enzymes, lipases, esterases, fatty acid modifying enzymes, proteases, and catalases, etc. and the presence of various surface components e.g. teichoic acid, peptidoglycans, protein A and capsule, etc. contribute to *S. aureus* pathogenesis. Toxin production is also one of the key virulence factors associated with *S. aureus* pathogenesis (Vasconcelos & Cunha, 2010). *S. aureus* produces a variety of toxins such as staphylococcal enterotoxins (SEs), leucocidin, exfoliatin, hemo that could have participated in pathogenicity. SEs are heat-stable proteins that are accountable for 95 percent of staphylococcal food poisoning (Al-Tarazi *et al.,* 2009).

1.32.1 Alpha (α)-hemolysin

α-hemolysin, which is a pore-forming, cytolytic toxin produced by *S. aureus* (Diep & Otto, 2008) is one of the key virulence factors studied in pathogenesis of *S. aureus*. α-hemolysin has a cytolytic nature against a variety of host cells and may target various human cells like lymphocytes, epithelial cells, and keratinocytes (Wardenburg & Schneewind, 2008). α-hemolysin and Phenol-Soluble Modulin (PSMα) are less frequently present in HA-MRSA strains. They are also found in a large number of CA-MRSA lineages (Li *et al.,* 2009; Wang *et al.,* 2007). One study reported that α-hemolysin plays a crucial role in biofilm formation (Caiazza & O'toole, 2003) *S. aureus* strains carry α-hemolysin and PVL toxin that are involved in causing severe infections in humans (Caiazza & O'toole, 2003). The pore-forming alpha-toxin encoded by the *hla* gene found in 95% strains of *S. aureus* (Grumann *et al.,* 2014). Hassan *et al*, reported the prevalence of the *hla* gene in 56.9% of isolates while *hlb* gene was in 93% isolates (Hassan *et al.,* 2012).

1.32.2 Beta (β)-hemolysin

β-hemolysin, also known as the Hot-Cold toxin shows substantial lysis activity against human monocytes and sheep, cattle and cow erythrocytes (Larsen *et al.,*

2002). Studies from Moscow, Idaho and India stated that β-hemolysin is sphingomyelinase which hydrolyzes the unique lipids present in the plasma membrane known as sphingomyelin and does not create a pore in the cell membrane (Huseby *et al.,* 2007; Ira *et al.,* 2013). Studies have reported the β-hemolysin gene in 42.5% from Bulgaria (Rusenova *et al.,* 2013), 85.88% from Iraq (Degaim *et al.,* 2015), 11.59% from Iran (Motamedi *et al.,* 2018) and 37.4% isolates from Iran (Mir *et al.,* 2019).

1.32.3 Gamma (γ)-hemolysin

A bi-component exotoxin which is composed of 6 multiple proteins such as leukocidins shows a superior hemolysis activity against horse's erythrocytes (Dinges, Orwin, & Schlievert, 2000). There are numerous pore-forming toxins (PFTs) that exist in *S. aureus.* Other than **γ**-hemolysin including 2 polypeptides, S and F proteins of 32–35 kDa that have been recognized as Pore-forming toxins. These polypeptides are classified according to differences in their electrophoretic capacities and they are involved in the construction of mature pore. A similar structural constitution is observed in bi-component pore-forming toxins produced by *S. aureus* (DuMont *et al.,* 2011). γ-hemolysin is composed of an F component i.e. *hlg* B which is associated with either *hlg* C or *hlg* A, that are examples of S component. Hemolysin genes such as *hla*, *hlb,* and *hld* are commonly found in most strains of *S. aureus*. However, there is a conflict about frequency of *lukED* gene. In one study, from New York, USA the incidence of *lukED* genes was reported in 87% cases of *S. aureus* isolates (Alonzo *et al.,* 2012). One study showed that most of the bloodstream infections found in animals and humans were usually associated with hemolysins produced by *S. aureus* (Takeuchi *et al.,* 2001). Nahar *et al*. reported that 63.33% of isolates harbored *hla* gene, 75% of isolates were positive for *hld* gene, 15% carried *hlb* gene, while 11.67% for *hlg* gene(Nahar *et al.,* 2017).

1.32.4 Panton-Valentine Leucocidin (PVL) toxin

PVL toxin comprises of two co-transcribed genes, LukFPV and LukSPV (Panton, Came, & Valentine, 1932), LukGH also called LukAB (DuMont *et al.,* 2011) and LukED, which are examples of Pore-forming Toxins (PFTs) (Gravet *et al.,* 1998;

Morinaga *et al.,* 2003). PVL is a leukocidin which is present in both MRSA and MSSA (Kaltsas *et al.,* 2011). PVL is generally linked with *S. aureus* involved in community-acquired infections, while this toxin is not significantly observed in nosocomial infections. Approximately all *S. aureus* showing resistance against methicillin have PVL in them, responsible for community-acquired infections, such as pneumonia, lungs tissues, SSTI but not involved in enterocolitis, mediastinitis, nosocomial pneumonia, carditis, urinary tract infection and those related to TSS.

USA300 and USA400 strains which are methicillin-resistant and associated with the community have PVL toxin, cause SSTI in humans, while nosocomial MRSA lack PVL toxin (Chua *et al.,* 2014). Studies from Pakistan have reported the prevalence of PVL in 33.33% in MSSA from Peshawar (Khan *et al.,* 2018), 46% in MSSA from Lahore (Iqbal *et al.,* 2018) and 40% in MRSA isolates from Lahore (Muhamad Sohail & Zakia Latif, 2018)

1.33 *Galleria mellonella* (*G. mellonella*) infection assay

Invertebrate models have been used as a valuable tool to assess either fungal or bacterial pathogenesis. *G. mellonella* belongs to family Pyralidae of Lepidopteran order (Paddock, 1918). This greater wax moth is now used as an emerging and facile host model. Its natural habitat is beehives, as it feeds on honey, beeswax, and pollen. It often come across several pathogenes, and established its own immune response. *G. mellonella* host model provides a useful platform for microbial pathogenesis. In *S. aureus* pathogenesis the introduction of *G. mellonella* proves a useful host model (Kaito *et al.,* 2002). The *agr* system mainly controls a variety of virulence factors in *S. aureus,* while *agr* mutants strains exhibit attenuated virulence in mammalian models, and demonstrate reduced virulence capacity in the *G. mellonella* model (Peleg *et al.,* 2009).

1.33.1 Benefits of *G. mellonella* host model

G. mellonella has many advantages to use as a model host for a variety of pathogens, as compared to other invertebrate host models. For example, insect larva can be maintained over a diverge array of temperatures ranging from 25°C to 37°C, other invertebrate models such as *Drosophila melanogaster* and *Caenorhabditis*

elegans do not facilitate testing at 37°C. Wax moth larva infection model also has many other benefits as compared to other non-mammalian models. First, the insect has the same innate immune response as mammals and share the same killing mechanism of action (Kavanagh & Reeves, 2004). Second, the wax moth model showed good physiological responses at 37°C as do the human body, as many virulence factors expresses at this temperature. (Konkel & Tilly, 2000). Third, an exact amount of pathogen inoculum can be injected directly iside the larvae. Previously *G. mellonella* (wax moth model) used for the screening to know about the effectiveness of antimicrobial agents (Coughlan *et al.,* 2010; Mylonakis *et al.,* 2005; Peleg *et al.,* 2009). The model has fast reproduction time, no ethical approval was required, and is inexpensive and simple. An exact amount of bacterial dose inoculation is done, resulting in consistent survival rates. The pathogen is delivered directly inside the larva , which meticulously mimics the conventional route of administration applied in mammalian models (Tsai *et al.,* 2016).

1.33.2 *S. aureus* infection assay

G. mellonella seems to be specifically and actively killed by *S. aureus*. Higher post inoculation temperature also greatly affects the motility of the wax moth model (Desbois & Coote, 2011). Some of the bacteria have temperature-regulated virulence-associated factors, that could be considerfor these observations, and their existence in *S. aureus* (Ziebandt *et al.,* 2004). However, when bacteria grow and divide, they produce their virulence factors quickly at 37ºC as compared to 30ºC and 25°C, which promotes larva killing. The exact mechanism of *S. aureus* pathogenesis in wax moth larva remains mysterious, and it is vital to recognize the important virulence factors utmost relevant in this model. The small lifespan of *G. mellonella* model makes them an ideal model for high throughput studies. An adaptive immune response has been developed in invertebratea, while their innate immune response show resemblances with the insect's immune response (Browne *et al.,* 2013).

1.33.3 *G. mellonella* used as a model to study the virulence factors of Gram-positive bacteria

This model has been widely used to examine various Gram-positive and Gram negative bacteria, also *S. aureus* (Cotter *et al.,* 2000; Peleg *et al.,* 2009; Quiblier *et*

al., 2013), *Enterococcus faecalis* (Evans & Rozen, 2012; La Rosa *et al.,* 2013), *Streptococcus pyogenes* (group A streptococcus) (Olsen *et al.,* 2011), *Enterococcus faecium* (Junior *et al.,* 2013), *Streptococcus pneumoniae* (pneumococcus) (Evans & Rozen, 2012) and *Listeria monocytogenes*. One study noticed that killing of *S. aureus* in *G. mellonella* larvae work in a dose-dependent manner where $1x10^7$/CFU cell of *S. aureus* was sufficient for the complete killing of larvae after 24 hours of infection, whereas infection with $1x10^5$/CFU killed only 20% of larva after 120 hours. More killing was observed at high temperature while less at decreasing temperature showed the temperature-dependent process, when tested at 37ºC other than 25ºC and 30°C. (Desbois & Coote, 2011).

1.33.4 *G. mellonalla* immune system

The wax moth model consists of 2 main i-e the cellular and antibody mediated immune response. Phagocytic cells mainly play a main part in cellular response, known as hemocytes, found in hemolymph, act as a similar functions as the mammalian blood, but also help in clotting and encapsulation while in case of humoral response the effector molecules kill or immobilize the pathogen, contain melanin, , and antimicrobial peptides (Tsai *et al.,* 2016).

1.33.5 Cellular immune response

In insects, almost 8 types of hemocytes are found (Boman & Hultmark, 1987). Different hemocytes have been recognized in the *G. mellonella* model (Boman & Hultmark, 1987). These include spherulocytes, oenocytoids, plasmatocytes, granular cells, prohemocytes, and coagulocytes. In *G. mellonella*, granular cells and plasmatocytes play a significant role in cellular defense and also in nodule formation, phagocytosis, and encapsulation (Tojo *et al.,* 2000).

In mammals and insects, phagocytosis is supposed to be very analogous and involves both granular cells and plasmatocytes (Tojo *et al.,* 2000). Once phagocytized, several mechanisms are involved in the killing of the pathogen including ROS (reactive oxygen species) e.g., superoxide generated by the oxidative burst, initiated by the NADPH oxidase complex (Choi *et al.,* 2002).

1.33.6 Humoral immune response

Several plasma proteins in *G. mellonella* are produced which serve as opsonins, binding with conserved microbial components that follow a similar mechanism as pattern recognition receptors in mammals. Apolipophorin-III (apoLp-III), act as a pattern recognition molecule in the case of the innate immune response of the larva model. ApoLp-III reveals a high affinity for lipoteichoic acid (LTA) and bacterial lipopolysaccharide (LPS). Immunoglobulin protein known as hemolin, binds with the LTA and LPS and associates with the hemocytes (Choi *et al.,* 2002).

In *G. mellonella* 18 putative or known antimicrobial peptides are found in hemolymph including, galiomycin, heliocin-like peptide, Gm anionic peptide 1 and 2, 2 cecropins 5, gallerimycin, Gm proline-rich peptides 1 and 2, x-tox, moricin-like peptides, gloverin, lysozyme, inducible serine protease inhibitor 2 (Brown, Howard, Kasprzak, Gordon, & East, 2009). Other antimicrobial peptides, known as galleria defensin defending, an insect defensin, purified from larval hemolymph acts against *E. coli* (Kim *et al.,* 2004). Brown *et al*, first identified in *G. mellonella* that moricin like peptides show good activity against filamentous fungi (Brown *et al.,* 2008).

1.34 *In vitro* infection assay (macrophage infection assay)

Classically *S. aureus* was considered as an extracellular pathogen but it has also the capability to survive inside macrophages and neutrophils, and other non-phagocytic cells (Strobel *et al.,* 2016). Adhesion proteins such as fibronectin-binding proteins (FnBPs) play a major role in the uptake of bacteria by non-professional phagocytes, indirectly cross-link with the α5β1 integrins host cell receptor, by forming fibronectin bridges (Fraunholz & Sinha, 2012). However, integral endothelia or epithelia commonly never expose α5β1 integrins, since these molecules somewhat are set up on the basolateral membrane in the tissues. *S. aureus* holds different tactics that permit rupturing of barriers irrespective of the activation state of its key virulence regulator, the *agr* system (quorum-sensing), active *agr* have a role in up-regulation of α-toxin production, while in case of protein A *agr* is inactive (Gomes-Fernandes *et al.,* 2017).

1.34.1 Small colony variants (SCVs) and intracellular persistence of *S. aureus*

Once bacteria enter inside the host cell, it provides a niche for its prolonged survival (Kubica *et al.,* 2008). Such long-term survival supports *S. aureus* to establish chronic infection within the host cell. Another feature of *S. aureus* is the formation of SCVs by making changes in the proteome and transcriptome of the pathogen (Garzoni *et al.,* 2007). These SCVs are often weakly pigmented, non-cytotoxic, non-hemolytic and have thick cell walls (Proctor *et al.,* 1994). Less cytotoxicity and reduced hemolysis of SCVs result in the low expression of *agr*, lead to fewer virulence factors secretion, and overproduction of cell wall adhesins (Tuchscherr *et al.,* 2015). Besides *agr,* the sigma factor σB have role in facilitating the shift from the toxin-producing phenotype to the metabolically dormant stage, continuing SCVs phenotype. σB mutants in an infection model were incapable of producing SCVs, which are cleared within days by the host immune system. (Tuchscherr *et al.,* 2015). One study reported that about 25% of the initial inoculum of normal *S. aureus* used for infection switches to a SCVs phenotype (Tuchscherr *et al.,* 2011) in addition to cell line models as well as*in vivo* mice model within bones and kidneys and are challenged intravenously with *S. aureus* (Horst *et al.,* 2012).

1.34.2 *S. aureus* survival within phagocytes

S. aureus lives and proliferates inside professional phagocytes, escape from the phagosome and is involved in host cell death (Gresham *et al.,* 2000). *S. aureus* can live inside vacuoles for 3-4 days of human-derived macrophage (Kubica *et al.,* 2008) and for 5-7 days within osteoblast (Hamza & Li, 2014) prior to escape into the cytoplasm and killing of the host cell. Macrophages could not sustain antibacterial activity as time goes on, bacteria were injested by the phagocytes , phagosome acidification was diminish which was consistent manimize the stimulation of cathepsin D and diminished bactericidal activity. This uninterrupted lysis cycle and ingestion sustain a pool of intracellular bacteria gradually over time, and trigger the *S. aureus* persistence in murine model (Jubrail *et al.,* 2016).

Previously published literature reported that Transmission electron microscopy (TEM) was used to study the localization of intracellular *S. aureus* in RAW 264.7

macrophages. MRSA (ATCC43300) and MSSA (ATCC25923), and MDR strain of *spa* type (t034) (X. Wang *et al.,* 2018) results showed that without damaging the host cell these three pathogens entered the RAW 264.7 macrophages and were contained in small vacuoles (tight phagosome). Other studies also reported the localization of intracellular *S. aureus* (Seral *et al.,* 2003), and in J774 and THP-1 macrophages (Barcia-Macay, 2006).

One study reported that RAW 264.7 macrophage and HL-60 human cells had the capability of killing microbes after differentiation (Nüsing *et al.,* 1993). Another study found that *S. aureus* sustainability diminished rapidly in RAW 264.7 displaying no signs of intracellular persistence or proliferation (Seto *et al.,* 2011).

S. aureus is an important pathogen which can escape from phagosomes and proliferate within the host cell cytoplasm. One study reported that *S. aureus* was engulfed by RAW264.7 macrophages but not digested by the cells, proposing that the maturation of phagosomes was blocked in macrophages. Further investigation showed that peptidoglycan (PG) induced the process of phagosome maturation into macrophages, subsequently leading to the eradication of *S. aureus*. Due to PG, the Janus kinase- signal transducer and activator of transcription (JAK-STAT) pathway was activated during the process of phagosome maturation of macrophages against *S. aureus*. These results showed that for phagosome maturation, JAK-STAT pathway activation was mandatory (Zhu *et al.,* 2015). Defense against the pathogen is the requirement of phagocytosis, yet the exact mechanism of phagocytosis against *S. aureus* remains unknown.

In the current study, presented in this thesis, we analyzed the rate of MRSA infections, because of the deficiency of comprehensive studies in our local hospital settings. We also examined phenotypic antibiotic resistance and antibiotic resistance genes, molecular typing, clonal relatedness, and pathogenicity of clinical *S. aureus* isolates. It is a comprehensive study which contributes to the knowledge of antibiotic resistance in MRSA and MSSA isolates, molecular typing, and pathogenicity in our local *S. aureus* isolates.

Aim and objectives of this study

This study aimed to investigate the antibiotic resistance, molecular characterization and typing, and pathogenicity of multi-drug resistant *S. aureus* isolates.

Objectives of the study
The objectives of the study were as following:

- To study the prevalence and antibiotic susceptibility profiles of multi-drug resistant *S. aureus* from the Pakistan Institute of Medical Sciences (PIMS) Hospital
- Molecular detection/characterization of antibiotic resistance genes (*bla*(Z), *mec*(A), *mec*(C), *vat*(A), *aacph*D1, *tet*M, *erm*(A), *tet*K, *erm*(C), *vat*(B), *fos*(A), *fos*(B), *gyr*(A), *gyr*(B), *grl*(A), *vat*(C), *grl*(B), *rpo*(B), and *dfr*(B))
- To evaluate the molecular types of the study isolates using *agr,* HVR*, spa*, SCC*mec*, MLST and the clonal types by PFGE
- Detection of hemolysins (*hla, hlb, hld,and hlg*), and other virulence genes such as, *saeS, codY, rot, spo*VG*, yyc*G*, arls, sar*A*, clf*A*, sas*G*, fnb*B*, fnb*A, PVL, *sigB, sea, seb, sec* in study isolates
- To determine the intracellular survival and pathogenicity of selected *S. aureus* strains using *in vitro* Raw 264.7 cells and *in vivo G. mellonella* infection model

Chapter 2

Materials and Methods

2.1 Sample collection

A total of 1528 clinical staphylococci isolates were collected from the Microbiology laboratory of the Pakistan Institute of Medical Sciences (PIMS), Islamabad over a period of 16 months, from April 2015 to July 2016. Study isolates were collected from different clinical specimen including invasive isolates such as, pus, blood, catheter tip, cerebrospinal fluid, central venous pressure (CVP) tip, and non-invasive isolates such as, sputum, tip of drain, tracheal secretions and urine) from patients of both genders and varying ages. Information regarding patient age, gender, ward, and clinical specimen was also collected from the hospital database.

2.2 Ethics

Ethical approval (No. F. 1-1/2015/ERB/SZABMU) was obtained from Ethics Review Board of PIMS, Islamabad.

2.3 Microbiological analysis of clinical *S. aureus* isolates

2.3.1 Gram staining

Vigilantly selected colonies from either nutrient or tryptic soy agar (TSA) were processed for Gram staining for further confirmation.

2.3.1.2 Smear preparation

For smear preparation, a drop of sterile normal saline was placed on a glass slide. Then using a sterile loop, a portion of an isolated colony from the aforementioned agar was transferred to normal saline and a thin smear was made. To make a uniform smear circular movement was used. The smear was left to air dry.

2.3.1.3 Smear fixation

The air-dried smear was then fixed with help of upper part of a flame. After the fixation, slide was kept cool.

2.3.1.4 Staining

The fixed smear was flood with crystal violet for 60 seconds, washed off with tap water then all the water was tipped off. Next, it was treated with Gram's iodine solution, waited for 60 seconds, wash with tap water and all the water was tipped off. Followed by decolorizing step with alcohol acetone for a few seconds and instantlywash with tap water, and counterstained for 30 seconds with safranin and then washed. . The slide was later allowed to air dry completely and was observed under a 100x (oil immersion) lens.

2.4 Strains Selection

Mannitol salt agar (MSA), (Oxoid, UK) was used for this purpose, which is a selective as well as differential media. It has a high concentration of sodium chloride (NaCl), which makes it selective for *S. aureus* and the indicator phenol red is responsible for the change in colour of the medium. All the isolates were inoculated onto MSA and incubated at 37°C for 24 hours. Mannitol fermenting isolates produced acidic by-products, which produced yellow colonies and yellow zones in plates whereas non-fermenters did not change the color of the media. The primary isolates obtained from the hospital were grown on MSA to select *S. aureus*.

2.5 Biochemical Identification

S. aureus isolates were confirmed by following biochemical tests:

2.5.1 Catalase Test

It is used to differentiate *Staphylococcus* species from *Streptococcus* species. The presence of the catalase enzyme in the test organism was detected by using hydrogen peroxide (H_2O_2). *S. aureus* is catalase positive. A drop of three percent H_2O_2 was applied onto a sterile glass slide, pick a colony with sterile wire loop and mixed with H_2O_2, bubble production indicated a positive result, as catalase enzyme that converts H_2O_2 into O_2 and H_2O. No bubble formation indicated the catalase-negative organism.

2.5.2 Coagulase test

This test was done to differentiate *S. aureus* from other species of staphylococci. *S. aureus* produces coagulase enzyme to convert soluble fibrinogen to insoluble fibrin. *S. aureus* can produce both bound and free coagulase. For the identification of *S. aureus*, a test tube coagulase test was performed.

2.5.3 Tube coagulase test

A tube coagulase test was performed for the detection of free coagulase production. For tube coagulase, a single bacterial colony was inoculated in the test tube containing 500 µl sterile nutrient broth (NB) and incubated it at 37℃ for 18-24 hours. Two drops of human plasma were added to each test tube and incubated at 37℃ for 4-6 hours After incubation of 4 hours, coagulation of plasma indicated the presence of free coagulase. A positive coagulase test was considered as an indication of that the isolate was *S. aureus*.

2.5.4 DNase test

S. aureus can break down DNA by producing a deoxyribonuclease (DNase) enzyme. One of the confirmatory tests used for *S. aureus* identification was the DNase test. To detect DNase activity, a single line of bacterial culture was streaked on DNase agar and was incubated at 37℃ for 18-24 hours. DNase agar contains nucleotides in its composition. On overnight incubation, bacteria will grow and degrade DNA, after that 1 molar hydrochloric acid (HCl) was applied on the plate to detect a zone of clearing and precipitation from the agar. The formation of a clear zone is because individual nucleotides are soluble in HCl while DNA is not soluble and so rest will give precipitation on the plate. Those bacterial isolates, which were positive for mannitol salt fermentation, catalase, DNase, and tube coagulase tests were considered as *S. aureus*

2.6 Antibiotic Susceptibility profiling

2.6.1 Disk-diffusion method

Antibiotic susceptibility tests were performed using the Kirby-Bauer test (Bauer *et al.,* 1966), and results interpretation was done using the guidelines of the Clinical and Laboratory Standard Institute (CLSI) 2017. *S. aureus* ATCC 25923 was used as a reference strain. Mueller Hinton agar (MHA) (Oxoid, UK) was prepared according

to the manufacturer's instructions. A panel of antibiotics was tested including, linezolid (30µg), chloramphenicol (30µg), rifampicin (5µg), trimethoprim/sulfamethoxazole (25µg), cefoxitin (30µg), tetracycline (30µg), ciprofloxacin (5µg), quinupristin/dalfopristin (15µg), gentamicin (10µg), clindamycin (2µg), and erythromycin (15µg) (Oxoid, UK).

2.6.2 Protocol for Disk diffusion method

- MHA plates were prepared.
- 1 ml sterile normal saline was taken in a sterilized 1.5 ml Eppendorf tube.
- Three to 5 isolated colonies were picked with the help of a sterile cotton swab.
- Sterile swab was inoculated in the tube containing normal saline and well mixed.
- 0.5 McFarland solution was used to adjust the turbidity of this suspension.
- Then a sterile swab was dipped into the turbid solution and pushed against the walls of the tube to drain out extra fluid.
- The growth lawn was prepared with the help of a sterile swab. This was done within 15 min of the turbid solution preparation.
- With the help of sterilized forceps antibiotic disks were placed (within 15 min of the lawn preparation).
- The antibiotic disks were placed 24 mm apart from each other on the MHA media plates for sensitivity testing.
- Incubation was done for 18-24 hours at 37°C.
- The inhibition zones were measured with the help of a ruler and readings were recorded.

2.6.3 Screening for cefoxitin resistance (MRSA)

S. aureus isolates were screened for methicillin resistance by using cefoxitin (FOX) antibiotic of 30 µg concentration (FOX30) by the Kirby-Bauer disk diffusion method (Bauer *et al.,* 1966). The test organisms were streaked on MHA plates and incubated at 37°C. Zone diameters were measured after 18-24 hours, and isolates with clear zone diameter of 21mm or less were considered as MRSA. Results were interpreted following CLSI guidelines, 2017.

2.6.4 Clindamycin inducible resistance based on D-test in study isolates

2.6.4.1 Double Disk Diffusion test (D-test)

Strains that were resistant to erythromycin (zone size ≤ 13mm) and sensitive to clindamycin (zone size ≥ 23mm) with a D-shaped zone of inhibition around clindamycin (D-test +ve) were labeled as clindamycin inducible phenotype. Results of the D-test were interpreted according to CLSI guidelines, 2017.

2.6.4.2 Materials required for D-test

- MHA plates
- Clindamycin sensitive strains
- Clindamycin disks (2 µg)
- Erythromycin disks (15 µg)
- Sterile swabs, Normal saline (0.9%)
- 0.5 McFarland
- Sterile forceps

Sterile needle 2.6.4.3 Protocol

- Three to five isolated colonies were picked with the help of sterile swab
- Sterile swab was inoculated in the tube containing normal saline and well mixed
- Turbidity of this suspension was adjusted to 0.5 McFarland solution
- Prepared bacterial lawn on MHA plate
- Placed antibiotic disks with the help of needle
- Clindamycin and erythromycin disks were placed 15-20mm apart
- MHA plates were incubated at 37°C for 24 hours

2.7 Minimum Inhibitory Concentrations (MICs) Determination

For all the resistant isolates MICs were determined against various antibiotics including cefoxitin, ciprofloxacin, chloramphenicol, rifampicin, vancomycin, tetracycline, erythromycin, penicillin, gentamicin, and sulfamethoxazole/trimethoprim using the microbroth dilution method (Andrews, 2001), while agar dilution method for fosfomycin and results were interpreted following CLSI guidelines, 2017.

2.7.1 Micro Broth dilution Assay

- Desired strains were grown to OD625= 0.08 – 0.1 (0.5 McFarland)
- The range of antibiotics concentrations were prepared to encompass the breakpoints; 512 µg/ml, 256 µg/ml, 128 µg/ml, 64 µg/ml, 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml, 2 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.125 µg/ml
- A 512 µg/ml dilution of antibiotic was made in Mueller Hinton broth (MHB)
- Added 50 µL of inoculum in 11 wells. In 12th well, added inoculated MHB as a standard.
- Added 100 µL of the antibiotic solution in well No. 1. From this well transferred 50 µL to next well, mixed and added 50 µL from each well to the next well, till well No. 11.
- Carefully placed the lid and incubated overnight at 37°C.
- Read with a microtitre plate reader

2.8 Determination of multidrug-resistant (MDR) *S. aureus*

MDR was characterized using previously published criteria where bacteria showing resistance to three or more antimicrobial classes were termed as MDR (Cohen *et al.,* 2008; Critchley *et al.,* 2003; Pillar *et al.,* 2008).

2.9 Control strains used in this study

S. aureus ATCC 49476 strain for *mec*(A) gene PCR, *S. aureus* ATCC BAA-2312 for *mec*(C) gene PCR (Cicconi-Hogan *et al.,* 2014; Huygens *et al.,* 2002), *agr* mutant RN4220 strain for *in vitro* and *in vivo* study (Peleg *et al.,* 2009) and *S. aureus* ATCC 25923 as a reference strain for antibiotic susceptibility assay (Ham *et al.,* 2010) were used.

2.10 Culture preservation

Culture confirmed as *S. aureus* by biochemical identification and 16S rRNA were preserved at -80℃. For culture preservation, a single colony was inoculated in 500 µL in tryptic soy broth (TSB), taken in Eppendorf, labeled with sample number, and was grown overnight. Added 500 µL of 70% glycerol in TSB and the culture was vortexed for 30 seconds to mix evenly. Cultures were stored at -80℃.

2.11 Molecular identification

For this purpose, a series of steps and techniques were followed, starting from DNA extraction to PCR and finally gel electrophoresis.

2.11.1 Culture preparation for DNA isolation

Study isolates identified as *S. aureus* by microbiological and biochemical techniques were grown on MSA plates by streaking, followed by an incubation period of 18-24 hours at 37℃ under aerobic conditions.

2.11.2 Pellet formation for DNA isolation

A sterile loop was used to pick isolated colonies from agar plates. A loop full of bacterial growth was added to 2 ml Eppendorf tubes containing 1.5 ml of sterile TSB. Centrifugation was done at 14,000 rpm for 2 min, and the process was repeated 2-3 time to get a heavy pellet The supernatant was removed using a 100-1000 µL micropipette.

2.11.3 Genomic DNA extraction

DNA extraction was done by using the previously described protocol (Lu *et al.,* 2000).

Procedure

- An overnight culture of *S. aureus* was grown in 5 mL TSB.
- The cells were collected through centrifugation at 10,000 rpm for 2-3 min.
- After centrifugation washed the cells with 1 mL lysis buffer.
- Followed by centrifugation at 14,000 rpm for 3 min to get the bacterial pellet.
- Suspended the bacterial pellet in 100 µL of lysis buffer.
- Boiled suspension for 30 min at 95ºC in a hot water bath.
- Centrifuged at 14,000 rpm for 8-10 min to remove any cell debris.
- Transferred the supernatant to a new sterile Eppendorf tube and this was used for PCR as a DNA template.

2.11.4 DNA estimation and quantification

There are several ways to quantify nucleic acid solutions. Most common way to quantify DNA is ultraviolet–visible spectrophotometry (UV) absorbance method. The spectrophotometric procedures suggest the DNA concentration as well as the other contaminants. Ultraviolet (UV)-induced emission of fluorescence from intercalated ethidium bromide should be used.

2.11.5 Ethidium bromide fluorescence quantitation

Principle

The principle is based on the intercalation of nucleotides to Ethidium Bromide that fluoresce under UV radiation.

Procedure

For this purpose, 1% agarose gel (Sigma-Aldrich, St. Louis, MO, US) was prepared in 1X TBE Tris-borate-EDTA (TBE) buffer. Two to three µL of ethidium bromide solution was added. After gel polymerization, 5 µL of DNA was mixed with 2 µL of 6X loading dye (Thermo scientific) and was loaded into each well, next to a DNA marker (Thermo scientific 100 bp ladder). The gel was then run for 30-35 min at a voltage of 100 Volts in TBE buffer. The gel was observed under UV trans-illuminator to visualize DNA bands, and were photographed by the Bio-Rad gel documentation system.

2.12 Spectrophotometric quantitation

Principle

The principle of spectrophotometric analysis is based on the Lambert-Beer equation and the fact that nucleic acids have a maximum absorption (abs) at 260 nm.

Procedure

For spectrophotometric quantitation, genomic DNA was suspended in 1X TE buffer or autoclaved distilled H_2O to prepare different dilutions of DNA. Usually, A260 of 1.0 is equal to 50 ug/ml pure dsDNA. DNA concentration was calculated by using the following formula:

concentration µg/ml = A260 reading x dilution factor x 50 µg/ml.

2.13 PCR mixture

The scheme of volumes used while making PCR reaction mixture is illustrated in Table 2.1

Table 2.1: Scheme for the total volume of PCR reaction for SCC*mec* types, antibiotic resistance and virulence genes

PCR Reaction Components	**Stock Concentration**	**Final Concentration**	**Volume per Reaction**
PCR Master Mix	2X	1X	12.5 µL
Forward Primers Each	100 pmol/µL	2 pmol/µL	0.5 µL x 9 = 4.5 µL
Reverse Primers Each	100 pmol/µL	2 pmol/µL	0.5 µl x 9 = 4.5 µL
Nuclease Free Water	-	-	1.5 µL
Template DNA	-	-	2 µL
Total PCR Reaction Volume			**25 µL**

2.14 PCR optimization for molecular detection of *S. aureus*

For molecular detection of *S. aureus* isolates, PCR conditions were optimized to amplify the gene of interest. For this purpose, a known pair of primers of 420bp for 16S rRNA (Strommenger, Kettlitz, Werner, & Witte, 2003) and Nuclease *nuc* gene

(Brakstad, Aasbakk, & Maeland, 1992) were used. Primer sequences, gene names, and amplicon sizes are given in Table 2.2.

2.15 Detection of Antibiotic resistance genes through multiplex-PCR

Two multiplex PCR reactions was carried out to detect different antibiotic resistance genes (ARGs) according to already published criteria (Strommenger *et al.,* 2003). In one multiplex PCR the following gene were optimized; *tet*K, *tet*M, *gyr*(A), *gyr*(B), *grl*(A), *grl*(B), the second multiplex PCR was run for *rpo*(B), *aacph*D1, *fos*(B) *erm*(A), *erm*(C), *vat*(C), *vat*(B), while the singleplex PCRs were carried out for *bla*(Z), *fos*(A), *dfr*(B), *vat*(A), *mec*(A) and *mec*(C) genes. Primers for ARGs were chosen from already published sequences, *dfr*(B) (Nurjadi *et al.,* 2014), *mec*(A) (Geha *et al.,* 1994), *mec*(C) (Stegger *et al.,* 2012), *bla*(Z) (Martineau *et al.,* 2000), *gyr*(A), *gyr*(B), *grl*(A), *grl*(B) (Sierra *et al.,* 2002), *rpo*(B) (Mick *et al.,* 2010), *tet*K, *tet*M, *erm*(A), *vat*(C), *vat*(B), *vat*(A), *aacph*D1(Strommenger *et al.,* 2003), *fos*(A), *fos*(B) (Chen *et al.,* 2014), and *erm*(C) (Jensen *et al.,* 1999). Primer sequences, genes names, and amplicon sizes are given in Table 2.2. For amplification of the desired amplicons bands, PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of amplification at 95°C for 1 min, annealing at 55°C for 1 min, initial extension at 72°C for 1 min, and a final extension at 72°C for 10 min. First we optimized all the primers, the annealing temperature for ARGs *dfr*(B), *tet*K, *tet*M, *bla*(Z), *gyr*(A), *gyr*(B), *grl*(A), *grl*(B), *rpo*(B), *aacph*D1, *erm*(A), *fos*(A), *fos*(B), *vat*(C), *vat*(B), *vat*(A) and *erm*(C) was 55°C, which was same for all whereas for *mec*(A) and *mec*(C) it was 53°C. *S. aureus* ATCC 49476 strain for *mec*(A) gene PCR while *S. aureus* ATCC BAA-2312 for *mec*(C) gene PCR (Cicconi-Hogan *et al.,* 2014; Huygens *et al.,* 2002) were used as controls. PCR products were purified before sequencing through GFX™ PCR DNA and Gel Band Purification Kit (Sigma-Aldrich). We also performed Sanger sequencing for the 10 different antibiotic resistance genes (*aacph*D1, *bla*(Z), *erm*(A), *grl*(A), *gyr*(A), *mec*(A), *mec*(C), *rpo*(B), *tet*K, and *tet*M) to find mutations.

Table 2.2: List of primers used in this study

Gene name	Amplicon Size	Reference	Sequences (5' to 3')
erm(C)	295	(Jensen *et al.,* 1999)	F: ATCTTTGAAATCGGCTCAGG R: CAAACCCGTATTCCACGATT
rpo(B)	432	(Mick *et al.,* 2010)	F: GTCGTTTACGTTCTGTAGGTG R: TCAACTTTACGATATGGTGTTTC
*aacph*D1	227	(Strommenger *et al.,* 2003)	F: TAATCCAAGAGCAATAAGGGC R: GCCACACTATCATAACCACTA
erm(A)	190	(Strommenger *et al.,* 2003)	F: AAGCGGTAAACCCCTCTGA R: TTCGCAAATCCCTTCTCAAC
*tet*K	360	(Strommenger *et al.,* 2003)	F: GTAGCGACAATAGGTAATAGT R: GTAGTGACAATAAACCTCCTA
*tet*M	158	(Strommenger *et al.,* 2003)	F: AGTGGAGCGATTACAGAA R: CATATGTCCTGGCGTGTCTA
vat(A)	467	(Strommenger *et al.,* 2003)	F: TGGTCCCGGAACAACATTTAT R: TCCACCGACAATAGAATAGGG
vat(B)	136	(Strommenger *et al.,* 2003)	F: GCTGCGAATTCAGTTGTTACA R: CTGACCAATCCCACCATTTTA
vat(C)	170	(Strommenger *et al.,* 2003)	F: AAGGCCCCAATCCAGAAGAA R: TCAACGTTCTTTGTCACAACC
16S rRNA	420	(Strommenger *et al.,* 2003)	F: CAGCTCGTGTCGTGAGATGT R: AATCATTTGTCCCACCTTCG
gyr(A)	398	(Sierra *et al.,* 2002)	F: ATGGCT GAATTACCTCAATC R: GTGTGATTTTAGTCATACGC
grl(A)	330	(Sierra *et al.,* 2002)	F: CAGTCGGTGATGTTATTGGT R: CCTTGA ATAATACCACCAGT

gyr(B)	469	(Sierra *et al.,* 2002)	F: GAAGCTGCTACGCATGAA R: GCTCCATCCICATCGGCATC
mec(A)	310	(Geha *et al.,* 1994)	F: GTAGAAATGACTGAACGTCCGATAA R: CCAATTCCACATTGTTTCGGTCTAA
mec(C)	138	(Stegger *et al.,* 2012)	F: GAAAAAAAGGCTTAGAACGCCTC R: GAAGATCTTTTCCGTTTTCAGC
grl(B)	363	(Sierra *et al.,* 2002)	F: GIGAAGCIGCACGTAA R: TCIGTATCIGCATCAGTCAT
bla(Z)	420	(Martineau *et al.,* 2000)	F: ACTTCAACACCTGCTGCTTTC R: TGACCACTTTTATCAGCAACC
dfr (B)	230	(Nurjadi *et al.,* 2014)	F: AATTGTGTTAAATTAAAGATAACTT R: TAAGTATTCTTTAGATAAATCGGAT
fos(A)	180	(Chen *et al.,* 2014)	F: GCTGCACGCCCGCTGGAATA R: CGACGCCCCCTCGCTTTTGT
fos(B)	232	(Chen *et al.,* 2014)	F: CAGAGATATTTTAGGGGCTGACA R: CTCAATCTATCTTCTAAACTTCCTG
SCC*mec* Type I(CIF2)	495	(Oliveira and Lencastre, 2002)	F: TTCGAGTTGCTGATGAAGAAGG R: ATTTACCACAAGGACTACCAGC
SCC*mec* Type II	284	(Oliveira and Lencastre, 2002)	F: AATCATCTGCCATTGGTGATGC R: CGAATGAAGTGAAAGAAAGTGG
SCC*mec* Type II, III	209	(Oliveira and Lencastre, 2002)	F: ATCAAGACTTGCATTCAGGC R: GCGGTTTCAATTCACTTGTC
SCC*mec* Type I,II,IV,VI	342	(Oliveira and Lencastre, 2002)	F: CATCCTATGATAGCTTGGTC R: CTAAATCATAGCCATGACCG
SCC*mec* Type III	414	(Milheiriço *et al.,* 2007)	F: TTCTTAAGTACACGCTGAATCG R: GTCACAGTAATTCCATCAATGC

SCC*mec* Type II, IV	311	(Milheiriço *et al.,* 2007)	F: AGTTTCTCAGAATTCGAACG R: AGTTTCTCAGAATTCGAACG
pan-agr		(Shopsin *et al.,* 2003)	F: ATGCACATGGTGCACATGC
agr I	440		R: GTCACAAGTACTATAAGCTGCGAT
agr II	572		R:GTATTACTAATTGAAAAGTGCCATAGC
agr III	406		R: CTGTTGAAAAAGTCAACTAAAAG
agr IV	588		R: CGATAATGCCG TAATAC CCG
HVR	Variable sizes	(Nishi *et al.,* 1995b)	F: ACTATTCCCTCAGGCGTCC R: GGAGTTAATCTACGTCTCATC
nuc	295	(Brakstad *et al.,* 1992)	F: GCGATTGATGGTGATACGGTT R: AGCCAAGCCTTGACGAACTAAAGC
hla	209	(Jarraud *et al.,* 2002)	F: CTGATTACTATCCAAGAAATTCG R: CTTTCCAGCCTACTTTTTTATCT
PVL	151	(Al-Talib *et al.,* 2009)	F: CAGGAGGTAATGGTTCATTT R: ATGTCCAGACATTTTACCTAA
hlb	833	(Booth *et al.,* 2001)	F: GCC AAA GCC GAA TCT AAG R: GCG ATA TAC ATC CCA TGG C
hlg	535	(Jarraud *et al.,* 2002)	F: GTCAYAGAGTCCATAATGCATTTAA R: CACCAAATGTATAGCCTAAAGTG
hld	444	(Rosec & Gigaud, 2002)	F: ATGGCAGCAGATATCATTTC R: CGTGAGCTTGGGAGAGAC
spa	Variable sizes	(Ma *et al.,* 2012)	F: GCGCAACACGATGAAGCTCAACAA R: ACGTTAGCACTTTGGCTTGGATCA
*sig*B	156	(Ma *et al.,* 2012)	F: TCAGCGGTTAGTTCATCGCTCACT R: GTCCTTTGAACGGAAGTTTGAAGCC
*cod*Y	120	(Ma *et al.,* 2012)	F: AAAGAAGCGCGCGATAAAGCTG R: TGCGATTAATAGGCCTTCCGTACC

*fnb*A	126	(Ferreira *et al.,* 2013)	F: ACTTGATTTTGTGTAGCCTTTTT R: GAAGAAGCACCAAAAGCAGTA
*fnb*B	118	(Ferreira *et al.,* 2013)	F: CGTTATTTGTAGTTGTTTGTGTT R: TGGAATGGGACAAGAAAAAGAA
*clf*A	151	(Xue *et al.,* 2012)	F: CGGTTTTGGACTACTCAGCA R: GCTACTGCCGATAAACTA
*sar*A	173	(White *et al.,* 2014)	F: CCTCGCAACTGATAATCCTTATG R: ACGAATTTCACTGCCTAATTTGA
*arl*S	103	(Truong-Bolduc *et al.,* 2011)	F: TGGAATACCAATTCCATGATCT R: TGCAATCAAATATGATGTGAAGAA
*spo*VG	Variable size	(Bischoff *et al.,* 2016)	F: TGTTCGTTGCAATGCCAAGT R: TGTCGCGGAATTCACCATC
saeS	110	(Wozniak *et al.,* 2012)	F: ATCCGAACAACAAGAAAAAACAG R: TGATTATACCATCACGTAGTCCTTCA
Rot	185	(Mootz *et al.,* 2015)	F: AAGAGCGTCCTGTTGACGAT R: TTTGCATTGCTGTTGCTCTA
Sae	102	(Mehrotra *et al.,* 2000)	F: GGTTATCAATGTGCGGGTGG R: CGGCACTTTTTTCTCTTCGG
Seb	164	(Mehrotra *et al.,* 2000)	F: GTATGGTGGTGTAACTGAGC R: CCAAATAGTGACGAGTTAGG
Sec	451	(Mehrotra *et al.,* 2000)	F: AGATGAAGTAGTTGATGTGTATGG R: CACACTTTTAGAATCAACCG
yycG	198	(Dubrac *et al.,* 2007)	F:TACAATCCCTTCATACTAAACTTGTAATTG R:GTGCATTTACGGAGCCCTTTTCGTCATATAC
arc up	456	(Enright *et al.,* 2000)	5' TTG ATTCACCAGCGCGTATTGTC-3'
arc dn			5' AGG TATCTGCTTCAATCAGCG -3'

aro up	456	(Enright *et al.,* 2000)	5' ATCGGAAATCCTATTTCACATTC -3'
aro dn			5'GGTGTTGTATTAATAACGATATC-3'
glp up	465	(Enright *et al.,* 2000)	5'CTAGGAACTGCAATCTTAATCC-3'
glp dn			5'TGGTAAAATCGCATGTCCAATTC-3'
gmk up	429	(Enright *et al.,* 2000)	5'ATCGTTTTATCGGGACCATC-3'
gmk dn			5'TCATTAACTACAACGTAATCGTA-3'
pta up	474	(Enright *et al.,* 2000)	5'GTTAAAATCGTATTACCTGAAGG-3'
pta dn			5'GACCCTTTTGTTGAAAAGCTTAA-3'
tpi up	402	(Enright *et al.,* 2000)	5'TCGTTCATTCTGAACGTCGTGAA-3'
tpi dn			5'TTTGCACCTTCTAACAATTGTAC-3'
yqi up	516	(Enright *et al.,* 2000)	5'CAGCATACAGGACACCTATTGGC-3'
yqi dn			5'CGTTGAGGAATCGATACTGGAAC-3'

2.16 Molecular typing

PCR was used to identify the *agr* specificity groups of the *agr* locus by amplification of the hypervariable domain using primers specific for all the 4 major specificity groups (Mullarky *et al.,* 2001). *S. aureus* isolates could be grouped into 4 mainly *agr* groups based on autoinducing peptide specificity for its membrane sensor (AgrC) (Mayville *et al.,* 1999). All isolates were characterized by *agr* typing (Shopsin *et al.,* 2003), and *mec*(A) positive isolates were also typed by SCC*mec* typing (Milheiriço *et al.,* 2007; Oliveira & de Lencastre, 2002). SCC*mec* element based on class of *mec* gene and the type of cassette chromosome recombinases (*ccr*) can be typed into following types; type I (Class B *mec* and type I *ccr*), type II (Class A *mec* and type 2 *ccr*), type III (Class A *mec* and type 3 *ccr*), type IV (Class B *mec* and type 2 *ccr*), type V (Class C 2 *mec* and type 5 *ccr*), type VI (Class B *mec* and type 2 *ccr*) (Ito *et al.,* 2001). SCC*mec* types I, II and III are assigned for HA-MRSA while types IV, V and VI for CA-MRSA. HVR typing is based on the polymorphism in the HVR region, which is a region of DNA between *mec*(A) gene and insertion sequences (IS431 *mec*) and responsible for methicillin resistance (Nishi *et al.,* 1995). *Spa* typing examines the type and number of point mutations of the *spa* gene repeat region. *Spa* typing was performed for 25 MRSA and 25 MSSA isolates. Isolates were selected from various specimen sources for *spa* typing. Primers of accession No. J01786; spa-1113f [10921113] & spa-1514r [1534-1514] were used for PCR amplification (Harmsen *et al.,* 2003).

PCR products were purified and sequenced using the ABI Prism Big Dye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems). *Spa* sequence data was analyzed using Ridom StaphType TM (Ridom GmbH, Würzburg, Germany) and the *spa* types were assigned using Ridom *spa* Server (http://spaserver.ridom.de). MLST genotyping method analyze single nucleotide variants within housekeeping genes such as phosphate acetyltransferase (*pta*), glycerol kinase (*glp*), triosephosphate isomerase (*tpi*), carbamate kinase (*arcC*), guanylate kinase (*gmk*), , shikimate dehydrogenase (*aroE*), , and acetyl coenzyme A acetyltransferase (*yqiL*) to a reference database, providing a sequence type (ST).

MLST allele names and ST were derived from the MLST database (http://www.mlst.net/dbqry/saureus/htm).

Mark Enright developed the MLST scheme in the laboratory of Brian Sratt Imperial College London, in collaboration with the laboratories of Sharon Peacock and Nick Day (Enright *et al.,* 2000). MLST and PFGE was performed on 5 representative isolates where one was MSSA, while 4 were MRSA (1 isolate carrying both *mec*(A) and *mec*(C) genes, 2 isolates with *mec*(C) gene, and 1 isolate was positive for *mec*(A) only). MLST was performed for 5 representative isolates where one was MSSA, while 4 were MRSA. Primers used for MLST genes *aro*(E), *tpi*, *yqi*, *pta*, *arc*(C), *gmk*, *glp*(F), were taken from a previously published report (Enright *et al.,* 2000). Data were analyzed using eBURST (http://eburst.mlst.net). PFGE is highly discrininative method, based on the variable movement of big DNA restriction fragments in an electrical field of alternating polarity. PFGE was also performed on these 5 isolates using a published methodology (McDougal *et al.,* 2003) and visual analysis was done using previously published criteria (Tenover *et al.,* 1995). *S. aureus* chromosomal DNA was prepared in agarose blocks and was cut with *Sma*I as descried previously (Bannerman *et al.,* 1995). The 1% agarose gel was used for samples run in 0.5% TBE buffer on a CHEF DR-III PFGE system (Bio-Rad, Hemel Hempstead, Hertsfordshire, United Kingdom) applying 1 second initial switching time which was increased to 5 seconds for the duration of 12 hours, further 12 hours for 15 seconds of an initial switching, that was increased to 30 seconds ofvoltage of 6 V cm^{-1} (Cookson *et al.,* 1996). Concatenated bacteriophage lambda DNA (New England Biolabs, Beverly, Mass) was used as a marker. *Spa* typing, MLST typing and PFGE for the isolates were done using commercial services of ID Genomics, USA.

2.17 Gel electrophoresis

After amplification, three to five µL of the PCR product was mixed with 2-3 µL loading dye and was loaded into wells. In one well a DNA marker (Thermo scientific 100bp ladder) was also loaded. The 1% gel was then run for 55 min at 90 Volts in 1X TBE buffer in the gel tank (Bio-Rad). After the defined time, PCR

products was observed under a UV trans-illuminator. Bands were photographed using Bio-Rad Gel Documentation system.

2.18 Virulence factors

2.18.1 Phenotypic test for the detection of virulence gene

2.18.1.2 Hemolysis Test

Hemolysis assay on sheep blood agar was done for all isolates using the method as previously described (Quinn *et al.,* 1994) to investigate the type of hemolysin produced by clinical *S. aureus.* Screening of hemolysin genes such as *hla, hlb, hlg,* and *hld* was done in phenotypically positive isolates by PCR.

Principle

S. aureus can produce several toxins such as hemolysin that is toxic to a range of host cells capable of causing hemolysis of erythrocytes.

Procedure

- The test sample was inoculated on a plate of MSA and incubated for 18-24 hours at 37°C.
- To check the hemolytic activity of *S. aureus*, 5% sheep blood agar was prepared. The sheep blood agar base was prepared according to the manufacturer's instructions (Oxoid, England). Agar base was allowed to cool down to 45°C to 50°C, then aseptically 5% sheep blood was added and mixed well. The blood agar plates were incubated at 37°C overnight to check sterility.
- To check hemolysis, *S. aureus* isolates were re-inoculated on sheep blood agar and incubated at 37°C for overnight.

Interpretation

- Hemolysin production or hemolysis of erythrocytes was detected upon the formation of a complete clearing zone around colonies in the medium.
- Incomplete clearing (green hemolysis) indicated α-hemolysis.

- Complete clearing was taken as β-hemolysis.

2.19 Detection of various virulence factors and other regulatory factors of *S. aureus* through multiplex PCR

Two multiplex PCR was performed for virulence factors, one multiplex PCR was performed for *fnbB*, *sae*, *sarA*, YycG, *clfA, and sec* gene, while the second multiplex PCR was for *rot, arls, cod*Y, *sig*B, *sae* and *seb* genes. Single plex PCR was performed for PVL and *spoVG* gene. Other virulence factors were screened through multiplex PCR such as the regulatory factors such as alternative sigma factor (*sig*B), Gene expression regulator (*cod*Y) (Ma *et al.,* 2012), staphylococcal accessory regulator (Biofilm-related) (*sarA*) (White *et al.,* 2014), Transcriptional regulator (*rot*) (Mootz *et al.,* 2015), WalK/WalR (YycG/YycF) autolytic activity (*yycG*) (Dubrac *et al.,* 2007), the stage V sporulation protein G (yabJ-spoVG operon, (*spoVG*) (Bischoff *et al.,* 2016), *arls* (Truong-Bolduc *et al.,* 2011), and TCS (phosphorylated response regulators) (Histidine protein kinase) (*sae*S) (Wozniak *et al.,* 2012), and virulence factors Fibronectin-binding protein A (*fnbA*), Fibronectin-binding protein B (*fnbB*) (Ferreira *et al.,* 2013), Clumping factor A (*clfA*) (Xue *et al.,* 2012), PVL toxin (Al-Talib *et al.,* 2009), Surface and secreted protein for bacterial aggregation (*spa*) (Ma *et al.,* 2012), and enterotoxin genes such as *sea*, *seb* and *sec* (Mehrotra *et al.,* 2000). Primer sequences are mentioned in Table 2.2.

The amplification conditions were following; initial denaturation was carried out for 5 min at 95°C, then for 10 cycles, each cycle consisting of 60 seconds at 95°C, 60 seconds at 51°C, and 60 seconds at 72°C and this was followed by 20 cycles each consisted of 60 seconds at 94°C, 60 seconds at 51°C, and 60 seconds at 72°C with a final extension at 72°C for 10 min. Annealing temperature for *hla, hlb, hlg*, and *hld* genes was 56°C, 58°C, and 47°C respectively, while all PCR conditions including initial and final denaturation, initial and final extension temperature and the number of cycles remained same for all genes.

2.20 Gel electrophoresis for the identification of PCR products

For this purpose, a 2% agarose gel (Sigma) was prepared. Five µL of PCR products were resolved in 2% gel along with 100bp DNA marker (Vivantis) for 45min at 90V in a horizontal gel electrophoresis apparatus.

2.21 Real-Time Quantitative PCR (RT-qPCR) Assays

An overnight culture of clinical MRSA strain was grown in TSB and inoculated into new MHB. The bacterial cells were harvested, when the OD_{600} was reached to 0.6. RNA isolation was done using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The detailed protocol is described below:

- The bacteria were disrupted and homogenized by bead-milling in a guanidine–thiocyanate–containing lysis buffer
- Centrifuged at 6000 revolutions per minutes (rpm), decanted the supernatant
- Added 700 µL of buffer RLT, vertexed vigorously for 5-10 seconds. Transferred the suspension to a new sterile tube of 2 mL, disrupted the cells for 5 minutes at maximum speed and transferred the supernatant into a new sterile tube
- Again, centrifuged at maximum speed for 10 seconds, transferred supernatant into a new tube
- Determined the volume of supernatant, added an equivalent volume of 70 percent ethanol, mixed gently by pipetting. At this step, do not centrifuge.
- Transferred up to 700 µL lysate to a RNeasy spin column put down in a 2 mL collection tube. Closed tightly and centrifuged at 10,000 rpm for 15 seconds.
- Discarded the flow through. Added buffer RW1 of 700 µL to the RNeasy spin column, centrifugation was done at 10,000 rpm to washing out the spin column membrane. Discarded the flow through.
- Then added RPE buffer of 500 µL to the spin column. Closed the lid smoothly and centrifuged at 10,000 rpm for 2 minutes for washing the spin column membrane.

- Placed the RNeasy spin column in a new 1.5mL collection tube. Added 30-50 µL RNase free water directly to the spin column membrane.
- The lid closed gently and centrifuged for 1 minute at 10,000 rpm for elution of the RNA.

For cDNA synthesis the Verso cDNA synthesis kit (Thermo Fisher Scientific, MA, USA) was used. RT-qPCR was done as described in the manufacturer's instructions (Bio-Rad, CA, USA) using the previously reported primers (Ma *et al.,* 2012; Qiu *et al.,* 2010) and the iCycler iQ real-time detection system (Bio-Rad). No-RT control was included as a negative control. According to (Mitchell *et al.,* 2011), the relative expression ratios were calculated as follows: n-fold expression = 2-ΔΔCt, ΔΔCt = ΔCt, where ΔCt represents the difference between the cycle threshold (Ct) of the gene studied and the Ct of housekeeping 16S rRNA gene (internal control). Three clinical isolates; one HA-MRSA, one CA-MRSA, and a methicillin sensitive *S. aureus* were selected as a control for expression analysis.

Primer sequence with forward 5-GGGACCCGCACAAGCGGTGG-3 and Reverse 5-GGGTTGCGCTCGTTGCGGGA-3 for the 16S rRNA gene were obtained from previous publication (Atshan *et al.,* 2013). Expression of Accessory gene regulator A (*agrA*) (Qiu *et al.,* 2010) and *ica* (Ma *et al.,* 2012) were analyzed using forward primers 5-TGATAATCCTTATGAGGTGCTT-3 and reverse primer 5-CACTGTGACTCGTAACGAAAA-3 for *agr* while forward primer sequence for *ica* were ATCTAATACGCCTGAGGA and reverse TTCTTCCACTGCTCCAA from published sequences through real-time PCR**.**

A total of 20 µL reaction mixture was prepared, containing 4 µL of 5X cDNA synthesis buffer of 1X final concentration, 2 µL of dNTP mix 500 µM each, RNA primer 10 µM, RT enhancer 1 µL, verso Enzyme Mix 1 µL, template RNA 1-5 µL of 20 ng final concentration and adjusted final reaction mixture volume up to 20 µL by adding water, nuclease-free (R0581). Statistical significance was calculated using Student's t-test.

2.22 Macrophage Infection Assay

Macrophages are key cells of the immune system involved in innate immunity and help initiate adaptive immunity, adapt to host defense against infections. RAW 264.7 monocyte/macrophages originating from Abelson leukemia virus transformed cell line derived from BALB/c mice were used to evaluate the intracellular killing of selected *S. aureus* isolates. Assays were performed in triplicate as previously described (Schmitt *et al.,* 2013). Macrophages were cultured and maintained as described previously (Jayamani *et al.,* 2017).

2.22.1 Materials and Reagents

- RAW 264.7 (ATCC® TIB-71™) cells
- MRSA strain
- TSB broth (Beckon Dickinson, catalog number: 244610)
- TSA (Beckton Dickinson, catalog number: 214010)
- DMEM (Sigma-Aldrich, catalog number: 11875-0930)
- Gentamicin 50 mg/ml (Life Technologies, catalog number: 15750-102).
- Phosphate-buffered saline (PBS) (pH 7.4)
- TSA plates
- Certified, heat-inactivated fetal bovine serum (FBS) (Life Technologies, catalog number: 10082139)
- 100x penicillin, streptomycin and glutamine (PSG) (Life Technologies, catalog number: 10378-016)
- Saponin (Sigma-Aldrich, catalog number: 8047-15-2)

2.22.2 Equipment

1. 24 well plates (Corning, Costar, catalog number: 3524)
2. 0.2-micron filter (Millipore, catalog number: SCGP00525)
3. 37, 5% CO_2 incubator
4. Sorvall Legend RT tabletop centrifuge

2.22.3 Procedure

- RAW 264.7 cells were cultured in DMEM (Dulbecco's Modified Eagle's medium) form of medium with fetal bovine serum (which was heat inactivated in a water bath at 50°C for 30 min) and 1X penicillin-streptomycin and glutamine at 37℃ in a CO_2 incubator.
- RAW cells were harvested using the scraper and suspended in fresh DMEM. Five µL of cells were mixed with 95 µL of trypan blue and 10 µL was loaded in new baur counting chamber and cell were counted in all 8 squares. Then appropriate amount of cells were seeded in the infection assay plates. And by adding 1 ml per well in a 24 well plate, 5x 10^5 RAW cells/well were seeded and incubated in a CO_2 incubator for 24 hours.
- Overnight cultures of isolate in 3 ml of LB broth in a 10 ml polypropylene tube were taken and were incubated at 37℃, with shaking at 200 rpm.
- The multiplicity of infection (MOI) was determined by formula (MOI = number of bacteria per RAW 264.7 cell) and accordingly the inoculum preparation was changed. For a MOI of 10, 2 x 10^6 CFU of the isolate was added for a well containing 2 x 10^5 RAW cells.
- Three ml culture was centrifuged at 4,000rpm for 10 min in a Sorvall centrifuge and pellet was obtained. Pellet was resuspended in 3 ml PBS.
- OD_{600} of broth culture was adjusted to four in PBS by measuring the optical density of a 1:10 diluted culture in a spectrophotometer and calculating the optical density of the undiluted culture.
- Dilution 1:100 was made in PBS.
- Dilutions of 1:10 in DMEM were made without antibiotics.
- RAW cells were washed twice with PBS.
- 1 ml of diluted culture was added to each well; three replicates per sample for the entire experiment were processed as recommended.
- All plates were incubated at 37℃, 5% CO_2 for 30 min.
- The supernatant was aspirated and was washed 3 times with PBS.
- 1 ml of DMEM was added with gentamicin (200 µg/ml) per well.
- Plates were incubated at 37℃, 5% CO_2 for 2 hours.

- After 2 hours, the supernatant was removed entirely by aspiration.
- Macrophages were lysed by adding SDS to a final concentration of 0.02% Serial dilution of cell lysates were made and the CFUs counts were done by plating on TSA plates (Tharmalingam *et al.,* 2017).
- Spotting assay was done to confirm the intracellular survival of study isolates

2.23 *G. mellonella* experiment

2.23.1 Reagents and Culture media

All reagents and culture media were purchased from Sigma–Aldrich Ltd (Poole, UK). All solutions were made with sterile ultrapure deionized water (Maxima; Elga, High Wycombe, UK). Water, PBS, and media were sterilized by autoclaving at 121℃ for 15 min.

2.23.2 Inoculum preparation

Overnight cultures of representative clinical *S. aureus* isolates such as; MSSA, MRSA, CA-MRSA, HA-MRSA, VISA, and *agr* mutant strain RN4220 were cultured for inoculum preparation in 5 ml TSB broth. Bacterial cells were collected by centrifuging these broth cultures at 6000rpm for 2 min and were washed twice with PBS. Appropriate dilutions were made in PBS. Then the OD 0.5 at 600nm was adjusted.

2.23.3 Survival Assay

For survival assay, *S. aureus* were injected at 1.0 x 10^7 cells/larva. Before each experiment, needles were cleaned with a thread that was provided with the needles. Needles were sterilized before injections and were also sterilized again after every six injections or between microbial strains.

2.23.4 Inoculation

Selecting white *Galleria* was key to getting consistent results. Those which were grey or had black spots were not able to give consistent results as they had already suffered some damage. The syringe was filled with 10 µL of 1.0 x 10^7 cells/ml inoculum to infect with 1.0 x 10^6 cells/ larvae dose. The injection was given at the last, left pro-leg (Figure 2.1) using a Hamilton syringe and incubated at 37℃. Overnight culture of a previously characterized strain of HA-MRSA (SCC*mec* type

III), VISA (MIC of 8mg/L), CA-MRSA (SCC*mec* type IV), *S. aureus* strain (methicillin-sensitive) and *agr* mutant RN4220 cells were selected. The larvae were held with the left hand and were injected with the right hand. The needle penetrated at the last left pro-leg easily. Infected larvae were transferred to a petri dish with a kemwipe to recover for a few min. Larvae were transferred to a new petri dish and stored at relevant temperature conditions (Mostly the larvae were kept in the dark at 37°C). Larvae were observed daily, and the number of dead larvae was recorded. Sixteen *G. mellonella* larvae (Vanderhorst Wholesale, St. Mary's, OH, USA), selected randomly and weighing between 300 and 350 mg, were used for each group (Gibreel & Upton, 2013).

Seven test groups were included in the current study, and the same dose of bacteria was injected into the corresponding infection groups: (1) PBS alone (no bacteria control), (2) HA-MRSA cells, (3) VISA cells, (4) CA-MRSA cells, (5) no injection and no bacteria (quality control), (6) methicillin-sensitive *S. aureus* (7) and *agr* mutant cells RN4220. *G. mellonella* survival was assessed up to 144 hours, with larvae considered dead if unresponsive to touch using sterile tips.

In all experiments, two negative control groups: one group that underwent no manipulation, while the other group (uninfected control) was injected with PBS only. No deaths in these control groups were observed and there were never more than two deaths per control group per experiment. During experimentation, larvae were stored at 37°C for 144 hours in petri dishes. Larvae were examined every 24 hours and were considered dead if they did not move in response to touch. Killing curves and differences in survival were analyzed by the Kaplan–Meier method using STATA software. Statistical analysis was carried out, and $p < 0.05$ was considered significant.

To record the data an excel file was set up as follows:

- Fail time represented the time point for each observation.
- Fail represented the number dead at each time point.

Censored was recorded as 0 until the last time point. Recorded the number of *Galleria* that were still alive, as shown in Table 2.3.

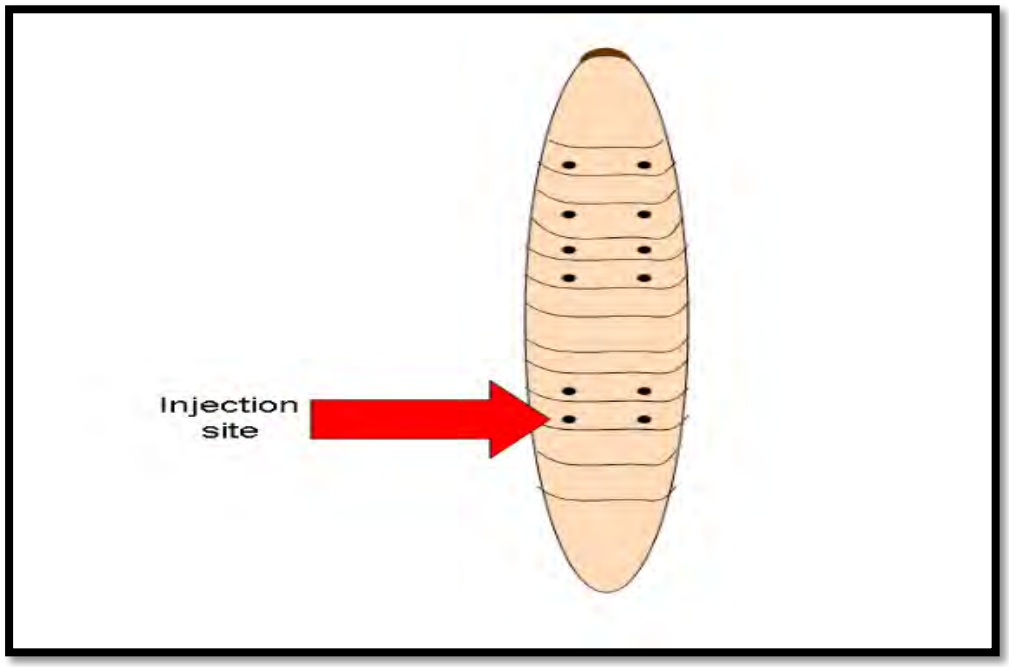


Figure 2.1: Arrow shows injection site in *Galleria* model

Table 2.3. Group represents the name of each test group

Group	Fail time	Fail	Censored
No injection	18	0	0
No injection	24	0	0
No injection	48	0	0
No injection	72	0	16
PBS	18	0	0
PBS	24	0	0
PBS	48	0	0
PBS	72	0	16
Test Group1	18	11	0
Test Group1	24	4	0
Test Group1	48	1	0
Test Group1	72	0	0
Test Group 2	18	3	0
Test Group 2	24	10	0
Test Group 2	48	2	0

Test Group 2	72	1	0
Test Group 3	18	10	0
Test Group 3	24	5	0
Test Group 3	48	1	0
Test Group 3	72	0	0

- Failtime represents the time point for each observation.
- Fail represents the number dead at each time point.
- Censored is recorded as 0 until the last time point. At the last time point record the number of *Galleria* that are still alive.

2.24 Hemocyte density assessment

The delivery of several microbes or drugs can affect the hemocytes density within the *Galleria* hemolymph. Variations in the hemocyte density showed immunomodulatory effect or systemic gratitude for pathogens. Within the hemolymph, six types of hemocytes existed: granulocytes spherulocyte, oenocytoids, prohemocytes, plasmacytes, and coagulocytes. While estimating hemocyte density, no differentiation was made between various kinds of hemocytes. Relatively, they were equaled/considered as a collective group (Bergin *et al.,* 2003, Fuchs *et al.,* 2016).

2.24.1 Collection of the *Galleria* hemocytes

Hemolymph was collected from the base of the last proleg of *G. mellonella* by bleeding into cold insect physiological saline (IPS). For cutting, No.10 scalpel was used and allowed the hemolymph to leak into chilled Eppendorf tube of 1.5 ml. A group of 4 larvae was selected into a single tube that was put on ice to collect hemolymph. An amount of 100 µL of collected material was transferred into a fresh Eppendorf microcentrifuge tube having 1:10 times diluted 900 µL of cold IPS. An aliquot of 10 µL was transferred to the hemocytometer to count hemocyte number. If hemocyte number was high, an additional dilution step was made. The number of

hemocytes present per ml was calculated. Control treatment groups were compared to larvae infected/treated with PBS.

This provided a baseline of the number of hemocytes present normally. There was not enough hemolymph captured in a single larva that could be reliably counted. Therefore, *G. mellonella* was pooled into groups of 4. Four replicates of 4 were used to generate the SD (standard Deviation) and an average number of hemocytes for a treatment/infection. More hemocyte indicates less virulence and less hemocyte indicates more virulence.

2.25 Statistical analysis

Chi-square test was performed to find any relationship among phenotypic antibiotic resistance and their respective ARGs, and the association of Panton valentine leucocidin (PVL) toxin with SCC*mec* types. A *p*-value of less than 0.05 was considered significant.

Chapter 3: Antibiotic Resistance in *Staphylococcus aureus*

3.1 Introduction

Staphylococcus aureus (*S. aureus*) is an important nosocomial pathogen and its multi-drug resistant strains, particularly methicillin-resistant *S. aureus* (MRSA), have been shown to pose a serious threat to public health due to its limited therapeutic options (Hamzah *et al.,* 2019). Resistance in *S. aureus* can develop either through the transfer of resistance determinants encoded by mobile genetic elements such as transposons, plasmids, and the staphylococcal cassette chromosome *mec* (SCC*mec*) types (I, II, III, IV, V, VI) or due to mutations in chromosomal genes (Foster, 2017). The morbidity and mortality associated with *S. aureus* are augmented when virulent strains acquire antibiotic resistance (Aslam *et al.,* 2018). Multidrug-resistant strains (MDR) *S. aureus* have spread globally with increasing frequency, leading to high levels of mortality and morbidity, and presenting an alarming situation for healthcare professionals (Wilson, *et al.,* 2016).

In this chapter, the susceptibility profiles of *S. aureus* isolates against a panel of antibiotics, frequency of multi-drug resistant (MDR) isolates and their respective antibiotic resistance genes (ARGs) among clinical *S. aureus* isolates have been described.

3.2 Results

3.2.1 Clinical Staphylococci isolates

A total of 1528 clinical staphylococci isolates were collected from the Microbiology Laboratory of Pakistan Institute of Medical Sciences (PIMS), Islamabad during April 2015-July 2016. Isolates were grown on selective and differential media to check their growth characteristics and were further processed for identification by biochemical tests.

3.2.2 Morphological Identification of Staphylococci

Mannitol salt agar (MSA) was used to differentiate *S. aureus* from other staphylococcal species. *S. aureus* isolates changed the media from pink to yellow, which were later identified as *S. aureus* as shown in Figure 3.1.

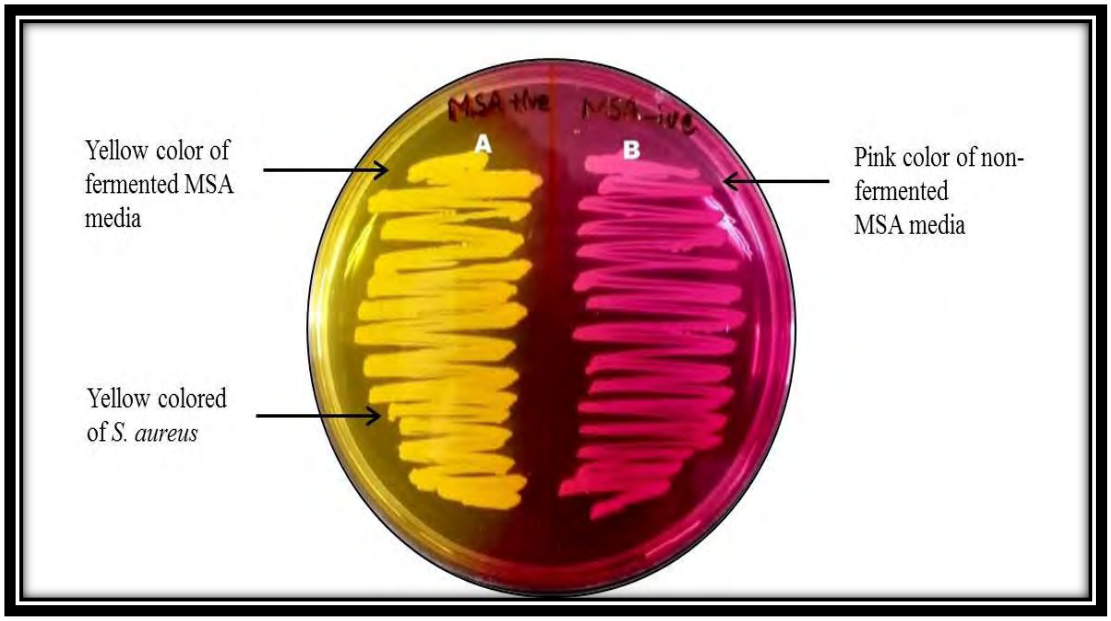


Figure 3.1: A representative Mannitol salt agar (MSA) plate with *S. aureus* culture. The *S. aureus* grows as a yellow colony with yellow zone fermenting the mannitol salt in MSA media.

3.2.3 Biochemical Identification

Staphylococcal isolates were identified based on their biochemical properties by performing three biochemical tests suah as DNase, catalase, and tube coagulase tests (Table 3.1). Out of 1528 staphylococci isolates, 485 isolates were confirmed as *S. aureus* based on the positive result of above mentioned biochemical tests. Representative images of the results of biochemical tests performed are shown in Figures 3.2 and 3.3.

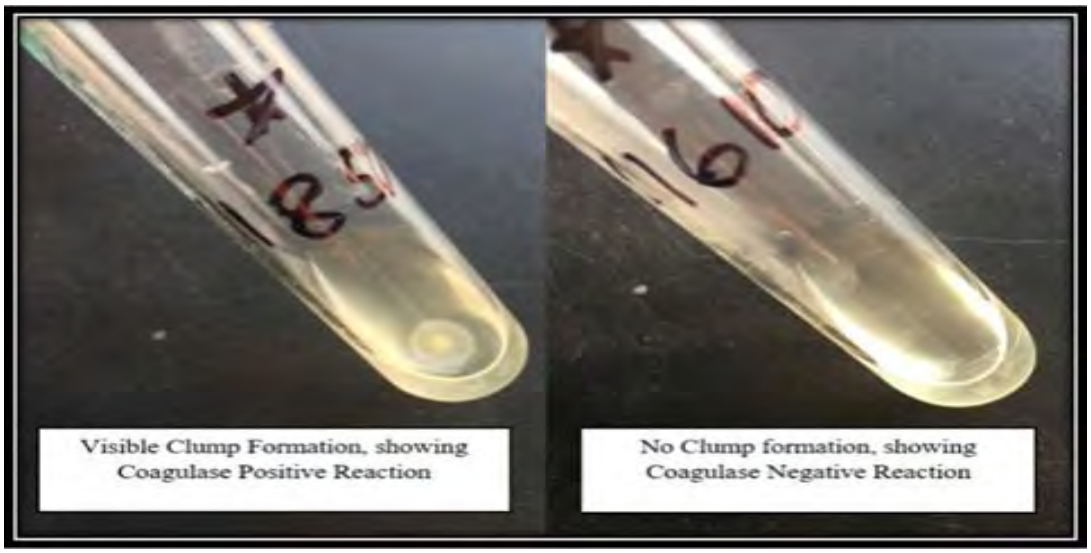


Figure 3.2: A representative image of Coagulase test: coagulase-positive and coagulase-negative reactions by clinical isolates

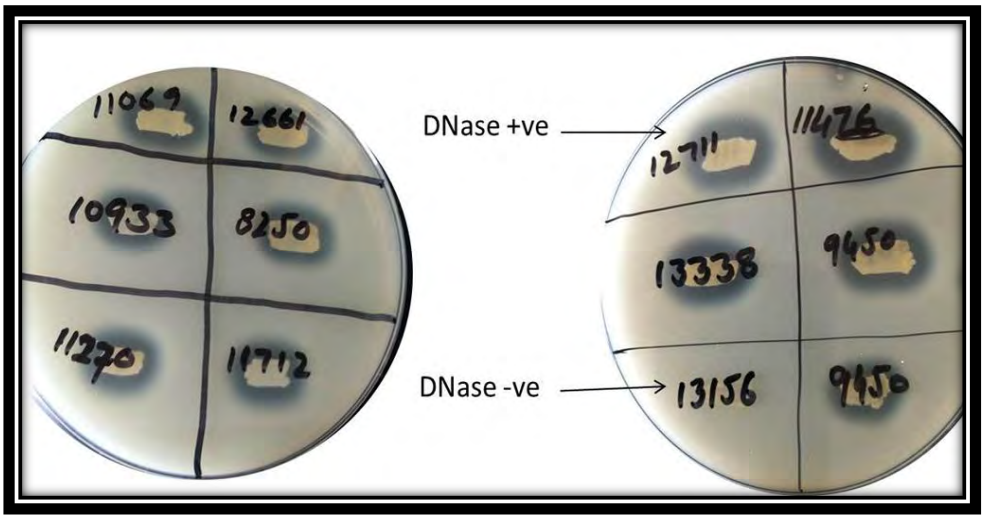


Figure 3.3: Representative images of DNase test. DNase positive isolates showed clear zones around *S. aureus* growth after the addition of 1 Molar HCl and DNase negative isolates showed no clear zones around their cultures.

Table 3.1: Biochemical tests results for clinical *S. aureus* isolates

Biochemical tests	**Results for *S. aureus***
Catalase	+
DNase	+
Tube coagulase	+

3.2.5 Molecular identification of *S. aureus* isolates

All the biochemically confirmed *S. aureus* isolates were screened for 16S rRNA and nuclease (*nuc*) genes by PCR specific for *S. aureus*. All *S. aureus* isolates were positive for the 16S rRNA and *nuc* genes. The representative gel images are shown in Figure 3.4.

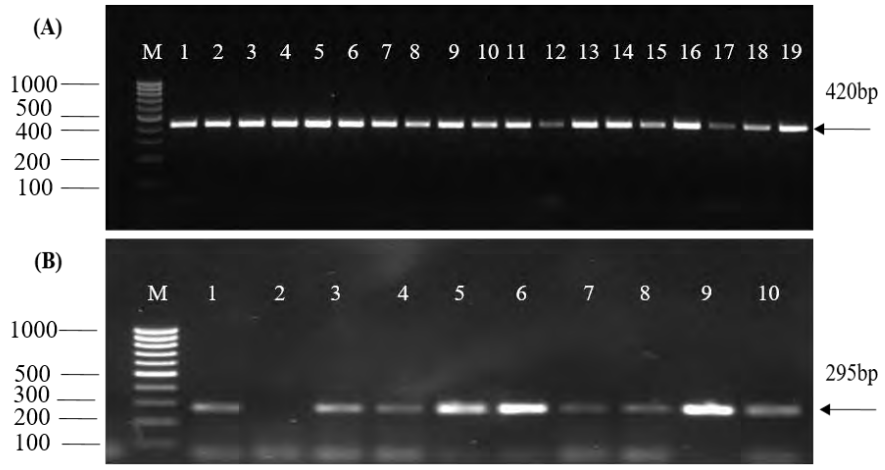


Figure 3.4: Representative gel images showing PCR amplification of 16S rRNA and *nuc* genes **(A)** Lane M shows the molecular weight marker of Gene Ruler 100bp (Thermo scientific). Lanes 2-19 show the amplified product of the 16S rRNA gene (420bp) of test isolates and Lane 1 shows positive control for 16S rRNA gene **(B)** Lane M shows the molecular weight marker of Gene Ruler 100bp (Thermo scientific). Lanes 3-10 show PCR product of the *nuc* gene (295bp) and Lane 1 shows positive control for *nuc* gene while lane 2 shows negative control.

3.2.6 Patient Demographic Details

Clinical *S. aureus* strains were isolated from a variety of specimens. A major source of isolates was pus (n=301, 62%), followed by urine (n=68, 14%) and blood (n=44, 9%). The distribution of *S. aureus* isolates from different specimens is shown in Figure 3.5 (A). According to gender *S. aureus* isolates were more common in males (n=277, 57%), as compared to female (n=208, 43%) patients as shown in Figure 3.5 (B). According to hospital ward, isolates mainly were from the emergency ward (n=184, 38%). The distribution of patients according to wards is shown in Figure 3.5 (C). The isolates were from patients of different ages and the age of the patients was divided into four different groups: less than 18 years, 18-35 years, 36-55 years and >55 years. It was observed that the highest rate of infection was in the age group of 18-35 years as shown in Figure 3.5 (D).

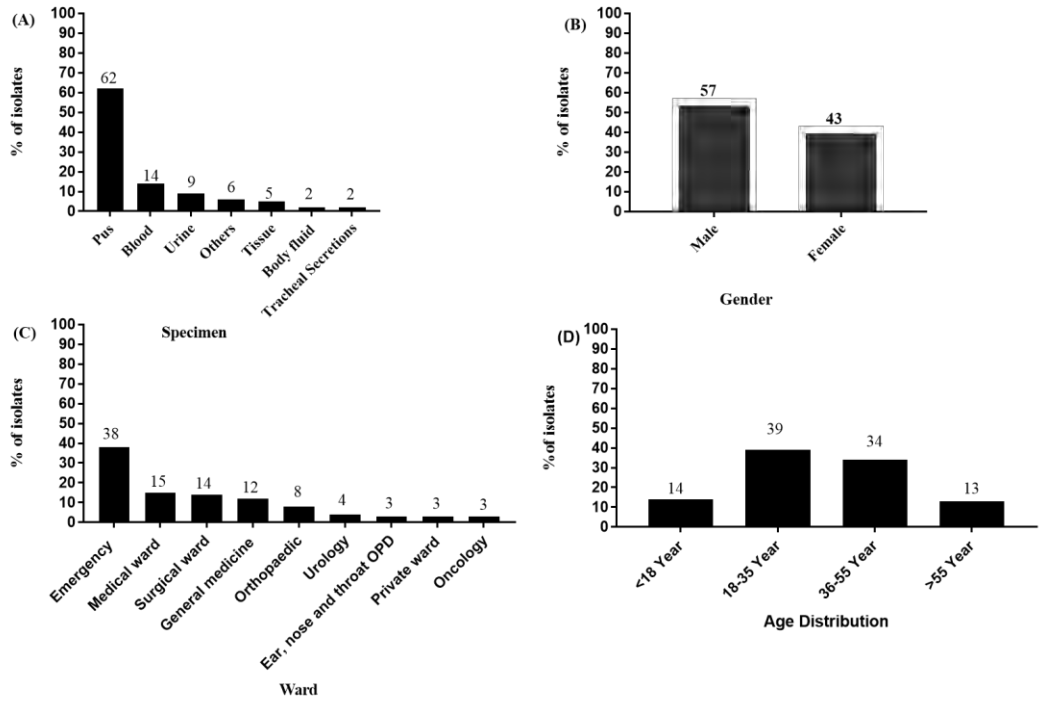


Figure 3.5: *S. aureus* distribution according to specimen, gender, ward and age **(A)** Distribution of *S. aureus* isolates from various specimen **(B)** Gender wise distribution of study isolates **(C)** *S. aureus* isolates from different wards of the hospital **(D)** Age-wise distribution of *S. aureus* isolates in different age groups (four groups)

3.3 Antibiotic susceptibility profiles of clinical *S. aureus* isolates

S. aureus isolates were highly resistant to clindamycin (n=336, 69%), ciprofloxacin (n=330, 68%), and cefoxitin (n=315, 65%), followed by gentamicin (n=224, 46%), erythromycin (n=219, 45%), tetracycline (n=150, 31%), while lower resistance levels to sulfamethoxazole/trimethoprim (n=102, 21%), rifampicin (n=73, 15%), chloramphenicol (n=34, 7%), linezolid and Quinupristin/dalfopristin (n=15, 3% each) were observed as shown in Figure 3.7 (A).

3.3.1 Antibiotic susceptibility profile of methicillin-resistant *S. aureus* (MRSA) isolates

Based on cefoxitin resistance, out of total 485 *S. aureus* isolates, 65% (n=315) were MRSA and 35% (n=170) were methicillin-susceptible *S. aureus* (MSSA). In the current study, 250 isolates (200 MRSA and 50 MSSA) were further processed. The D-test was performed to detect the inducible clindamycin resistance, using antibiotic discs of clindamycin (2µg) and erythromycin (15µg).

Among MRSA isolates, phenotypic clindamycin inducible resistance was observed in 4% (n=8) isolates (Figure 3.6). In MRSA maximum isolates 96% (n=192) were sensitive to linezolid while quinupristin-dalfopristin susceptibility was noticed in 95% (n=190) isolates. Chloramphenicol sensitivity was recorded in 88% (n=176), while 12% (n=24) isolates showed resistance. In the case of sulfamethoxazole/trimethoprim 26% (n=52) of isolates showed resistance. In the case of tetracycline, 36% (n=72) isolates were resistant, 5% (n=10) were intermediate, while 59% (n=118) were susceptible. Ciprofloxacin resistance was recorded in 85% (n=170) isolates. Resistance to rifampicin was noticed in 20% (n=40) isolates while 76% (n=156) isolates were sensitive. Gentamicin and clindamycin resistance was observed in 64% (n=128) and 26% (n=52) isolates respectively. Fifty percent (n=100) isolates were resistant to erythromycin, while 28% (n=56) were sensitive, and 22% (n=44) were intermediate, as shown in Figure 3.7 (B).

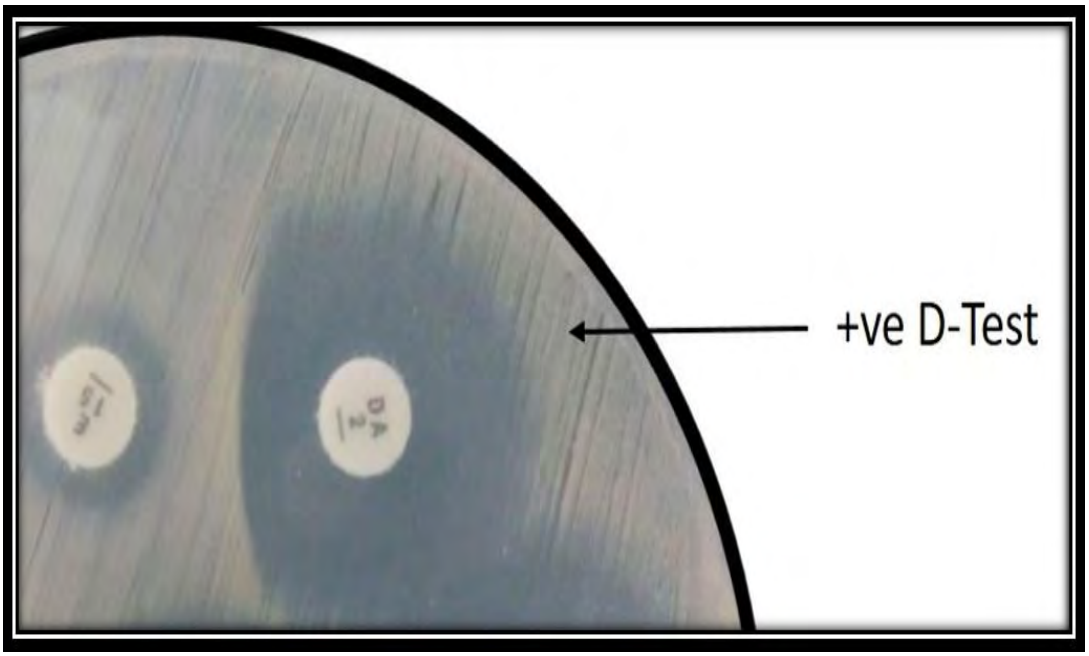
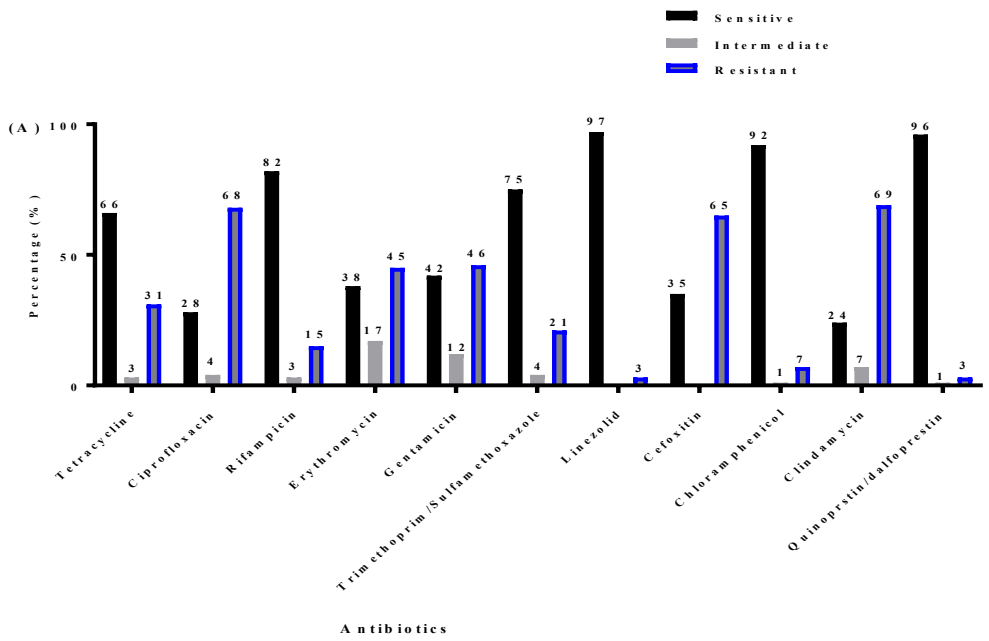
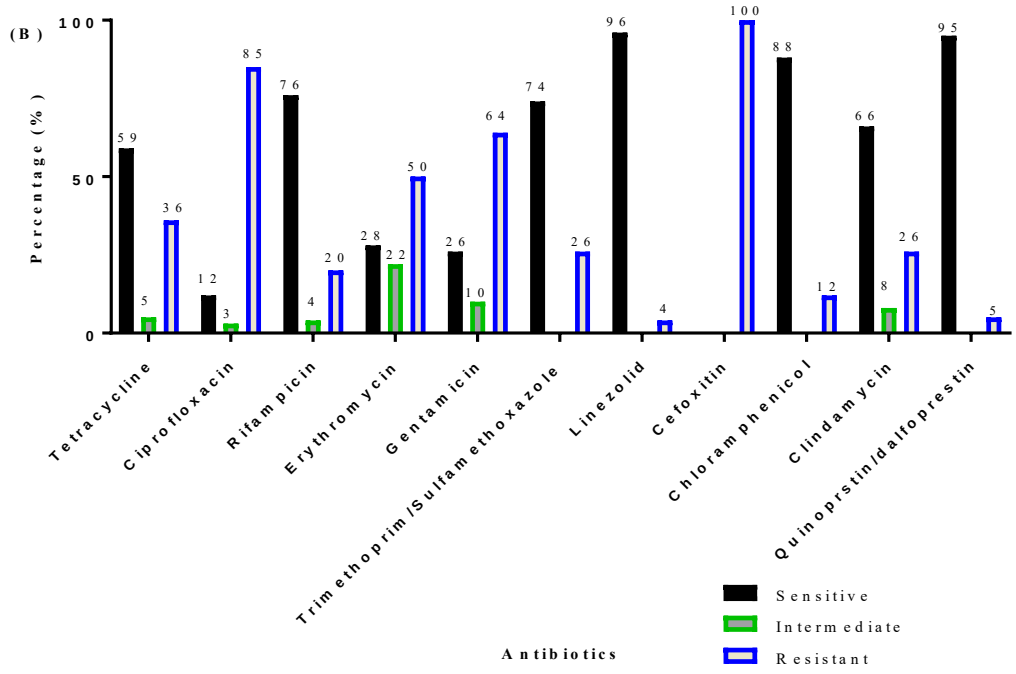


Figure 3.6: Mueller Hinton Agar plate showing a positive D-test, phenotypic confirmation of clindamycin inducible resistance (D-test) using erythromycins and clindamycin discs.

3.3.2 Antibiotic susceptibility profile of methicillin-sensitive *S. aureus* (MSSA) isolates

Antibiotic susceptibility patterns in MSSA isolates were recorded as follows: 32% (n=16) isolates were sulfamethoxazole/trimethoprim resistant while 68% (n=34) were sensitive. Tetracycline resistance was observed in 72% (n=36), 4% (n=2) were intermediate, while 24% (n=12) were sensitive. Ciprofloxacin resistance was observed in 80% (n=40) isolates while 20% (n=10) were sensitive. Only 12% (n=6) isolates were rifampicin-resistant while 88% (n=44) were sensitive. Gentamicin resistance was observed in 32% (n=16) isolates, 8% (n=4) were intermediate while 60% (n=30) were sensitive. Twenty percent (n=10) isolates were clindamycin resistant, 8% (n=4) were intermediate while 72% (n=36) were sensitive. Thirty-two percent (n=16) isolates were erythromycin-resistant, 16% (n=8) were intermediate, while 52% (n=26) were sensitive. All MSSA isolates were sensitive to linezolid, cefoxitin, quinupristin/dalfopristin, and chloramphenicol, as shown in Figure 3.7 (C).


Sensitive
Intermediate
Resistant
(A)
Percentage (%)
100
50
0
66
3
31
28
4
68
82
3
15
38
17
45
42
12
46
75
4
21
97
3
35
65
92
1
7
24
7
69
96
1
3
Tetracycline
Ciprofloxacin
Rifampicin
Erythromycin
Gentamicin
Trimethoprim/Sulfamethoxazole
Linezolid
Cefoxitin
Chloramphenicol
Clindamycin
Quinoprstin/dalfoprestin
Antibiotics


(B)
Percentage (%)
100
50
0
59
5
36
12
3
85
76
4
20
28
22
50
26
10
64
74
26
96
4
100
88
12
66
8
26
95
5
Tetracycline
Ciprofloxacin
Rifampicin
Erythromycin
Gentamicin
Trimethoprim/Sulfamethoxazole
Linezolid
Cefoxitin
Chloramphenicol
Clindamycin
Quinoprstin/dalfoprestin
Antibiotics
Sensitive
Intermediate
Resistant

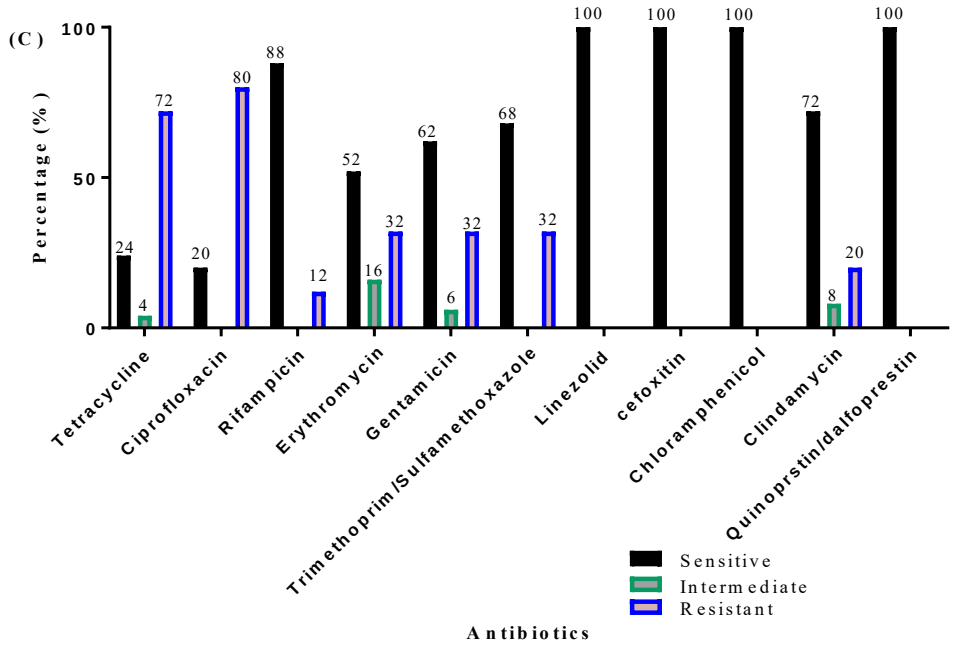


Figure 3.7: Antibiotic susceptibility profiles of study isolates **(A)** antibiotic susceptibility profiles of *S. aureus* isolates **(B)** Antibiotic susceptibility profiles of MRSA isolates **(C)** Antibiotic susceptibility profiles of MSSA isolates

3.4 Prevalence of MDR *S. aureus* isolates

In this study, the most predominant bacteria were MRSA (65%) while 35% were MSSA. Bacteria resistant to three or more antimicrobial classes were characterized as MDR, as previously described (A. L. Cohen *et al.,* 2008; Pillar *et al.,* 2008; Seas *et al.,* 2006). Overall, 85% *S. aureus* isolates were MDR. The prevalence of MDR-MRSA was 83% (n=166) while 20% (n= 10) of isolates were identified as MDR-MSSA.

3.5 Minimum inhibitory concentration (MICs)

In case of MRSA isolates, 80% (n=160) isolates resistant to cefoxitin had MICs >512µg/ml, while 12% (n=24) had MICs of 256µg/ml, 6% (n=12) had 128µg/ml, and 2% (n=4) had 64µg/ml. In case of ciprofloxacin, 85% isolates were resistant where 80% (n=136) isolates showed MICs >512µg/ml, while 12% (n=20) had MICs of 256µg/ml, 6% (n=10) had 128µg/ml, and 2% (n=4) had 64µg/ml. Resistance to tetracycline were recorded in 36% (n=72) isolates, 21% (n=15) isolates had MICs of

512µg/ml, 60% (n=43) had MICs of 256µg/ml, and 19% (n=14) had MICs of 128µg/ml. Resistance to erythromycin were recorded in 50% (n=100) isolates, 20% (n=20) isolates had MICs of 512µg/ml, 60% (n=60) had MICs of 256µg/ml, and 20% (n=20) had MICs of 128µg/ml. Resistance to rifampicin were noted in 20% (n=40) isolates where 60% (n=24) isolates had MICs of 512µg/ml, 20% (n=8) had 256µg/ml, 10% (n=4) had 128µg/ml, 5% (n=2) had 64µg/ml, and 5% (n=2) isolates had MICs of 32µg/ml. Gentamicin resistance was recorded in 64% (n=128) isolates where 90% (n=115) isolates had MICs >512µg/ml, while 10% (n=13) had MICs of 512µg/ml. Fosfomycin resistance was observed in 28% (n=56) isolates, 75% (n=42) isolates had a MIC of 512µg/ml while 25% (n=14) had a MIC of 256µg/ml. None of the isolates was resistant to vancomycin while 10 isolates exhibited intermediate resistance having MICs of 4µg/ml (n=5) and 8µg/ml (n=5).

In case of MSSA isolates, all MSSA isolates were sensitive to linezolid, chloramphenicol, cefoxitin, vancomycin and fosfomycin. Ciprofloxacin resistance was noticed in 80% (n=40) isolates, of these 80% (n=32,) had MICs of 512µg/ml, 15% (n=6) had 128µg/ml and 5% (n=2) had 64µg/ml. Rifampicin resistance was observed in 12% (n=6) isolates, and 67% (n=4) isolates had MICs of 512µg/ml, one isolate had 256µg/ml, and one had 128µg/ml. Resistance to tetracycline was noticed in 72% (n=36) isolates, in which 19% (n=7) isolates had MICs of 512µg/ml, 62% (n=22) had 128µg/ml, while 19% (n=7) had 64µg/ml. Resistance to erythromycin was noticed in 32% (n=16) isolates, in which 19% (n=3) isolates had MICs of 512µg/ml, 62% (n=10) had 128µg/ml, while 19% (n=3) had 64µg/ml. Gentamicin resistance was recorded in 32% (n=16) isolates, and 88% (n=14) of isolates had MICs of 512µg/ml, while one isolate each had MICs of 256µg/ml, and 128µg/ml.

3.6 Molecular detection of antibiotic resistance genes in phenotypically resistant MRSA isolates

All MRSA isolates that showed resistance to cefoxitin were screened for the presence of *mec*(A) and *mec*(C) genes (Figure 3.8 (A)). All MSSA isolates were also screened for the presence of *mec*(A) gene (Figure 3.8 (B)).

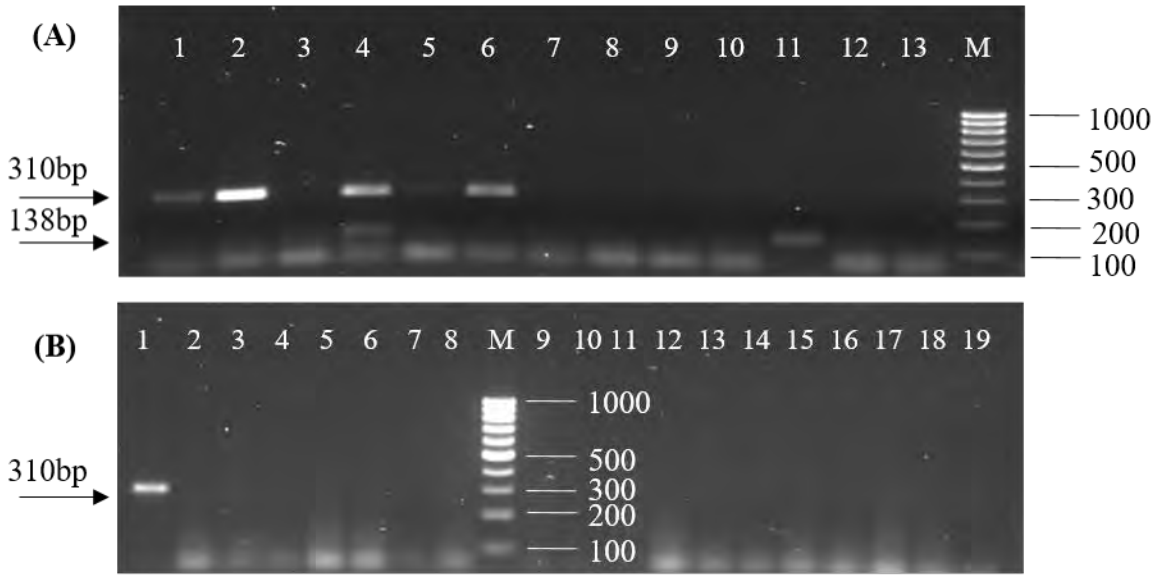


Figure 3.8: Representative gel images showing PCR amplification of *mec*(A) and *mec*(C) genes **(A)** Lane M shows the molecular weight marker of Gene Ruler 100bp (Thermo scientific). Lanes 1, 2, 4, 5, 6 show PCR product of *mec*(A) gene (310bp) and Lane 1 shows positive control for *mec*(A) gene while Lane 11 shows positive control for *mec*(C) gene (138bp), Lane 4 shows PCR product of *mec*(C) gene (138bp) **(B)** Lane M shows the molecular weight marker of Gene Ruler 100bp (Thermo scientific). Lanes 1-19 show amplification of the *mec*(A) gene (310bp) and Lane 1 shows positive control for *mec*(A) gene while lane 2 shows negative control.

3.7 Prevalence of *mec*(A) and *mec*(C) gene in MRSA isolates

In cefoxitin resistant isolates (phenotypically confirmed MRSA) *mec*(A) gene was found in 54% (n=108), and *mec*(C) gene in 3% (n=6) isolates, while only one MRSA harboured both *mec*(A) and *mec*(C) genes (Figure 3.8 (A)).

3.8 Multiplex PCR for molecular detection of antibiotic resistance genes in study isolates

All the resistant isolates were screened for 17 different antibiotic resistance genes using multiplex PCR. The tested genes were tetracycline resistance genes, *tet*K, *tet*M, penicillin resistance gene *bla*(Z), ciprofloxacin resistance genes *gyr*(A), *gyr*(B), *grl*(A) and *grl*(B), rifampicin resistance gene *rpo*(B), gentamicin resistance gene *aacph*D1, fosfomycin resistance genes, *fos*(A) and *fos*(B), erythromycin resistance gene *erm*(A), clindamycin inducible resistance gene *erm*(C), trimethoprim

resistance gene *dfr*(B), streptogramins A resistance genes *vat*(C), *vat*(B) and *vat*(A). The amplification products of the genes are shown in Figure 3.9.

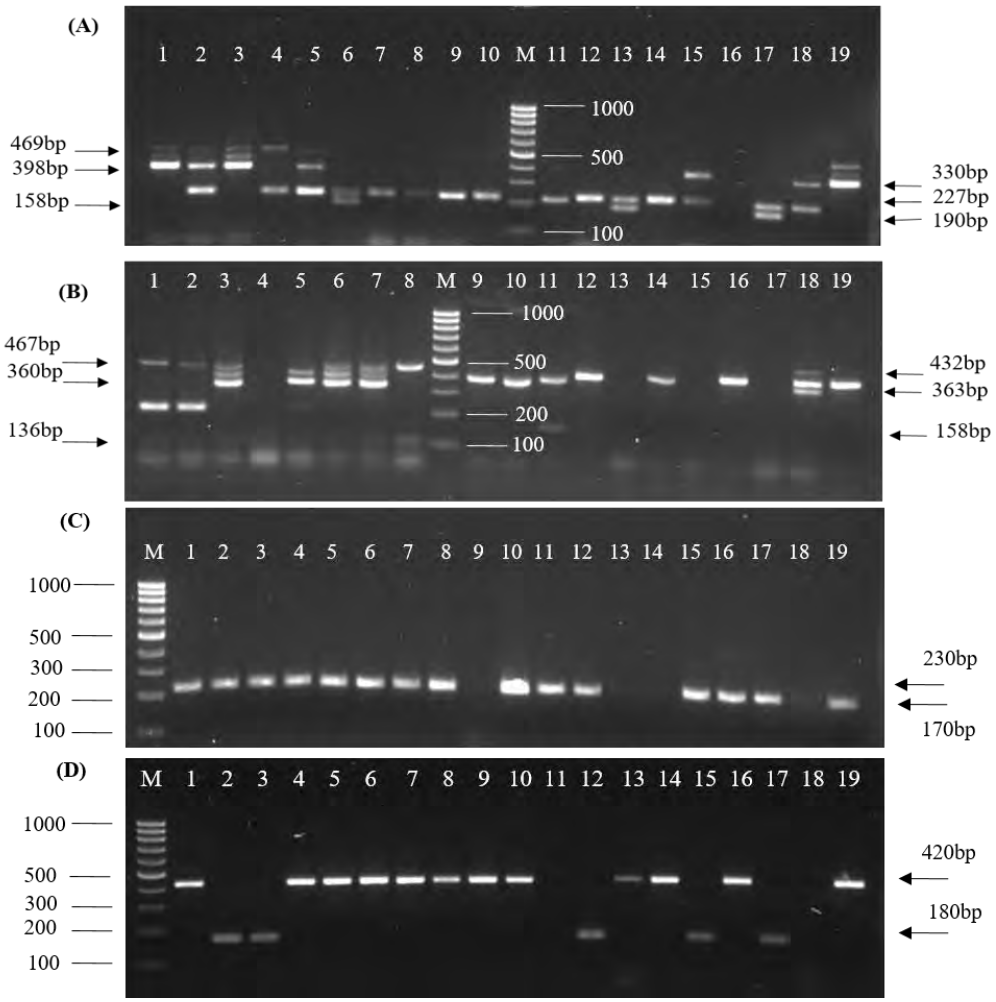


Figure 3.9: Representative gel images showing PCR amplification of 17 different antibiotic resistance genes such as; *tet*K, *tet*M, *bla*(Z), *gyr*(A), *gyr*(B), *grl*(A), *grl*(B), *rpo*(B), *aacph*D1, *fos*(A), *fos* (B) *erm*(A), *erm*(C), *dfr*(B), *vat*(C), *vat*(B) and *vat*(A) **(A)** Lane M shows the molecular weight marker of Gene Ruler 100bp (Thermo scientific). Lanes 1, 2, 3 show PCR product of *gyr*(A) (398bp), *gyr*(B) (469bp), Lane 6 shows *tet*M gene (158bp), Lane 11 shows *aacph*D1, gene (227bp), Lane 13 shows *erm*(C) gene (295bp), Lane 18 shows *erm*(A) (190bp), Lane 19 shows *grl*(A) (330bp) **(B)** Lane M shows the molecular weight marker of Gene Ruler 100bp (Thermo scientific). Lane 1 shows *vat*(A) gene (467bp), Lane 3 shows *tetK* gene (360bp), Lane 8 shows *vat* (B) gene (136bp), Lane 18 shows *grl*(B) (363bp), Lane 19 shows *rpo*(B), (432bp) **(C)** Lane M shows the molecular weight marker of Gene Ruler 100bp (Thermo scientific). Lanes 1-8, 10-13, 15-17 show *dfr*(B) (230bp), Lane 19 shows *vat* (C), (170bp) **(D)** Lane M shows the molecular weight marker of Gene Ruler 100bp (Thermo scientific). Lanes 1, 4-10, 13, 14, 16 and 19 show *bla*(Z) (420bp), Lanes 2, 3, 12, 15 and 17 show *fos*(A) (180bp).

3.9: Prevalence of antibiotic resistance genes (ARGs) in MRSA isolates

All the resistant isolates were screened for the detection of ARGs through multiplex PCR. In MRSA isolates ARGs were observed with the following percentages; tetracycline resistant genes, *tet*K 87% (n=62) was dominant followed by *tet*M 81% (n=58), while 81% (n=58) isolates were positive for both *tet*M and *tet*K genes. Penicillin resistance gene *bla*(Z) was observed in all isolates 100% (n=200). *gyr*(A) gene was in 95% (n=161), *gyr*(B) 33% (n=56), *grl*(A) 9% (n=15), and *grl*(B) with 6% (n=10) were recorded. Two isolates carried both *gyr*(A) and *grl*(B) genes. Among ciprofloxacin-resistant isolates, 31% (n=26) isolates were positive for both *gyr*(A) and *gyr*(B) genes, while 2 isolates had both *gyr*(A) and *grl*(A) genes. *rpo*(B) gene was observed in 80% (n=32) isolates. *aacph*D1 gene was recorded in 75% (n=96) isolates. *erm*(A) gene was found in 13% (n=26). *fos*(A) gene was found in 81% (n=45) isolates, while none of the isolates harbored *fos*(B) gene. *erm*(C) gene was found in all phenotypically (D-test) positive isolates (100%). *Dfr*(B) gene was noted in 80% (n=40) isolates. Streptogramins A resistant genes such as *vat*(C), *vat*(B), *vat*(A) were observed in 56% (n=112), 29% (n=58), and 4% (n=8) isolates respectively (Figure 3.10 (A).

3.10 Antibiotic resistance genes in MSSA isolates

In MSSA isolates the percentages of antibiotic resistance genes were as follows; *tet*K with 31% (n=11), while none of the isolates showed *tet*M. *gyr*(A) was observed in 52% (n=21) isolates. *rpo*(B) gene was found in 67% (n=4) isolates. *aacph*D1 gene was recorded in 75% (n=12) isolates. *erm*(A) gene was found in 75% (n=12) isolates. *Dfr*(B) gene was found in 75% (n=12) isolates. *vat*(C) gene was found in 50% (n=20), and *vat*(B) in 2% (n=1) isolates, while none of the isolates showed *vat*(A) gene. *erm*(C) gene was found in all phenotypically positive D-test isolates. All MSSA isolates were found negative for *mec*(A) gene. As compared to MSSA antibiotic resistance genes were more common in MRSA isolates and the data was statistically significant (p=0.001).

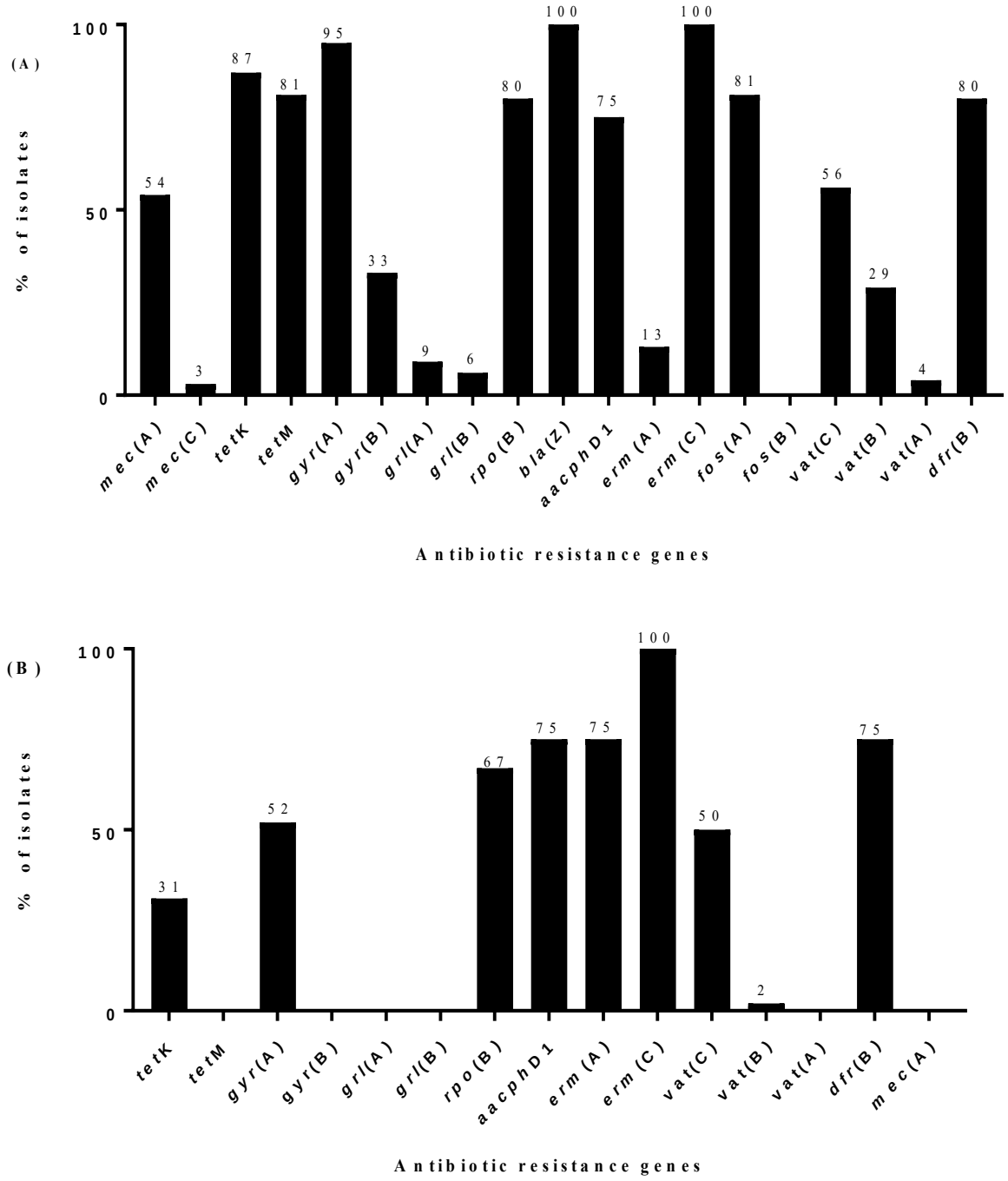


Figure 3.10: Antibiotic resistance genes carried by *S. aureus* isolates **(A)** shows the antibiotic resistance genes in MRSA isolates **(B)** antibiotic resistance genes in MSSA isolates

3.11 Correlation between antibiotic susceptibility and resistance genes

In *mec*(A) positive isolates, the susceptibility result determined by the disk diffusion and broth microdilution method correlated with the result of multiplex PCR for the detection of ARGs. MRSA isolates having MICs ranging from 64µg/ml to >512µg/ml to cefoxitin were positive for *mec*(A) gene. Fifty-eight (81%) positives isolates for both *tet*M and *tet*K genes having MICs range from 256µg/ml to 512µg/ml correlated with the PCR results.

Table 3.2: Correlation between antibiotic susceptibility, MICs values and resistance genes

Isolate	Disk diffusion method	MICs value	Antibiotic resistance genes	*p*-value
MRSA	cefoxitin (R)	64µg/ml	*mec*(A)	0.001
MRSA	cefoxitin (R)	128µg/ml	*mec*(A)	0.001
MRSA	cefoxitin (R)	512µg/ml	*mec*(A)	0.0001
MRSA	tetracycline (R)	512µg/ml	*tet*M and *tet*K	0.0001
MRSA	tetracycline (R)	256µg/ml	*tet*M	0.001

R; Resistant

3.12: Discussion

The present study describes more occurrence of MRSA in males (57%) as compared to female (43%) patients. Similar findings have been observed from Pakistan in different studies where MRSA was more dominant in male (54.7%) as compared to female (45.3%) (Brohi & Noor, 2017; Rafiq *et al.,* 2015), while in another, among the infected patients 65% were male and 35% were female (Muhamad Sohail & Zakia Latif, 2018a). As compared to this study, one report from Iran described MRSA prevalence in females (39.8%) higher than in males (34%) (Alfatemi *et al.,* 2014). In this study, *S. aureus* infections were more common in the age group of 18-35 years, and the same findings were also observed in other studies conducted in Pakistan with more infection reports in age group of 16-35 years (Taj *et al.,* 2016) and age group of 21-30 years (Siddiqui *et al.,* 2017).

In the current study, more isolates of MRSA were found from pus specimen (62%) followed by urine (14%) and blood (9%), while the least occurrence was in sputum (1%). The same finding was observed in other studies, where 70% MRSA was from pus specimen (Bhatta *et al.,* 2015) while one study reported only 17% (Brohi & Noor, 2017) MRSA from pus specimen. The more occurrence of MRSA from pus could be due to the opportunistic nature of *S. aureus* as their existence as normal flora of the host. In contrast, few other studies within the country have shown that MRSA was more in blood with isolation rate of 48.7% (Ullah *et al.,* 2016) and 20% (Siddiqui *et al.,* 2017). Higher rate of MRSA causing *S. aureus* bloodstream infection have also been reported from Greece, UK, Portugal, and Ireland (Humphreys, 2008).

In this study, the prevalence of MDR-MRSA was 83% while 20% of isolates were identified as MDR-MSSA. One study from Lahore, Pakistan reported that among MSSA, 37.84% isolates were MDR, while in the case of MRSA all isolates (100%) were found to be MDR (Taj *et al.,* 2016). Other studies from Pakistan have reported 54% (Ahmad *et al.,* 2014), 53.3% (Khan *et al.,* 2014), 15.84% (Ullah *et al.,* 2016) and 68% as MDR-MRSA (Siddiqui *et al.,* 2017). These differences could be due to the misuse of antibiotics, immune status of patients, different geographical location, sample size, hospital management, number of samples and severity of infections.

In this study, the resistance for MRSA was high for penicillin (100%), ciprofloxacin (85%), followed by gentamicin (64%), erythromycin (50%), tetracycline (36%), while least resistance was observed for clindamycin (26%) and rifampicin (20%), whereas one study from Pakistan noticed high resistance profiles in MRSA isolates against rifampicin (50%), clindamycin (79%), cotrimoxazole (59%), tetracycline (82%), with minimum resistance to fusidic acid (9%) and chloramphenicol (10%) (Idrees *et al.,* 2009). In the current study, high resistance was noticed in MSSA against ciprofloxacin (80%) and tetracycline (72%), gentamicin (32%) and low resistance to clindamycin (20%), while in a study sample collected during January 2013-2014 from Lahore reported low resistance profiles in case of MSSA isolates to ciprofloxacin (26.5%) and gentamicin (12.5%) and ciprofloxacin (76.5%) in MRSA isolates (Saeed *et al.,* 2016). One study from Karachi reported that, 28% of isolates were resistant to rifampicin, 65% to erythromycin, and 42% to tetracycline (Siddiqui *et al.,* 2017). The result of this study shows that all MSSA and MRSA isolates were linezolid susceptible, and similar findings were observed previously from India, a total of 232 *S. aureus* samples were collected during June to November 2012 (Trivedi, *et al.,* 2015). In this study, MSSA was noticed to be more sensitive to antibiotics as compared to MRSA and the data was statistically significant ($p< 0.05$).

Variation within the country in resistance profiles could be due to date of isolation, misuse and uncontrolled use of antibiotics, hospital infection control strategies, self-medication of antibiotics and nature of strains. The most effective drug was linezolid as observed in this study, as all isolates were linezolid susceptible and same findings (100%) were observed previously (Sipahi *et al.,* 2013; Vijayamohan & Nair, 2014). While a study from Pakistan has also reported linezolid resistance in 8% isolates (Brohi & Noor, 2017). These results disagree with the current finding. Previous study conducted in Faisalabad, Pakistan reported *cfr* gene in 78% of linezolid resistant *S. aureus* isolates (Azhar *et al.,* 2017). These results are not in line with the current study. The increase in resistance to linezolid in Pakistan might be due to the misuse of this drug or the transfer of plasmid (pSCFS1) encoded *cfr* gene to linezolid sensitive strains, which need to be investigated.

In this study, 88% MRSA isolates were chloramphenicol susceptible, and a similar finding was also observed from Hyderabad, Pakistan previously where 96% isolates were found to be chloramphenicol susceptible, 176 isolates were collected from March 2013 to June 2014 (Brohi & Noor, 2017). A similar result for chloramphenicol activity against MRSA was also reported (Bhutia *et al.,* 2015) while a contrasting study from Pakistan recorded chloramphenicol susceptibility in 75.86% isolates with MICs of 8µg/ml, 2.30% were intermediate had MIC of 16µg/ml and 21.84% were resistant with MIC of 32µg/ml (Fayyaz *et al.,* 2013).

In this study, sulfamethoxazole/trimethoprim resistance was noticed in 26% isolates which relate to the results of another study from Pakistan where 19% isolates were resistant (Ahmad *et al.,* 2014). One study from Pakistan reported that 41.67% isolates were trimethoprim resistant (Taj *et al.,* 2016), while other studies reported higher resistance rate ranging from 64 to 96% (Bukhari *et al.,* 2011; Bukhari, 2006; Kumar *et al.,* 2012). One study from Africa observed that 54% of isolates were resistant to trimethoprim and found that 94% of trimethoprim resistance was due to *dfr*(B) gene (Nurjadi *et al.,* 2014). In this study, the *dfr*(B) gene was found in 80% MRSA and 75% MSSA isolates. Other studies reported the *dfr*(B) gene in 92% (Nurjadi *et al.,* 2014), and 30% *S. aureus* isolates (Bojang *et al.,* 2017). These differences could be due to different geographical location, presence of *dfr*(B) gene on transposable elements and different sample size.

In Pakistan, MRSA prevalence has increased enormously in the last decades. The prevalence of MRSA in this study was 65% which is higher than previous reports from Pakistan where a range of 5% in 1989 and up to 52% in 2017 have been reported (Ashiq & Tareen, 1989; Siddiqui *et al.,* 2017), although one study has reported similar rate (67%) as to our findings (Hussain *et al.,* 2019). This indicates a continuous increase in circulation of this organism in clinical settings. Other studies within the country have reported wide variation and increase over the time in MRSA prevalence. Looking back at the prevalence of MRSA in different cities of Pakistan, these have been variable rates as follows: Karachi (67%, sample collection during Jan to June 2017) (Hussain *et al.,* 2019), Rawalpindi (55%, sample collection during

April 2011 to June 2013) (Perveen *et al.,* 2013), Karachi (52%, sample collection during, Jan 2015 to June 2017) (Siddiqui *et al.,* 2017), Lahore (46%, sample collection during March to September 2012) (Asghar, 2014), Kohat (44%, sample collection during Jan to December 2012) (Hussain *et al.,* 2013), Karachi (43%, sample collection during March 2004 to February 2005) (Perwaiz *et al.,* 2007), Lahore (38.5%, sample collection during June 2000 to December 2000) (Khatoon *et al.,* 2002), Hyderabad (36.5%, sample collection during July 2011 to December 2011) (Bano *et al.,* 2015), Peshawar (36%, sample collection during September 2012 to September 2013) (Ullah *et al.,* 2016) and in Sargodha (23%, sample collection during December 1995 to November 1996) (Siddiqui *et al.,* 1999). These differences could be due to different city, different sample size, hospital management, number of samples studied and varying host and environmental factors.

In this study, the phenotypic detection of MRSA by the disk diffusion method and MIC was 65%, while the presence of *mec*(A) and *mec*(C) gene was observed in 54% and 3% isolates respectively. To the best of our knowledge, this is the first report of clinical MRSA strains from Pakistan carrying *mec*(C) alone and in combination with *mec*(A) gene. There is limited data available on the molecular identification of the *mec*(A) gene in Pakistan as most of the studies limit up to susceptibility testing for MRSA. However, it has been reported from Pakistan in few studies and a range of 29.9%-87% (Brohi & Noor, 2017; Bukhari *et al.,* 2011; Fatima *et al.,* 2019; Siddiqui *et al.,* 2018; Taj *et al.,* 2015; Ullah *et al.,* 2016) has been reported. Worldwide various studies have reported the prevalence of *mec*(A) gene in MRSA isolates. Some studies from Iran reported the prevalence of *mec*(A) gene in 74.5% (Khorvash *et al.,* 2008), 87.36% (Khosravi *et al.,* 2012), and in 87.36% isolates (Hawraa *et al.,* 2014). One study from Saudi Arabia revealed that 92.8% of isolates were *mec*(A) positive (Al-Khulaifi *et al.,* 2009), whereas from Iraq *mec*(A) gene was found in 71.05% isolates (Aziz *et al.,* 2014).

In the literature, *mec*(A) gene-negative MRSA has been reported (Ba *et al.,* 2013), and a similar finding was observed in this study. In one study from Tehran, Iran

78.2% isolates were MRSA, and 15% were phenotypically MRSA were negative for *mec*(A) gene (Sahebnasagh *et al.,* 2014). In this study, 43% of phenotypically MRSA were negative for *mec*(A) gene. However, in the literature, studies have reported less prevalence of *mec*(A) gene in phenotypically positive MRSA (Alfatemi *et al.,* 2014; Bhatta *et al.,* 2016; Bukhari *et al.,* 2011). The variation in the prevalence of *mec*(A) gene could be attributed to additional multiple mechanisms of methicillin resistance such as acquiring *mec*(B) gene, overexpression of PBP and mutation in PBP.

MRSA carrying the *mec*(C) gene can cause a variety of infections, especially in community-acquired isolates (Petersen *et al.,* 2013). In the current study, the *mec*(C) gene was detected in 3% MRSA isolates while no previous *mec*(C) gene data is available from human clinical isolates from Pakistan. However, some studies from other parts of the world have reported the prevalence of *mec*(C) gene in MRSA isolates. One study from Egypt reported that all MRSA isolates were negative for *mec*(C) gene (Hefzy *et al.,* 2016). One study from Sweden reported the MRSA strain with *mec*(C) gene in human clinical samples (Swedish Institute for Communicable Disease Control 2012) whereas the study conducted in Belgium reported low occurrence of *mec*(C) MRSA in less than 1% from human clinical samples (Deplano *et al.,* 2014). These results agreed with the current finding as *mec*(C) gene was found in 3% isolates.

In this study, resistance to ciprofloxacin was recorded in 85% isolates, the same finding (87%) was also observed previously (Pandya *et al.,* 2014). As compared to the current study, low resistance to ciprofloxacin was observed in other studies from Pakistan with rates as 17% (Ahmad *et al.,* 2014), 44% (Kaleem *et al.,* 2010), 47% (Ullah *et al.,* 2016), and in 48% of isolates (Hussain *et al.,* 2019). These differences within the country may be due to different location, sample size, hospital management, number of samples and varying host genetic factors and other reasons could be that the antibiotic resistance profiles vary with time and drug stimulus. In the current study, 95% of isolates were positive for *gyr*A, 33% for *gyr*B, 9% for *grl*A and 6% *grlB*. These results agree with previously reported reports where one

study from Egypt reported *gyr*A gene in 63% and *gyr*B gene in 70.4% of clinical isolates (Osman *et al.,* 2016). One study from China reported *gyr*A gene in 73%, *gyr*B in 60%, *grl*A in 63% while *grl*B in 64% of isolates (Liu *et al.,* 2018). Another study from China reported *gyr*A in 36%, *grl*A in 20.4%, *gyr*B in 0.97%, and *grl*B in 10.68% *S. aureus* isolates (Liang *et al.,* 2019).

In the current study, 80% isolates were rifampicin sensitive, and other studies have reported similar findings in MRSA isolates (Bhutia *et al.,* 2015; Bukhari *et al.,* 2011; Bukhari, 2006). However some studies from Pakistan reported rifampicin susceptibility in 94% (Ahmad *et al.,* 2014), 14% (Taj *et al.,* 2010), 18.8% (Idrees *et al.,* 2009) and in 28% isolates (Siddiqui *et al.,* 2017). As compared to our current finding, high resistance was reported in China, where out of 88 isolates, 94.3% were rifampicin-resistant with MIC of ≥8µg/ml, 5.7% isolates had a low-level resistance to rifampicin (MIC, 2 to 4µg/ml) (Zhou *et al.,* 2012). *rpo*B gene encodes the DNA-dependent RNA polymerase which is essential for *S. aureus* survival. Rifampicin resistance is caused due to mutation in the highly conserved part of *rpo*B gene known as rifampicin resistance-determining region (Bongiorno *et al.,* 2018). In the present study, the low prevalence of *rpo*B gene in MRSA (80%) and 67% in MSSA isolates could be due to the poor efficiency of PCR.

In this study, resistance to tetracycline was observed in 36% isolates. Other studies from Pakistan have reported high tetracycline resistance in 51% (Akhtar, 2010), 82% (Hussain *et al.,* 2013), 42% (T. Siddiqui *et al.,* 2017) and 58% isolates (Hussain *et al.,* 2019). In this study, 87% isolates were found positive for *tet*K gene in the case of MRSA while 31% in MSSA. Other studies from Pakistan noticed *tet*K gene in 14.6% (Khattak *et al.,* 2015), 46% *tet*K gene in MRSA while 33% in MSSA isolates (Ullah *et al.,* 2012). In our isolates, *tet*M was absent in MSSA while both the *tet*K and *tet*M were almost equally prevalent in MRSA isolates having a rate of 87% and 81%. One study reported 33.33% isolates with resistance to oxytetracycline and carried either *tet*M or *tet*K or both resistance genes (Strommenger *et al.,* 2003) and another reported 9% isolates (Bojang *et al.,* 2017). These studies disagreed with the current finding as high percentages of *tet*M and

*tet*K genes was observed. Because of the association with Tn916, *tet*M is acquired in MRSA as a result of selective pressure and persists in these strains (Ong *et al.,* 2017). As we already know that the conjugative transposan Tn916 carry the *tet*M gene and capable of horizontal transfer (Devirgiliis *et al.,* 2009). As compared to current findings, 100% tetracycline resistance was reported in one study from Lahore, Pakistan (isolates collected during February to May 2008) (Ta *et al.,* 2010). Another study reported the prevalence of *tet*K gene in 86% isolates (Jones *et al.,* 2006), the same findings were observed in the current study. One study from Iran noticed *tet*K and *tet*M genes in 82.75% and 56.9% isolates respectively (Khoramrooz *et al.,* 2017).

Our findings is not similar with a study from Poland that noticed low prevalence of *tet*M and *tet*K genes in MRSA isolates with prevalence of 36.4% and 31.8%, respectively (Trzcinski *et al.,* 2000). As compared to this study, a report from Turkey described low prevalence of *tet*K (14.3%) and *tet*M (50%) genes in MRSA isolates respectively (Ardic *et al.,* 2005). A previous study from Malaysia that reported the prevalence of *tet*M and *tet*K in 49% and 21% isolates respectively (Lim *et al.,* 2012) also contradicts with our study. In this study, multiplex PCR results showed that 56% isolates were positive for *vat*(C) gene, 29% for *vat*(B) gene and 4% for *vat*(A) gene in MRSA isolates while in MSSA *vat*(C) and *vat*(B) gene was displayed by 50% and 2% isolates respectively. This is different from one study where all quinupristin/dalfopristin resistant isolates were positive for *vat*(B) gene (Strommenger *et al.,* 2003).

In this study, gentamicin resistance was noticed as 64% and 32% in MRSA and MSSA isolates respectively. Similar results were reported in 60.3% isolates in a study from Lahore, Pakistan, a total of 1102 isolates collected between February 2003 and March 2005 (Bukhari, 2006). Various studies from Pakistan have reported gentamicin resistance in *S. aureus* isolates. One study from Dera Ismail Khan, Pakistan reported 100% resistance against gentamicin in *S. aureus* isolates, collected during January 2011 to August 2011 (Iqbal *et al.,* 2011), while another study from Peshawar, Pakistan noticed 57% resistance in MRSA while 12% in

MSSA, a total of 280 *S. aureus* isolates collected during September 2012 to September 2013 (Ullah *et al.,* 2016). These findings are close to our current study in case of MRSA isolates. One study from Lahore, Pakistan reported 97.6% gentamicin resistance, in total of 1102 *S. aureus* isolates collected during April 2006-March 2008 (Bukhari *et al.,* 2011), which is not in accordance with the current study. Other studies from Abbottabad, Pakistan, reported gentamicin resistance in 45.83% of *S. aureus* isolates, a total of 98 isolates were collected during from 2007 to 2010 (Taj *et al.,* 2016) and another study from Rahim Yar Khan, Pakistan reported 40% amikacin resistance in MRSA isolates, in total of 100 clinical isolates collected from January to June 2017 (Hussain *et al.,* 2019), which is not similar to current study. These differences could be due to different number of isolates, improper usage of this drug, and different MRSA clone circulating in different hospitals in Pakistan.

In this study, 75% of aminoglycoside resistant isolates harbored *aacph*D1 gene; variable findings have been observed by other studies from Iran, Poland, Korea, and European countries, which reported *aac(6')-Ie-aph(2")* gene as the most prevalent one, with a range of 28.9 to 93.7% (Choi *et al.,* 2003; Emaneini *et al.,* 2013; Hauschild *et al.,* 2008; Mohammadi *et al.,* 2014; Schmitz *et al.,* 1999). This high variation is probably because of different circulating strains and pharmaceutical practices in different regions.

Some studies from India reported the *aac (6')-Ie-aph (2''),* gene in 55.4% (Perumal, Murugesan, & Krishnan, 2016), and 16.6% gentamicin resistant isolates (Bhutia *et al.,* 2015). These findings are not in accordance with this study. One study from Jordan reported *aac(6')/aph(2")* gene in 45% of *S. aureus* isolates, 34.8% in MSSA and 52.6% in MRSA (Bdour, 2012). Other studies reported the *aac(6')/aph(2")* gene in 48% (Fatholahzadeh *et al.,* 2009) and 28% clinical *S. aureus* isolates (Ardic *et al.,* 2006).Various other studies reported gentamicin susceptibility in 85.64% (Trivedi *et al.,* 2015), 86.05% (Akpaka *et al.,* 2006), 91.8% (Tertiary, 2013) and in 77.78% MSSA isolates (Goswami *et al.,* 2011). They don't have to be in accordance with the present study, as we found only 62% and 32% susceptibility against gentamicin in case of MSSA and MRSA respectively.

In this study, 50% of isolates showed resistance to erythromycin in MRSA while 32% in MSSA. Other studies within the country reported a wide variation in the prevalence of erythromycin resistance with 69% (Akhtar, 2010), 70% (Aghazadeh *et al.,* 2009), 100% prevalence in MRSA while 70% in case of MSSA (Ullah *et al.,* 2016), 65% (Siddiqui *et al.,* 2017) and in 46% MRSA isolates (Hussain *et al.,* 2019). Variation in erythromycin resistance pattern from different cities of Pakistan may be due to different sample size, hospital infection control program, self-medication and uncontrolled use of antibiotics.

One study from China reported erythromycin resistance in 76.92% *S. aureus* isolates, 95% of the MICs of the resistant strains were ≥128μg/mL (Ding *et al.,* 2012). Other studies, reported erythromycin susceptibility in 77.08% (Trivedi *et al.,* 2015), 82.8% (Tertiary, 2013) and 67.35% isolates (Akpaka *et al.,* 2006), while the current study represents the lower susceptibility rate (50%) in MRSA and 32% in MSSA. As compared to this study, previous report from Pakistan described high prevalence of *erm*(A) in 79.2% isolates (Khattak *et al.,* 2015). In this study, a low prevalence of *erm*(A) in MRSA isolates conflicts with the result from previous study (Petrelli *et al.,* 2008). Besides, the different genetic backgrounds of different MRSA strains may also contribute to the divergent antibiotic resistance profiles. The variation in the *erm*(A) gene in different studies might be due to the presence of this gene on mobile genetic elements.

In the present study, clindamycin showed moderately good activity towards MRSA isolates with a susceptibility rate of 66%. As compared to the current study, 32.4% susceptibility rates to clindamycin were reported (Bukhari *et al.,* 2011; Bukhari, 2006). Other studies from Pakistan reported clindamycin resistance in 79% (Akhter *et al.,* 2009), and 70% isolates (Hussain *et al.,* 2013) and compared to these, the current study noticed low resistance as 26% in MRSA while 20% in MSSA. This might indicate the restoring effectiveness of an antibiotic while switching to other antibiotics for a certain period. In this study, prevalence of clindamycin inducible resistance was observed to be 4% both in case of MRSA and MSSA, while as compared to our finding, a study from Karachi reported 72% inducible clindamycin

resistance phenotype in *S. aureus* isolates (Fasih *et al.,* 2010) and 15.84% from Peshawar in MRSA isolates (Ullah *et al.,* 2016). Globally clindamycin inducible resistance was recorded as, 12.1% from India (Juyal *et al.,* 2013), 25.4% from Turkey (Cetin *et al.,* 2008) and 92.1% from Korea (Lee *et al.,* 2010), 12.04% in MRSA and 24.44% in MSA respectively from India (Baragundi *et al.,* 2013), 22.2% in MSSA and 46.2% in MRSA from India (Reddy & Suresh, 2017), 43.56% in MRSA and 6.93 in MSSA from India (Pal *et al.,* 2010), 73% in MSSA and 70% in MRSA from Pakistan (Fasih *et al.,* 2010), 14.8% in MSSA and 24.4% in MRSA from Turkey (Yilmaz *et al.,* 2007) and 25% in MRSA isolates from India (Khara *et al.,* 2016). Varying clindamycin inducible resistance has been recorded from different countries as, 12.1% from India (Juyal *et al.,* 2013), 25.4% from Turkey (Cetin *et al.,* 2008) and 92.1% from Korea (Ji *et al.,* 2010), 12.04% in MRSA and 24.44% in MSSA respectively from India (Baragundi *et al.,* 2013), 22.2% in MSSA and 46.2% in MRSA (Reddy & Suresh, 2017), 43.56% in MRSA and 6.93 in MSSA (Pal *et al.,* 2010), 73% in MSSA and 70% in MRSA (Fasih *et al.,* 2010), 24.4% in MRSA and 14.8% in MSSA (Yilmaz *et al.,* 2007) and 25% in MRSA isolates (Khara *et al.,* 2016). These findings are not in accordance with our present study, as we found low prevalence rate. To overcome the problem of inducible clindamycin resistance, D-test must be performed as routine practice in diagnostic microbiology to avoid clindamycin therapeutic failure.

In the present study, the *erm*(C) gene was observed in all *S. aureus* isolates both in MRSA and MSSA, as compared to our current finding low prevalence of *erm*(C) gene was reported from Turkey with 5% in MRSA isolates (Aktas *et al.,* 2007). One study from China reported the *erm*(C) gene in 84.6% in *S. aureus* isolates (Ding *et al.,* 2012) and is in accordance with the current study.

In this study, fosfomycin susceptibility was noticed in 72% isolates. Some studies have reported non-susceptibility of the isolates for fosfomycin in 25% MRSA isolates (Saleem *et al.,* 2017), and in 31% MRSA isolates (Taj *et al.,* 2010), whereas low fosfomycin resistance (2.7%) was reported from Karachi in MRSA isolates (Sattar *et al.,* 2019). These results disagree with the current findings. In the

present study, *fos*(A) gene was noticed in 81% isolates, while all isolates were negative for *fos*(B) gene. In one study 13.43% fosfomycin resistant isolates with MIC of 128µg/ml were positive for *fos*(B) gene (Fu *et al.,* 2016), which is not in accordance with the current study finding.

In the current study 5% isolates were confirmed as VISA, while none of the isolates was vancomycin-resistant. One study from Pakistan reported VISA in 4% isolates (Bukhari *et al.,* 2004; Mehmood *et al.,* 2007), the same finding was noticed in the current study. Various studies from Pakistan reported variation in VISA isolates as 13% (Hakim *et al.,* 2007), 28% had MICs of 8µg/ml (Ahmad *et al.,* 2014), 8% had MICs of 4µg/ml (Arshad *et al.,* 2015) and 7 isolates with MICs of 4µg/ml (n=6) and 8µg/ml (n=1) (A. Ullah *et al.,* 2016). This variation may be due to the abundant use of vancomycin in complicated MRSA infection and may be due to different screening methods.

Chapter 4: Molecular Typing in clinical *S. aureus* isolates

4.1 Introduction

Several molecular typing methods are being used in epidemiological studies of MRSA. Such techniques provide relationships among different clones of MRSA circulating in a particular area during an outbreak or just generally. The two main categories of typing techniques are sequence-based typing and band-based typing. The former is the preferred technique because it generates reliable data. For MRSA, several molecular typing methods i-e band based methods include accessory gene regulator (*agr*) typing, hypervariable region (HVR) typing, staphylococcal cassette chromosome *mec* (SCC*mec*) typing and pulsed-field gel electrophoresis (PFGE) (de Sousa, 2004), while staphylococcal protein A (*spa*) typing, multi-locus sequence typing (MLST) are sequence based method. MRSA clones are usaually defined by incorporation of SCC*mec* typing information in combination other typing methods such as MLST and *spa* typing. SCC*mec* typing is a reliable tool for the proper definition of MRSA clones to identify the genetic relatedness and epidemiology of MRSA clones that are circulating worldwide. It is important in the case of CA-MRSA clones that are emerging and spreading frequently (McClure-Warnier, *et al.,* 2013).

In this chapter, typing of study isolates using different molecular typing methods such as *agr* group*,* HVR typing, *spa* typing, SCC*mec* typing, MLST, and PFGE and their link with antibiotic resistance has been described.

4.2 Results

4.3 *Agr* typing in *S. aureus* isolates

4.3.1 Prevalence of *agr* specific group in MRSA and MSSA

All study isolates (250 isolates; 200 MRSA and 50 MSSA) were processed for *agr* typing (Figures 4.1 and 4.2). In MRSA *agr* I was the most prevalent group (n= 44/200, 22%), while in MSSA *agr* III (n= 8/50, 16%) was the dominant group. The distribution of the *agr* group in MRSA was: *agr* I (n= 44/200, 22%), *agr* II (n= 14/200, 7%), *agr* III (n= 40/200, 20%), *agr* IV (n = 0/200, 0%), while in MSSA it was *agr* I (n= 2/50, 4%), *agr* II (n= 4/50, 8%), *agr* III (n= 8/50, 16%), *agr* IV (n= 2/50, 4%). Sixty eight percent (n=34/50) of MSSA and 51% (n=102/200) of MRSA isolates remained non-typeable (NT). *Agr* group's distribution in MRSA and MSSA isolates is shown in Table 4.1.

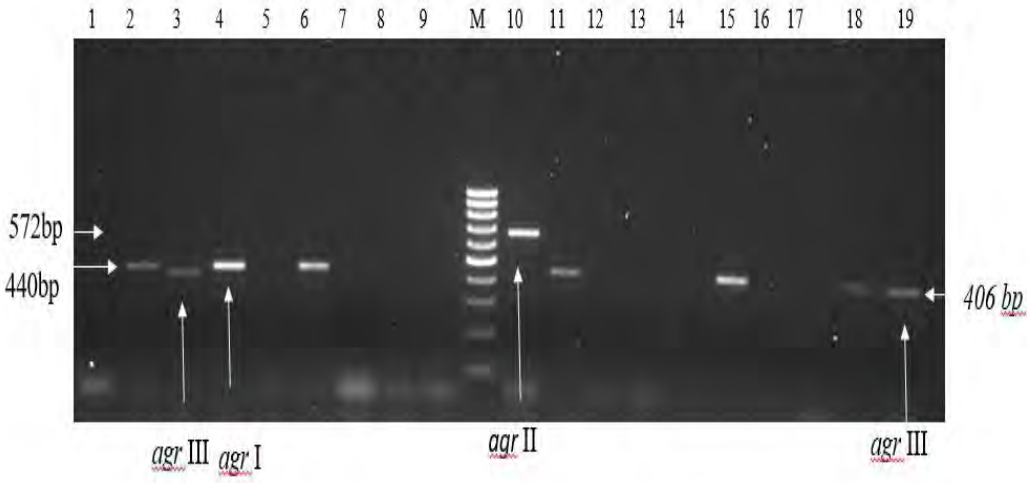


Figure 4.1: Representative gel image shows PCR amplification of *agr* groups in MRSA isolates. Lane 2 represents the *agr* group I of (440bp) positive control, lane 3 *agr* group III (406bp) positive control, lanes 4, 6, 11 and 15 represents *agr* I (440), lanes 18, 19 show *agr* III, and lane 10 represents *agr* II (572bp). Lanes 5, 8, 9 represent negative control for *agr* I, and II, lanes 1, 7, 12, 13, 14, 16 and 17 showed no amplification. Lane M shows the molecular weight marker of Gene Ruler 100bp (Thermo scientific).

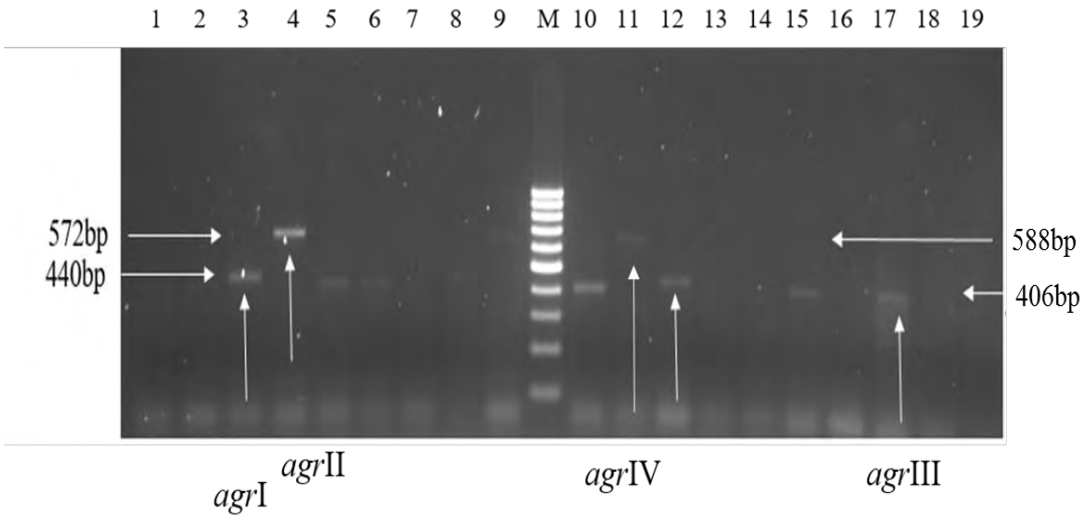


Figure 4.2: Representative gel image shows PCR amplification of *agr* groups in MSSA isolates, lane 3 represents the *agr* group I (440bp) positive control, lanes 8 and 12 show *agr* goup I, lane 4 represents *agr* group II (572bp) positive control, lane 9 shows *agr* group II, lane 5 represents *agr* III (406) positive control, lanes 6, 10, 15 and 17 show *agr* group III, lane 11 represents *agr* IV (588bp). Lanes 1, 2 and 19 showed no amplification. Lanes 7, 13, 14, and 18 show negative controls for *agr* I, II, III, and IV. Lane M shows the molecular weight marker of Gene Ruler 100bp (Thermo scientific).

Table 4.1: Prevalent *agr* groups in MRSA and MSSA

Agr groups	MRSA (n=200)	Source of sample	MSSA (n=50)	Source of sample
I	(n=44, 22%)	Pus (n=24), Urine (n=10), Tissue (n=4), Blood, Tracheal secretions, and throat swab (n=2 each)	(n=2, 4%)	Urine (n=1), Body fluid (n=1)
II	(n=14, 7%)	Pus (n=6), Blood (n=4), Tracheal secretions and tissue (n=2 each)	(n=4, 8%)	Pus (n=2), Blood (n=1), Tissue (n=1)
III	(n=40, 20%)	Pus (n=20), Tracheal secretions (n=8), Blood (n=6), Urine (n=4), Tissue (n=2)	(n=8, 16%)	Pus (n=6), Urine (n=1), Body fluid (n=1)
IV	(n=0, 0%)	Nil	(n=2, 4%)	Pus(n=1), Blood (n=1)
Non typeable (NT)	(n=102, 51%)	Pus (n=80), Tracheal secretions (n=8), Blood (n=6), Urine (n=4), Tissue (n=4)	(n=34, 68%)	Pus (n=24), Blood (n=4), Tracheal secretion (n=4), Tissue (n=2)
Total	100%		100%	

4.3.2 Distribution of *agr* groups in MRSA isolates according to patient gender, specimen, and wards

Among sample sources of MRSA, *agr* I was more common in pus samples (n=24/44, 54.54%) followed by urine (n=10/44, 22.72%), tissue (n=4/44, 9.09%), blood, tracheal secretions, and throat swab (n=2/44 each) (Figure 4.3 (A)). *Agr* I was more common in isolates from male (n=28/44, 63.63%) as compared to female patients (n=16/44, 36.36%) (Figure 4.3 (B)). MRSA isolates mainly were from emergency ward (n=16/44, 36.36%), followed by urology (n=8/44, 18.18%), surgery, orthopedic ward and general male ward (n=6/44, 13.63% each), and 4.54% (n=2/44) from dermatology (Figure 4.3 (C)).

Agr II was more common in pus (n=6/14, 42.85%) followed by blood (n=4/14, 28.57%), tracheal secretion and tissue (n=2/14, 14.28% each) (Figure 4.3 (D)). In case of gender distribution *agr* II was more common in males (n=10/14, 71%) as compared to females (n=4/14, 29%) (Figure 4.3 (E)). According to wards, these were from medical wards, surgical ward and emergency ward (n=4/14, 28.57% each) and 14.28% (n=2/14) from orthopedic ward (Figure 4.3 (F)), while in *agr* III MRSA isolates were found more in pus (n=20/40, 50%), followed by tracheal secretions (n=8/40, 20%), blood (n=6/40, 15%), urine (n=4/40, 10%) and 5% (n=2/40,) were from tissue (Figure 4.3 (G)). In case of gender distribution *agr* III was more common in males (n=24/40, 60%), as compared to female (n=16/40, 40%) (Figure 4.3 (H)). In *agr* III, MRSA isolates mainly were from emergency ward (n=12/40, 30%), followed by general OPD and medical ward (n=8/40, 20% each), general surgery (n=6/40, 15%), and 5% (n=2/40) from private ward, orthopedic and oncology each (Figure 4.3 (I)).

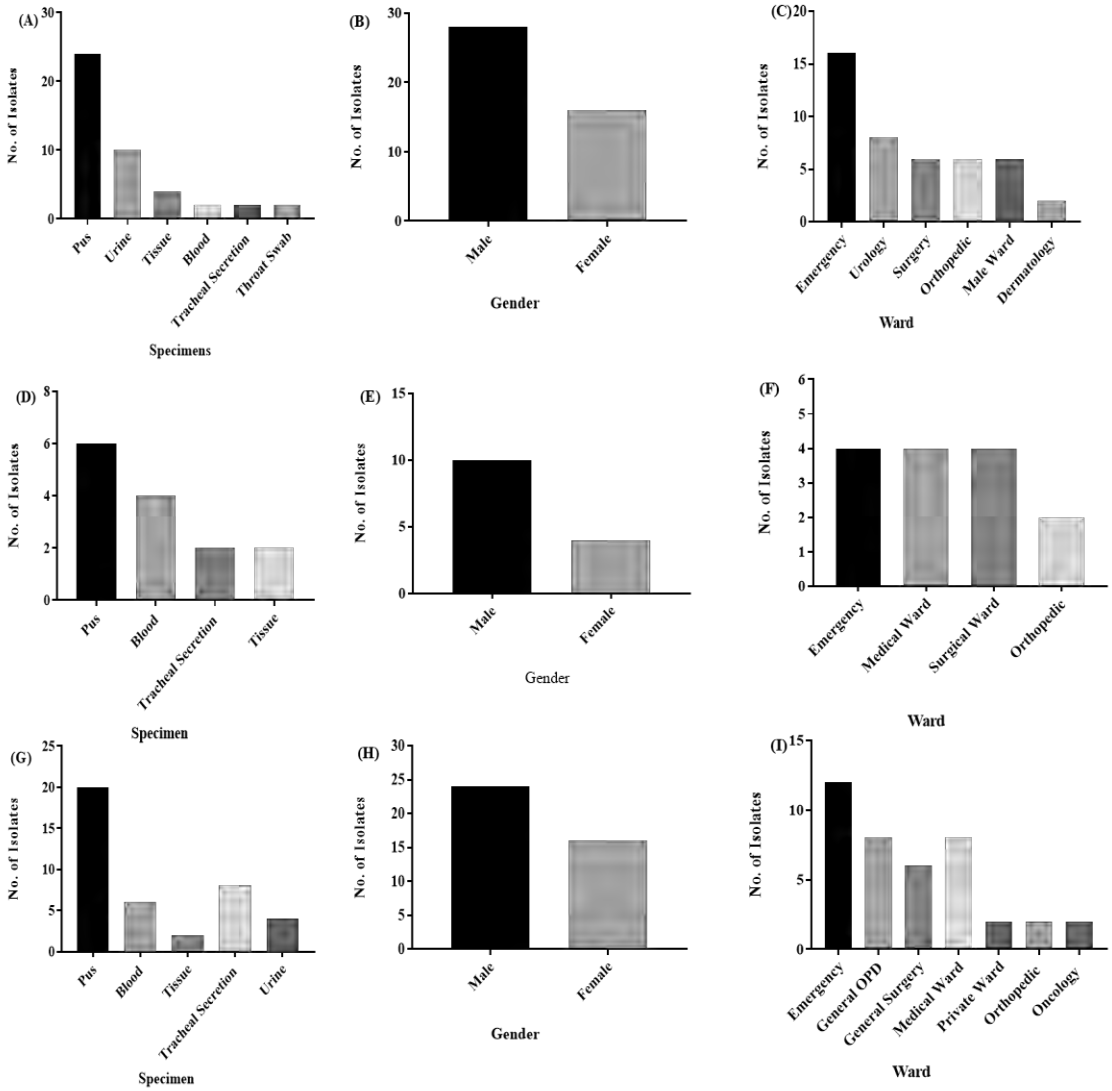


Figure 4.3: Distribution of *agr* groups according to specimen, gender, and wards in MRSA isolates, **(A-C)** shows specimen, gender and wards distribution of *agr* group I in MRSA isolates **(D-F)** shows the specimen, gender and ward distribution of *agr* group II in MRSA isolates **(G-I)** shows the specimen, gender and ward distribution of *agr* group III in MRSA isolates

4.3.4 Antibiotic resistance in *agr* groups in MRSA and MSSA isolates

In MRSA isolates *agr* I group showed 98% resistance to ciprofloxacin, followed by sulfamethoxazole/trimethoprim, erythromycin, tetracycline, gentamicin, and clindamycin. *agr* II group showed 98% resistance to ciprofloxacin while low resistance was observed against gentamicin and clindamycin. *agr* III group showed 100% resistance to ciprofloxacin while low resistance was observed in sulfamethoxazole/trimethoprim and clindamycin. *Agr* non-typeable (NT) groups lower resistance to erythromycin and clindamycin while 84% isolates were resistance to ciprofloxacin (Table 4.2). In MSSA isolates *agr* I group showed 100% sensitivity to tested antibiotics except for ciprofloxacin. *agr*II group showed 50% resistance to sulfamethoxazole/trimethoprim and tetracycline while was sensitive to the rest of the tested antibiotics. *Agr* III group showed highest resistance to tetracycline (90%) and lowest resistance to clindamycin. *Agr* IV group showed 12% resistance to tetracycline while the remaining were sensitive to all tested antibiotics. *Agr* NT group showed 20.58% resistance to tetracycline while low resistance to erythromycin and clindamycin (Table 4.2).

Table 4.2: Antibiotic resistance pattern in *agr* specific groups in MRSA and MSSA

Strain	Antibiotics	Phenotypic resistance	Genotypic resistance	*Agr* specific group Agr I	*Agr* II	*Agr* III	*Agr* IV	*Agr* Non-typeable	*p*-value
(MSSA)	Penicillin	100%	*bla*(Z) 100%	100%	100%	100%	100%	100%	>0.999
(MRSA)	Sulfamethozole-trimethoprim	26%	*Dfr*(B) 80%	14%	13%	20%	-	25%	<0.0001*
(MSSA)		34%	*Dfr*(B) 75%	0%	50%	11%	0%	0%	
(MRSA)	Erythromycin	50%	*erm*(A) 13%	57%	40%	50%		13%	<0.0001*
(MSSA)		32%	*erm*(A) 75%	0%	11%	0%	0%	12%	
(MRSA)	Tetracycline	36%	*tet*K, 87% and *tet*M 81%	40%	60%	30%	-	33%	<0.0001*
(MSSA)		72%	*tet*K, 31%	0%	50%	90%	12%	21%	
(MRSA)	Ciprofloxacin	85%		98%	98%	100%	-	84%	<0.0001*
(MSSA)		80%		1%	25%	0%	0%	36%	
(MRSA)	Gentamicin	64%	*aacph*D1 75%	50%	40%	50%		63%	ND
(MSSA)		32%	*aacph*D1 75%	0%	0%	0%	0%	21%	
(MRSA)	Clindamycin	26%		36%	20%	30%	-	19%	0.0002*
(MSSA)		20%		0%	0%	11%	0%	9%	

*Numbers are statistically significant ($p < 0.05$)
ND= Not determined

4.4 Detection of the hypervariable region (HVR) in MRSA (200) isolates

In MRSA isolates, 42% (n=84/200) were found to be positive for HVR (Figure 4.4) whereas 58% (n=116/200) isolates did not reveal the presence of HVR. In MRSA isolates 5 HVR types were observed, the most dominant being type 6 (n=50/200, 25%) followed by type 5 (n=14/200, 7%), type 7 (n=8/200, 4%), type 3 (n=8/200, 4%), and type 4 (n=4/200, 2%).

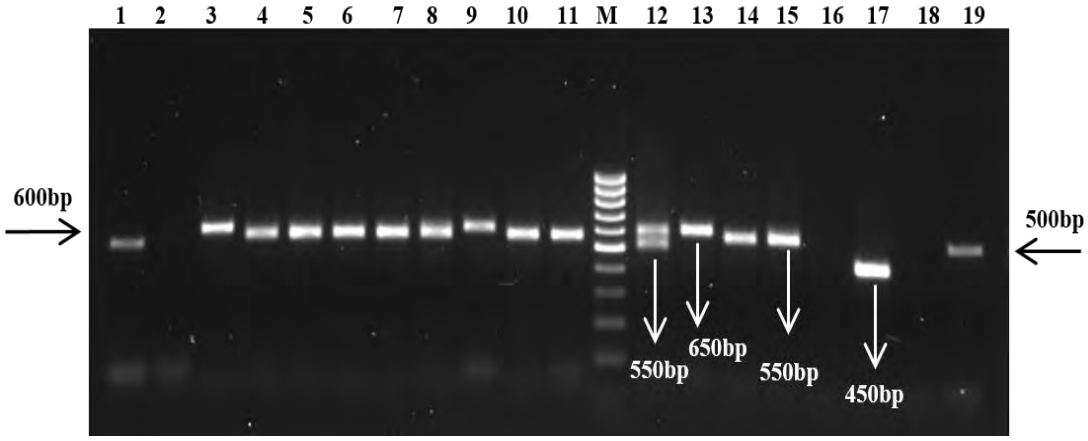


Figure 4.4: Representative gel image showing PCR amplification of the HVR gene. Lane M shows the DNA ladder of 100bp (Thermo scientific), Lanes 1-19 show amplification of HVR gene and lane 2, 16, and 18 showed no amplification for the HVR gene. Lanes 5, 9, and 14 show positive control for type 6, 5, and 7 respectively. Lanes 4, 6, 7, 8, 10 and 11 show amplification of HVR type 6 (600bp), Lane15 shows HVR type 5 (550bp), Lanes 12 and 13 show HVR type 7 (650bp), while Lanes 17 and 19 show positive results for 450bp and 500bp for HVR type 3 and 4 respectively. Lane 12 shows 2 HVR types of type 5 and 7 of 550bp and 650bp respectively.

4.4.1 Antibiotic resistance in HVR types in MRSA isolates

In MRSA isolates HVR type 6 showed 85%% resistance to ciprofloxacin followed by tetracycline (55%), gentamicin (70%), clindamycin (56%), fosfomycin (50%), and rifampicin (60%) while low resistance was observed to erythromycin (20%). HVR type 5 showed 80% resistance to ciprofloxacin followed by tetracycline (60%) and rifampicin (45%) while low resistance was observed against gentamicin (35%), and clindamycin (28%). HVR type 7 showed 82% resistance to ciprofloxacin followed by gentamicin (60%), fosfomycin (40%) while low resistance was observed to sulfamethoxazole/trimethoprim (26%), and clindamycin (20%).

HVR type 3 showed 80% resistance to ciprofloxacin followed by gentamicin (48%), clindamycin (40%) while low resistance was observed to tetracycline (26%) and sulfamethoxazole/trimethoprim (20%). HVR type 4 showed 100% resistance to ciprofloxacin, while 50% isolates were resistant against gentamicin and clindamycin while low resistance was observed to tetracycline and sulfamethoxazole/trimethoprim (25% each). HVR non-typeable isolates showed 80% resistance to ciprofloxacin followed by erythromycin (80%), tetracycline (55%), gentamicin (70%), sulfamethoxazole/trimethoprim (60%), clindamycin (56%), fosfomycin (60%), and rifampicin (60%).

4.5 SCC*mec* typing

The genomic DNA of *mec*(A) positive MRSA isolates was extracted by DNA lysis method, and subjected to SCC*mec* typing via multiplex PCR assay. The prevalence of SCC*mec* types I-VI were investigated and the correlation of the PVL gene with SCC*mec* types was also studied. PCR amplification resulted in different sized bands. SCC*mec* type I showed two bands of 342bp and 495bp. Type II showed four bands of 209bp, 284bp, 342bp, and 381bp. Type III displayed 4 bands of 209bp, 243bp, 303bp and 414bp, while type IV generated two band 342bp, 311bp, type VI displayed single band of 342bp. Also, PVL positive isolates produced an amplicon size of 151bp (Figure 4.5).

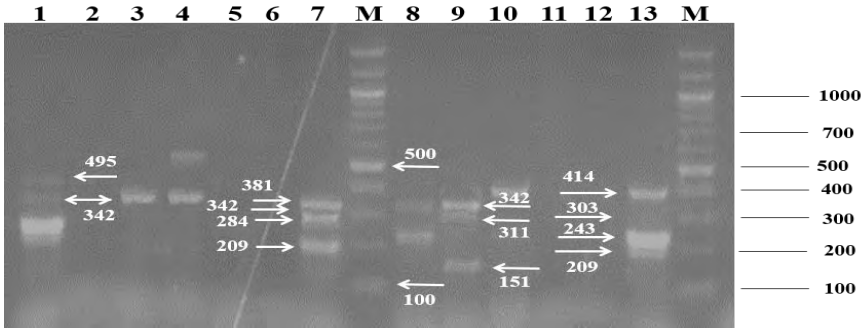


Figure 4.5: Representative gel image of various SCC*mec* types and the PVL gene. PCR products of varying sizes were observed as shown by the pointed arrows. Lane I; type I (495bp, 342bp). Lanes 3 and 4 shows type VI (342bp), Lane 7 shows type II (381bp, 342bp, 284bp, 209bp), lane 8 shows type VI (342bp), lane 9 shows type IV (342bp, 311bp) and PVL (151bp), lane 13 shows type III (414bp, 303bp, 243bp, 209bp). Lane M shows the molecular marker of 100bp (Vivantis).

4.5.1 Prevalence of SCC*mec* typing in MRSA (n=200) isolates

mec(A) positive MRSA isolates were processed for SCC*mec* typing; type III was observed as the most predominant (n=93, 86%) followed by types IV, VI, II, and type I. Non-typeablity was observed in 7.1% (Table 4.3) while type V was not detected in any of the isolates. Some MRSA isolates were also found to harbor multiple SCC*mec* elements. These combinations included Types I and III; Types II and III; Types I, III and IV; Types I and IV; Type III and IV; Types III and VI; and Types II, III and VI.

Table 4.3 Prevalence of SCC*mec* types in MRSA strains

S. No.	SCC*mec* Types	Number of Isolates	Percentage
1	Type I	15	14%
2	Type II	25	23%
3	Type III	93	86%
4	Type IV	43	40%
5	Type V	0	0%
6	Type VI	25	23%
7	Non-typeable	3	7.1%

4.5.2 Antibiotic resistance in SCC*mec* types in MRSA isolates

In MRSA isolates, 30% of SCC*mec* type III isolates exhibited resistance against up to 7 classes of antibiotics (CIP, TE, CN, DA, RD, FOS, E), followed by 30% to 6 (CIP, TE, CN, DA, E, SXT), 10% to 6 (FOX, SXT, CIP, TE, CN, DA), 10% to 4 (CIP, CN, TE, DA), and 20% to 4 classes of antibiotics (DA, CIP, CN). In SCC*mec* type II isolates, 26% exhibited resistance to 7 classes of antibiotics (CIP, TE, CN,

DA, E, FOS, E), followed by 32% to 6 (CIP, TE, CN, DA, SXT, E), and 42% to 3 classes of antibiotics (DA, CIP, CN). Among SCC*mec* type I isolates, 50% exhibited resistance to 7 classes of antibiotics (FOS, CIP, TE, CN, DA, RD, E), followed by 25% to 6 (CIP, TE, CN, DA, RD, SXT), and 25% to 3 classes of antibiotics (E, CIP, DA). In SCC*mec* type IV isolates, 52% showed resistance to 3 classes of antibiotics (CIP, TE, CN), followed by 44% to 2 (CN, CIP), while one isolate was resistant to 5 classes of antibiotics (DA, CIP, C, SXT, CN). In SCC*mec* type VI isolates, 50% exhibited resistance to 3 classes of antibiotics (CN, CIP, TE), followed by 25% to 2 (TE, CIP), and 25% to 4 classes of antibiotics (E, CIP, C, SXT).

4.5.3 PVL co-existence in SCC*mec* types

Panton-Valentine leukocidin (PVL) was detected in 24% (n=48) isolates with distribution in all prevalent SCC*mec* types but was strongly associated with types VI (n=17/48, 35.41%), IV (n=7/48, 14.58%) and II (n=7/48, 14.58%). However, PVL was also associated with SCC*mec* Types III (n=13/48, 27.08%) and I (n=4/48, 8.33%). PVL co-existence with SCC*mec* types was found significant in SCC*mec* types VI and III (*p*-value, 0.0001, and 0.026).

4.5.4 PVL distribution in different clinical specimen

Regarding PVL distribution in different clinical specimen, the highest occurrence was observed in pus (n=40/48, 83.33%) followed by tissue (n=4/48, 8.33%). The lowest incidence of PVL was found in urine and body fluids samples with prevalence of 4.16% (n= 2/48) each, while PVL was not detected in blood, CSF and sputum specimen as shown in Table 4.4.

Table 4.4: PVL distribution in different clinical specimens of MRSA

S. No.	Specimens	Number of Isolates		Total
		PVL +ve	PVL –ve	
1	Blood	-	18	18
2	CSF*	-	3	3
3	CVP Tip**	-	2	2
4	Body Fluid	2	2	4
5	Pacemaker	-	2	2
6	Pus	40	84	124
7	Sputum	-	2	2
8	Tip of Drain	-	3	3
9	Tissue	4	6	10
10	Tracheal Section	-	4	4
11	Urine	2	26	28
Grand Total		**48**	**152**	**200**

***Cerebrospinal Fluid **Central Venous Pressure Tip Culture**

4.5.5 Existence of SCC*mec* types with relation to sample sources

The existence of SCC*mec* types was also analyzed in association with sample sources. All 5 types of SCC*mec* types were more prevalent in pus. This was followed by urine and blood, both of which were linked to 4 SCC*mec* types, while

no *mec* element was detected in isolates from sputum specimen as shown in Table 4.5.

Table 4.5: Specimen-wise distribution of SCC*mec* types in MRSA isolates

Specimens	Number of Specimens	Number of SCC*mec* Types				
		I	II	III	IV	VI
Blood	11	-	2	7	1	1
Cerebrospinal Fluid	3	-	-	3	-	-
Fluid	3	-	-	3	-	-
Pus	65	4	11	26	15	9
Sputum	2	-	-	-	-	-
Tissue	7	-	2	3	2	-
Urine	17	3	-	10	2	2
Total		7	15	52	20	12

4.6 *Spa* typing in MRSA (200) and MSSA (50) isolates

A total of 25/200 MRSA and 25/50 MSSA were selected from various specimen sources for *spa* typing. Out of a total of 21 *spa* types, 15 *spa* types were observed in MSSA while 10 in MRSA. MRSA isolates exhibited different *spa* types with t657 as the most prevalent (n=6/25, 24%), followed by t5414 (n=5/25, 20%), t008 (n=4/25, 16%), t632, t064, t314 (n=2/25, 8% each), t030, t5598, t127, t345 (n=1/25, 4% each) (Figure 4.6 (B)). As compared to MRSA isolates the *spa* types in MSSA were t021 (n=6/25, 24%), t657 (n=5/25, 20%), t409 (n=2/25, 8%), t712, t12595, t11981, NEW *spa* type, t632, t1149, t304, t9248, t442, t5598 and t345 (n=1/25, 4%) each (Figure 4.6 (A)). In MSSA the new *spa* type was 7,12,21,21,17,13,13,13,34,34,34,34,34.

Same *spa* types found both in MRSA and MSSA isolates were t657, t314, t632 and t5598 (Figure 4.6 (C)).

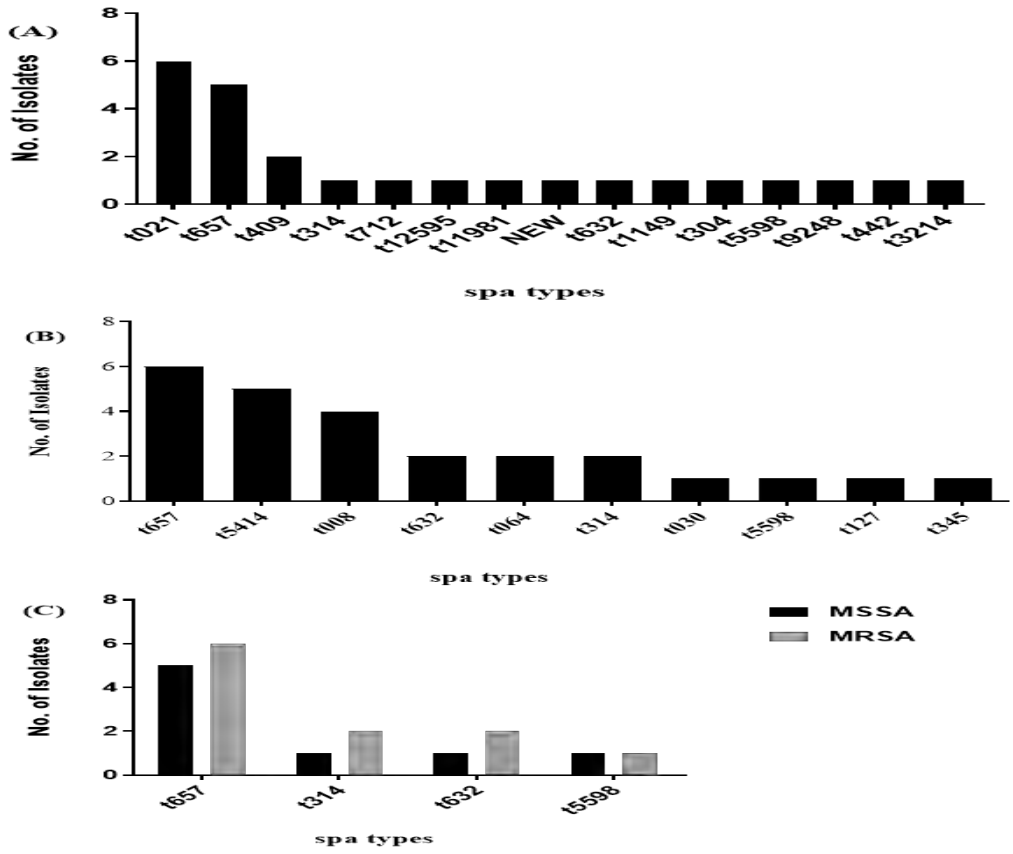


Figure 4.6: The distribution of different *spa* types, **(A)** shows the *spa* types in MSSA isolates **(B)** *spa* types in MRSA **(C)** Shared *spa* types in MRSA and MSSA isolates

4.6.1 MRSA *spa* type, source of sample and the *spa* type repeats

The *spa* types were obtained from different clinical samples. Five isolates had *spa* type t657 which were from pus while one was observed from blood specimen. In the case of *spa* type t5414, two isolates were from blood and pus respectively while one was observed from a urine specimen. *Spa* type t008 was observed in 3 isolates from pus while one from urine specimen. One *spa* type t064 was recovered from urine while one from pus specimen. Two isolates had *spa* type t632 and one t314 was from tracheal secretion specimen. Two isolates had *spa* types t314 and t127 and were from blood and tissue specimen respectively. Repeats were assigned a

numerical code using a *spa* server (spaServer.ridom.de) and their source of the sample is shown in Table 4.6.

Table 4.6: MRSA *spa* type repeats, the *spa* types and their source of sample

S.No.	Gender	Source of sample	*Spa* type	*Spa* repeat
1	Male	Pus	t657	26-23-13-21-17-34-33-34
2	Female	Pus	t657	26-23-13-21-17-34-33-34
3		Pus	t657	26-23-13-21-17-34-33-34
4	Male	Pus	t657	26-23-13-21-17-34-33-34
5	Male	Blood	t657	26-23-13-21-17-34-33-34
6	Female	Pus	t657	26-23-13-21-17-34-33-34
7	Male	Urine	t5414	26-16-23-13-21-17-34-33-34
8	Female	Blood	t5414	26-16-23-13-21-17-34-33-34
9	Female	Pus	t5414	26-16-23-13-21-17-34-33-34
10	Female	Blood	t5414	26-16-23-13-21-17-34-33-34
11	Male	Pus	t5414	26-16-23-13-21-17-34-33-34
12	Female	Pus	t008	11-19-12-21-17-34-24-34-22-25
13	Female	Urine	t008	11-19-12-21-17-34-24-34-22-25
14	Female	Pus	t008	11-19-12-21-17-34-24-34-22-25
15	Male	Pus	t008	11-19-12-21-17-34-24-34-22-25
16	Female	Urine	t064	11-19-12-05-17-34-24-34-22-25
17	Male	Pus	t064	11-19-12-05-17-34-24-34-22-25
18	Female	Tracheal secretion	t632	08-16-02-24-24
19	Female	Tracheal secretion	t632	08-16-02-24-24
20	Male	Urine	t030	15-12-16-02-24-24
21	Male	Pus	t5598	08-16-34-24-34-17
22	Male	Pus	t345	26-23-13-21-17-34-34-33-34
23	Female	Tissue	t127	07-23-21-16-34-33-13

24	Female	Tracheal secretion	t314	08-17-23-18-17
25	Female	Blood	t314	08-17-23-18-17

4.6.2 MSSA *spa* type repeats, the *spa* type and their source of sample

Four isolates had *spa* type t657 where 3 were from pus while one was observed from fluid specimen. In case of *spa* type t021, five isolates were from pus, while one was observed from blood specimen. *Spa* types t314, t409, t12595, t11981, t3214, t1149 and t9248 were observed from pus specimen. One isolate had *spa* type t632 from catheter tip while four isolates with *spa* types t712, t304, t442, and t5598 were from blood specimen. One isolate from tracheal secretion had NEW *spa* type as shown in Table 4.7. Repeats were assigned a numerical code using a *spa* server (*spa*Server.ridom.de) and their source of the sample is shown in Table 4.7.

Table 4.7: MSSA *spa* types, their source, and *spa* type repeats

S.No.	Gender	Source of sample	*Spa* type	*Spa* repeat
1	Female	Pus	t657	26-23-13-21-17-34-33-34
2	Female	Pus	t657	26-23-13-21-17-34-33-34
3	Male	Pus	t657	26-23-13-21-17-34-33-34
4	Male	Pus	t657	26-23-13-21-17-34-33-34
5	Female	Fluid	t657	26-23-13-21-17-34-33-34
6	Male	Pus	t021	15-12-16-02-16-02-25-17-24
7	Male	Pus	t021	15-12-16-02-16-02-25-17-24
8	Male	Pus	t021	15-12-16-02-16-02-25-17-24
9	Male	Pus	t021	15-12-16-02-16-02-25-17-24
10	Male	Pus	t021	15-12-16-02-16-02-25-17-24
11	Male	Blood	t021	15-12-16-02-16-02-25-17-24
12	Female	Pus	t314	08-17-23-18-17
13	Male	Blood	t712	26-23-13-23-05-05-17-25-17-25-16-28
14	Male	Tissue	t409	60-61-34-22-34-17
15	Male	Pus	t409	60-61-34-22-34-17
16	Male	Pus	t12595	04-17-34-17-32-23-24
17	Male	Pus	t11981	04-17-34-17-32-23-24
18	Male	Tracheal Secretion	NEW	7-12-31-17-13-13-13-34-34-34-34-34-34-34
19	Female	Catheter Tip	t632	08-16-02-24-24
20	Male	Pus	t3214	04-44-24-33-31-12-16-34-16-12-25-22-22-34
21	Male	Pus	t1149	08-16-34-24-34-17-17

22	Male	Blood	t304	11-10-21-17-34-24-34-22-25
23	Female	Pus	t9248	26-23-13-21-17-17-34-33-34
24	Male	Blood	t442	35-17-34-17-20-17-12-17-16
25	Male	Blood	t5598	08-16-34-24-34-17

4.6.3 Association of *spa* types with antibiotic resistance and their respective antibiotic resistance genes in MRSA isolates

All t657 *spa* types shared the same resistance pattern for CIP, and CN. One *spa* type t657 showed resistance to 7 classes of antibiotics such as CIP, CN, RD, TE, DA, E, and FOS, while the other one showed resistance to 7 classes of antibiotics (Table 4.9). In t008 *spa* types, 2 isolates shared the same resistance pattern against CIP, and E, while one isolate exhibited resistance to 7 classes of antibiotics. *Spa* type t632 shared the same resistance pattern such as TE, CIP, and CN. One isolate displayed resistance against 5 classes of antibiotics. *Spa* type t064 showed resistance against 5 classes of antibiotics. One t064 was resistant to C, SXT, CIP, CN, and one t064 to TE, CIP, CN, and FOS. t030 *spa* type showed resistance to 6 classes of antibiotics. t5598 showed resistance to TE, and CN while t127 showed resistance to CIP, TE, and E. t314 shared the same resistance pattern to CIP, and CN. t345 *spa* type showed resistance to 4 classes of antibiotics. All the *spa* types were recorded as MDR. Their phenotypic antibiotic resistance and respective ARGs are shown in Table 4.8.

Table 4.8: Association of *spa* types with resistant phenotype and their ARGs in MRSA isolates

S.No.	*Spa* type	Resistant phenotypes	Antibiotic resistance genes
1	t657	CIP, CN	*aacph*D1, *mec*(A)
2	t657	CIP, CN, RD, TE, DA, E, FOS	*aacph*D1, *tet*M, *tet*K, *mec*(A), *fos*(A), *erm(*A)
3	t657	CIP, CN, RD, TE, DA, E,	*aacph*D1, *tet*M, *mec*(A), *bla*(Z)
4	t657	C, CIP, CN	*aacph*D1, *mec*(A), *bla*(Z)
5	t657	CN, TE, CIP	*aacph*D1, *tet*K, *mec*(C)
6	t657	CIP, CN, E	*aacph*D1, *mec*(A), *mec*(C), *erm*(A)
7	t5414	TE, CIP,	*tet*M, *mec*(A)
8	t5414	CIP, CN, E	*aacph*D1, *mec*(A), *erm*(A)
9	t5414	TE, CN, CIP	*aacph*D1, *tet*M, *mec*(A)
10	t5414	CIP, CN, E	*aacph*D1, *tet*M, *mec*(A), *erm*(A)
11	t5414	CIP, RD, E	*mec*(A)
12	t008	CIP, E	*erm*(A), *mec*(A)

13	t008	CIP, E	*mec*(A), *erm*(A)
14	t008	CIP, CN	*aacph*D1, *mec*(A)
15	t008	CIP, RD, SXT, C, DA, E	*mec*(A), *dfr*(B), *erm*(A)
16	t632	CIP, RD, CN	*aacph*D1, *mec*(A)
17	t632	TE, CIP, CN, FOS	*tet*K, *mec*(A), *fos*(A), *aacph*D1
18	t064	C, SXT, CIP, CN	*aacph*D1, *dfr*(B), *mec*(A)
19	t064	TE, CIP, CN, FOS	*aacph*D1, *tet*M, *mec*(A), *fos*(A)
20	t030	TE, CIP, RD, CN, FOS	*aacph*D1, *tet*M, *mec*(A), *fos*(A)
21	t5598	TE, CN	*aacph*D1, *tet*K, *mec*(A)
22	t127	CIP, TE, E	*tet*M, *mec*(A), *bla*(Z), *erm*(A)
23	t314	CIP, CN	*mec*(C), *aacph*D1
24	t314	CN, CIP, TE	*aacph*D1, *tet*M, *mec*(A)
25	t345	TE, CIP, RD	*mec*(A), *tet*k

C; chloramphenicol, TE; Tetracycline, SXT; sulfamethoxazole/trimethoprim, CIP; Ciprofloxacin, RD; Rifampicin, CN; Gentamicin, DA; Clindamycin, E; Erythromycin, FOS; Fosfomycin

4.6.4 Association of *spa* types with resistant phenotypes and their ARGs in MSSA isolates

All t021 *spa* types shared the same resistance pattern for TE, while one *spa* type remained sensitive to all antibiotics. *Spa* types t657 showed different antibiotic resistance patterns; one isolate showed resistance only to CIP and one isolate showed resistance to CN. *Spa* types t409 and t314 remained susceptible to all of the

tested antibiotics. t12595 showed resistance against CIP. t11981 showed resistance towards SXT, CIP. t632 showed resistance against SXT, E, CN. t1149, t9248, t442 showed resistance only to RD. t304 showed resistance to RD and CIP. t5598 showed resistance to RD, E, and SXT. t3214 showed resistance against CIP, CN, and E. As compared to MRSA isolates, MSSA showed less antibiotic resistance in their *spa* types, as shown in Table 4.9.

Table 4.9: Association of *spa* types, with resistant phenotypes and their ARGs in MSSA isolates

S.No.	*Spa* type	Resistant phenotypes	Antibiotic resistance genes
1	t657	CIP	
2	t021	TE, CIP	*tet*K
3	t657	CN	*aacph*D1
4	t021		
5	t021	TE,CIP	*tet*K
6	t409		
7	tT021	TE, CIP	*tet*K
8	t314		
9	t712	TE	*tet*K
10	t021	TE, SXT	*tet*k, *dfr*(B)
11	t12595	CIP	
12	t11981	SXT, CIP	*dfr*(B)

13	NEW	TE	*tet*K
14	t657	SXT,CIP,TE	*tet*K, *dfr*(B)
15	t632	SXT, E, CN	*aacph*D1, *erm*(A), *dfr*(B)
16	t1149	RD	
17	t304	RD, CIP	*gyr*(A)
18	t657	SXT,CN	*aacph*D1, *dfr*(B)
19	t9248	RD	
20	t409	CIP	*gyr*(A)
21	t657	RD,CN,CIP	*aacph*D1
22	t021	RD,SXT, E, TE,CIP	*tet*k, *erm*(A)
23	t442	RD	
24	t5598	SXT,RD, E	*dfr*(B), *erm*(A)
25	t3214	CIP, CN, E	*aacph*D1, *erm*(A)

TE; Tetracycline, SXT; sulfamethoxazole/trimethoprim, CIP; Ciprofloxacin, RD; Rifampicin, CN; Gentamicin, E; Erythromycin,

4.7 MLST and PFGE

Five representative isolates (4/200 MRSA and 1/50 MSSA) of *mec*(A) and *mec*(C) carriage were selected for clonal complex analysis and multi-locus sequence typing. The reason of focusing on these five isolates was that 2 MRSA with *mec*(C) genes, one MRSA which carried both *mec*(A) and *mec*(C) genes were novel and first time reported from Pakistan from clinical isolates. One MRSA with *mec*(A) gene, one MSSA negative for *mec*(A) gene was also included to know about the genetic relatedness of the isolates and they were further processed for MLST and PFGE analysis. Among these 5 isolates, 1 was MSSA, while 4 were MRSA where 1 isolate

carried both *mec*(A) and *mec*(C) genes, 2 isolates had *mec*(C) gene, and 1 isolate was positive for *mec*(A) only. Patient demographic details of these isolates are shown in Table 4.10.

Table 4.10: Demographic data of five representative isolates

S.No.	Isolates	Genes	Sex	Age	Specimen	Ward
	MRSA (4/200)					
1	32	*mec*(A)	Female	51	Pus	General-surgery
2	202	*mec*(C)	Female	36	Pus	General-surgery
3	314	*mec*(C)	Female	22	Tissue	Emergency
4	369	*mec*(A)+*mec*(C)	Female	2	Blood	Emergency
	MSSA (1/50)					
5	58		Female	18	Urine	Urology

4.8 MLST results

The amplification of 7 housekeeping genes such as, *pta, gmK, glp*, *arcC*, *yqiL, aroE*, and *tpi* through PCR of five representative isolates (Table 4.10) was analyzed. Sequences of 7 housekeeping genes and their association with other sequences available in the MLST database (www.pubmlst.org/*S.aureus*) was done. It was observed that the most prominent ST in MRSA was ST772 (n=3/200) followed by ST1 (n=1/200) while 1/50 MSSA isolate belonged to ST1535. The difference of one allele makes the sequence type different as in the case of ST1, as shown in Table 4.11. ST1535 is the subfounder of clonal comlex CC15. CC15 is also large CC and contains a total of 911 STs but consists mainly of methicillin-sensitive *S. aureus*. ST2434, ST15, ST582, ST1659, and ST2750 represent the further subfounders of this clonal complex. ST772 is single locus variant of ST1 and belonged to CC1. CC1

is large and contains a total of 911 STs. ST1, ST567, ST1920, ST2312, ST610, ST852, ST472, ST81, ST573, and ST4768 represent the further subfounders of this clonal complex (Figure 4.8 (A) and (B)). All the STs of *S. aureus* and sub-founder of the obtained STs found using software PHYLOVIZ 2.0 are shown in Figure 4.7.

Table 4.11. Allelic profile of seven housekeeping genes of *S. aureus* isolates

Sample ID	aroC	aroE	glpF	gmK	Pta	Tpi	Yqil	ST	CC
32	1	1	1	1	22	1	1	772	CC1
314	1	1	1	1	1	1	1	1	CC 1
369	1	1	1	1	22	1	1	772	CC 1
202	1	1	1	1	22	1	1	772	CC1
58	13	13	1	1	81	11	13	1535	CC15

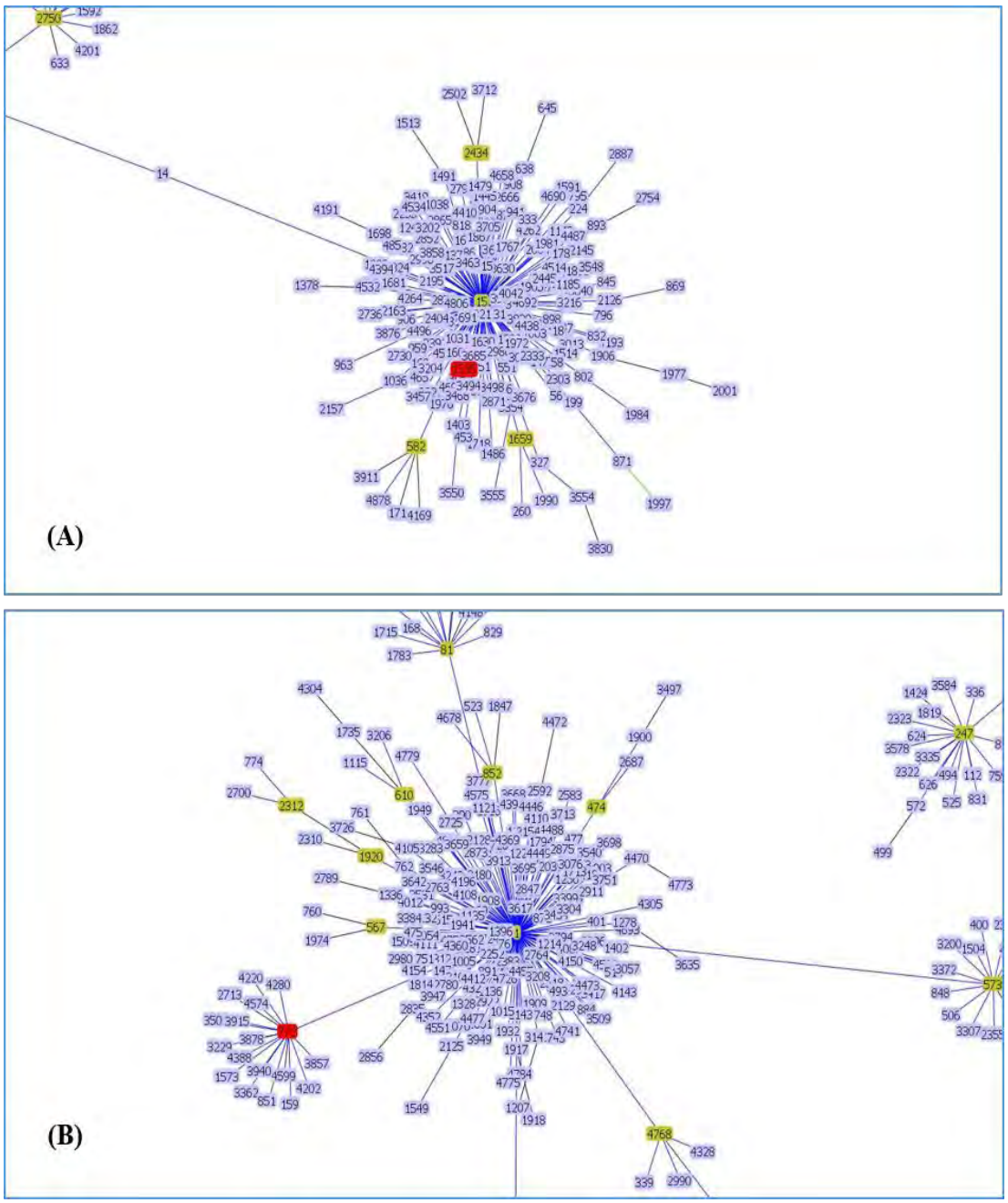


Figure 4.7: The images generated using PHYLOVIZ 2.0 gives a closer look at the STs found in CC15 and SLVs. **(A)**: ST1535 (red circle) at the center CC15 is the subfounder of this clonal complex. ST2434, ST15, ST582, ST1659, and ST2750 represent the further subfounders of this clonal complex, CC15 is characteristic of MSSA and a single MSSA isolate was investigated for ST analysis in this study **(B)** ST772 (red circle) belonged to the CC1 at the center is the subfounder of this clonal complex. ST1, ST567, ST1920, ST2312, ST610, ST852, ST472, ST81, ST573, and ST4768 represent the further subfounders of this clonal complex.

4.9 Clonal analysis of representative isolates

4.9.1 Interpretation of *S. aureus* PFGE restriction patterns

PFGE was performed for 5 *S.aureus* representative isolates (4 out of 200 MRSA while one out of 50 MSSA). Chromosomal restriction patterns of *S. aureus* were interpreted by visual analysis. The banding patterns were clustered into one group based on band similarity. Type A had 4 subtypes (A1, A2, A3, A4). A3 was further divided into subtypes A3(I)/ A3 (II). Clonal analysis of representative isolates showed that 2 isolates Atype A3(I) and A type A3(II) looked exactly the same i-e 100% similarity index, 85% genetic similarity were shown by A1 and A2 and Atype A4 with 75% similarity as shown in Figure 4.8.

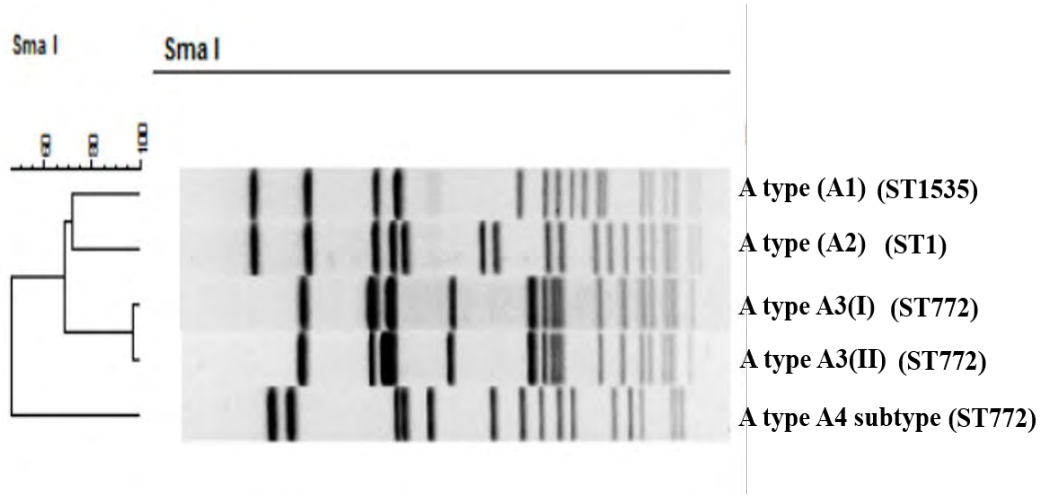


Figure 4.8: Dendrogram Similarity Index Type A had 4 subtypes (A1, A2, A3, A4). A3 was further divided into subtypes A3(I)/ A3 (II) which were same clones.

4.10 Comparative analysis of different typing methods and their antibiotic-resistance determinants in 5 representative isolates

Comparative analysis of five (4/200 MRSA and 1/50 MSSA) representative isolates including four MRSA and one MSSA showed that two MRSA isolates carrying the *mec*(C) gene were of the same clone. Three MRSA had same sequence type ST772 while the other one (ST1) was different. MSSA isolate had different ST1535 and *spa* type. The detailed comparison of these isolates' clinical source, *spa* type, *agr* type, SCC*mec* types, clonal analysis, and ARGs is given in Table 4.12.

Table 4.12: Comparative molecular typing analysis of five representative isolates

Strain	ST CC	PFGE A type	*Spa* type	PVL	*Agr*	SCC*mec*	Antibiotic-resistance genes
32	772 (CC1)	A4 subtype	t657	–	NT	NT	*aacphD*1, *tet*M, *mec*(A), *bla*(Z), *rpo*(B), *fos*(A)
202	772 (CC1)	A type A3(II)	t657	–	NT	IV	*tet*M, *mec*(C), *gyr*(A), *gyr*(B), *vat*(A), *vat*(B), *vat*(C)
369	772 (CC1)	A_3(I)	t657	+	NT	NT	*aacphD*1, *mec*(A), *mec*(C), *gyr*(A), *gyr*(B)
58	1535 (CC15)	A type (A1)	t3214	–	NT	NT	*aacphD*1, *erm*(A)
314	1 (CC1)	A type (A2)	t127	+	NT	NT	*mec*(C), *tet*M, *gyr*(A)

ST (sequence type) , NT (Nontypeable)

4.11 Discussion

S. aureus is subdivided into four *agr* specificity groups based on *agr* locus polymorphisms (Lyon *et al.,* 2000). In this study, *agr* I (22%, n=44) followed by *agr* III (20%, n=40) was the predominant group in MRSA while in MSSA it was *agr* III (16%, n=8) followed by *agr* II (8%, n=4) whereas *agr* IV was absent in MRSA isolates while present in 4% (n=2) MSSA. One study from Pakistan reported the *agr* groups where *agr* I (n=22, 45.8%) was most prevalent, followed by *agr* III (n=14, 29.1%) and *agr* II (4.1%), while no *agr* IV was found in MRSA isolates (Khan *et al.,* 2014), and the results of the current study show that the percent prevalence of the groups is different but trend is same. According to another study, from Netherlands, among 192 *S. aureus* isolates, *agr* I (92.2%) was the dominant one followed by *agr* II (6%) and *agr* III (5%) and these results are in accordance with our present findings, but with a low percentage in MRSA isolates (van *et al.,* 2000). One study from Riyadh processed 100 isolates collected from January 2004 to June 2006, reported that in MRSA isolates (n=9, 16.4%) belonged to *agr* I, (n=8, 14.5%) to *agr* II, and (n=38, 69.1%) to *agr* IlI. While in case of MSSA, (n=10, 22.2%) isolates belonged to *agr* I, (n=8,17.8%) to *agr* II and (n=27, 60%) to *agr* III (Ayed *et al.,* 2008). These findings are also in accordance with current findings, where *agr* I is dominant in MRSA and *agr* III in MSSA but with reduced prevalence.

However, a study from Belgium has reported *agr* group in 511 MRSA isolates as *agr* I (42%), *agr* II (24%), *agr* III (34%) (Hallin *et al.,* 2007), a study from Tehran reported *agr* group in 182 *S. aureus* as *agr* I (n=117, 55.1%), *agr* II (n=36, 16.9%) and *agr* III (n=35, 16.5%) (Peerayeh *et al.,* 2009), another from Netherlands reported *agr* I (92.2%), *agr* II (6%) and *agr* III (5%)(van Leeuwen *et al.,* 2000), and another study from Pakistan reported *agr* groups in 420 MRSA isolates as *agr* I (n= 256, 61%) as the most predominant followed by *agr* II (n=55, 13%) and *agr* III (n=42, 10%) (Muhammad Sohail & Zakia Latif, 2018); the percent prevalence of the groups of above mentioned studies is different but trend is the same in the present study. Other studies from Iran reported the *agr* I as the most dominant in 31 MRSA isolates with prevalence of 54.8% (n=17) (Mohsenzadeh *et al.,* 2015), 64.3% (n=89) in 128 MRSA (Goudarzi *et al.,* 2017) and 54.66% (n=82) in 150 isolates collected

during May to November 2017 (Javdan *et al.,* 2019) while one study from India reported 86% (n=43) in 50 isolates collected during Feb 2015 to Jan 2017 (Jain *et al.,* 2019). These reports are supported by our observation but the prevalence of *agr* group I is low in MRSA isolates in the current study. *Agr* groups III and I are closely associated having 80 percent sequence homology that could suggest exclusive genetic characteristics of our study isolates and selection for the coexistence of *S. aureus* strains in the population.

The *agr* system plays a vital role in *S. aureus* pathogenesis (Arvidson & Tegmark, 2001). Our study demonstrated that only 14 isolates (7%) were typed as *agr* II in 200 MRSA, which is contradictory to previous findings where 29 out of total of 92 isolates (31.52%) (Indrawattana *et al.,* 2013) and 36 isolates (16.9%) out of total of 212 (Peerayeh *et al.,* 2009) were reported to be of *agr* II type and this difference could be due to regional variations.

agr IV was only noticed in case of MSSA while it was not detected in MRSA isolates close findings was observed in few other studies (Ayed *et al.,* 2006; Shopsin *et al.,* 2003). While previous study from Pakistan has reported *agr* group IV in 16% in a total of 420 MRSA isolates (Muhamad Sohail & Zakia Latif, 2018), the absence of group IV isolates in MRSA from this study and other studies (Moore & Lindsay, 2001; van Leeuwen *et al.,* 2000) may be due to ecological and geographical differences.

In this study, (n=102, 51%) and (n=34, 68%) non-typeability was observed in MRSA and MSSA isolates respectively, and a similar finding was observed in one study from Germany, where 49% (n=96) out of a total of 195 isolates were non-typeable (Fischer, Lee, Peters, & Kahl, 2014). Our findings are not in accordance with the previous findings where 40% (n=23) in 57 MRSA (Ayed *et al.,* 2006), 18.88% (n=17) in 90 MRSA (khan *et al.,* 2014) and 14% (n=26) in 182 MRSA were non-typeable (Muhamad Sohail & Zakia Latif, 2018). It remains unknown which factors are involved in the loss of *agr* activity in clinical *S. aureus* isolates.

In this study, MRSA isolates subjected to HVR-PCR generated amplification products that ranged from 350bp to 650bp in size with 5 HVR types: HVR 3

(450bp), HVR 4 (500bp), HVR 5 (550bp), HVR 6 (600bp) and HVR 7 (650bp). HVT types vary by different country and time of strain isolation (Nia *et al.,* 2013, Mirkarami *et al.,* 2016, Schmitz *et al.,* 1998).

Current findings regarding the SCC*mec* types, revealed that the prevalent *mec* types in this region were type III (n=93, 86%), followed by IV (n=43, 40%), VI (n= 25, 23%), II (n=25, 23%), and I (n=14, 14%). Previous studies from Pakistan have reported SCC*mec* type III as the most prevalent one with prevalence of 47% (47/100) isolates during March to September 2012 (Asghar, 2014), 71% (78/126) isolates collected during 2006-2007 (Zafar *et al.,* 2011) and 77.27% (17/22), isolates collected from November1, 2012 to 31 January 2013 (Arshad *et al.,* 2015). As compared to our findings, other studies from Pakistan reported a low prevalence of SCC*mec* type III with prevalence of 5.8% out of total of 85 isolates during 2013 to 2016, from Karachi, Pakistan (Mirani *et al.,* 2017) and 22% out of 488 isolates from Lahore) (Muhamad Sohail & Zakia Latif, 2018). In addition, we investigated, SCC*mec* IV was (n=43, 40%) and similar levels (43%) were also reported previously from Pakistan. One study from Pakistan reported SCC*mec* IV (74.1%) out of 85 isolates as the dominant one (Mirani *et al.,* 2017), which is not similar to the current study.

Our study demonstrated, SCC*mec* II in (n=25, 23% isolates, the same finding (20%) out of 488 isolates was observed previously from Pakistan (Muhamad Sohail & Zakia Latif, 2018b). Another study from Iran reported SCC*mec* II in 20% (29/146) isolates collected during 2012 and 2013 (Ebrahim *et al.,* 2015), which is similar to the current study. A relatively higher percent of SCC*mec* II (28%, 14/50) was reported from Iran (Hashemizadeh *et al.,* 2019). Based on current study, SCC*mec* I was found in (n=15, 14% isolates and low prevalence (9%, 9/100) has been reported previously (Asghar, 2014). As compared to this study, low prevalence of SCC*mec* I (4%, 2/50) was reported from Iran (Hashemizadeh *et al.,* 2019). The spread of SCC*mec* types in MRSA strains has also been observed worldwide, SCC*mec* III, II were predominant in Asia IV, II in America and Europe while IV and V were mostly distributed in Africa (Asadollahi *et al.,* 2018). The distribution of different SCC*mec*

types varies from region to region. These differences in the prevalence of SCC*mec* types could be due to different genetic background of MRSA strains circulating in a particular region and antibiotic pressure that enables the MRSA to acquire antibiotic resistance through the transfer of MGEs like SCC*mec*, plasmids, and transposons.

Our findings noticed the prevalence of SCC*mec* NT in (n=3,7.1%) MRSA isolates. Similar results 2 out of a total of 26 were reported previously (Oliveira & de Lencastre, 2002; Taherirad *et al.,* 2016). Our study demonstrated that SCC*mec* type III was more in PVL positive MRSA with 39% prevalence, which is in contrast to a study from India that reported 7 out of 97 MRSA having SCC*mec* III with PVL negative MRSA (D'Souza *et al.,* 2010). Another previous study from Florida reported type IV SCC*mec* MRSA with a prevalence rate of 41.7% (23/25), isolates were collected from March 2005 to November 2005 with PVL marker in 92% isolates (Moroney *et al.,* 2007). In our study, type IV was noticed in 40.5% isolates with a reduced prevalence of 52.9% of MRSA with PVL marker. In contrast to the current study findings, a study in Algeria reported the prevalence of SCC*mec* type IV in 50% (32/64) isolates, with only 29.7% (19/64) of the type IV isolates carrying PVL gene (Ouchenane *et al.,* 2011). Regarding PVL co-existence with SCC*mec* IV, the current results showed that 52% isolates carried SCC*mec* type IV with PVL gene in 52% isolates. This indicates that the prevalence of SCC*mec* type IV and the PVL gene carriage greatly varies in different locations of the world. Regarding type V incidence, our findings were according to the a previous study (0/100) (Asghar, 2014) while one study from Pakistan reported a low prevalence of SCC*mec* type V only in 2/50 (4%) isolates (Hannan *et al.,* 2015).

In this study, the prevalence of PVL was reported as (n=48/200, 24%) in MRSA and (n=5/50, 10%) in MSSA isolates. Our findings are in line with the results of a study from Pakistan where 21.62% (13/60) MRSA and 8.33% (5/60) MSSA were noticed as PVL positive (Haque *et al.,* 2017). However, as compared to this study, other studies from Pakistan have reported more prevalence of PVL in 33.33% (9/64) in MSSA (Khan *et al.,* 2018), 46% (52/127) in MSSA (MIqbal *et al.,* 2018) and 40% out of a total of 488 MRSA isolates (Muhamad Sohail & Zakia Latif, 2018).

Regarding the distribution of PVL in specimen types, current study noticed that (n=40/48, 83.33%) of the pus specimens were PVL positive. Less prevalence was reported in a study from England where 20.8% (22/106) of MRSA were PVL carrier (Shallcross *et al.,* 2010). One study from Nepal reported that PVL occurrence in pus specimen was high (75.5%, 74/98) while it was least in urine (16.6%, 2/12) (Bhatta *et al.,* 2016), and this finding closely relates to our present study. In addition, we found that PVL detection in male patients was high (n=30/48, 62.5%) as compared to females (n=18/48, 37.5%). A similar finding was reported from Nepal that PVL presence was high (54%, 75/139) in males as compared to females (46%, 64/139) (Bhatta *et al.,* 2016). In one study, PVL positive MRSA were reported in the patients of age group 20-39 (22/106) years (Shallcross *et al.,* 2010), while in current study, PVL positive MRSA were mainly isolated from patients in the age group of >40 years. In contrast to the present study findings, other studies have reported PVL prevalence in children with age of less than 14 years (Bhatta *et al.,* 2016; Bhutia & Singh, 2012). PVL is generally regarded as a genotypic marker for CA-MRSA but it is not present in nosocomial MRSA strains (Bukharie, 2010; Havaei *et al.,* 2010). One study reported PVL prevalence in 10% (10/100) CA-MRSA (Habeeb *et al.,* 2014). Whereas some studies revealed that about 8-75%, out of total of 86 of the MRSA isolates from nasal carriage had PVL toxin (Adwan *et al.,* 2013; Raymon *et al.,* 2002). Current findings noticed the SCC*mec* types and its co-existence with PVL clearly indicates that PVL is now also found in SCC*mec* types other than IV and VI. Thus, considering PVL as a marker for CA-MRSA can be debated in the future. Same results were also reported by another study (Bhutia *et al.,* 2015).

In our study, 74.7% (124/166) and (n=42/166, 25.3%) of MDR-MRSA were PVL negative and positive respectively, a highly significant difference was found between the PVL+ve MDR-MRSA and PVL-ve MDR MRSA with a *p*-value of <0.0001. A study also supported these findings that the antibiotic resistance was significantly higher in PVL negative as compared to PVL positive MRSA (Bhatta *et*

al., 2016). This indicates that the PVL gene harboring can compromise antibiotic resistance determinant carriage by a bacterium.

One study demonstrated that SCC*mec* types I, IV, and VI were resistant to β-lactam antibiotics while types III and II exhibited resistance to tetracycline, erythromycin and non β-lactam drugs (Ito *et al.,* 2003). However, in this study SCC*mec* types I, IV, and VI showed resistance to up to five classes of antibiotics and non-susceptibility to non β-lactam drugs. SCC*mec* II, III showed resistance to up to seven and 8 classes of antibiotics, respectively. These differences could be attributed to the fact that the current study found a large number of isolates simultaneously carrying multiple SCC*mec* elements.

In this current study, the most frequent *spa* type in MRSA was t657 (n=6/25), while in MSSA it was t021 (n=6/25). *Spa* types in MSSA (n=15) isolates were more diverse as compared to MRSA (n=10). One NEW *spa* type in MSSA isolates was also observed. The same *spa* types (n=4) were also shared both in MRSA and MSSA isolates such as t657, t632, t5598, and t345. In Pakistan, limited data is available about the *spa* types. One study from Pakistan recently reported t657 from table eggs out of 33 MRSA isolates (Syed *et al.,* 2018). Another study from Pakistan also reported *spa* types such as; t064, t030, t632, t987, t451, t021, t632 in 123 MRSA isolates (Yasrab Arfat, 2013), as compared to this study t657 (n=6/25) was more dominant in case of MRSA while t021 (n=6/25) in MSSA in the current study. Our current study finding is also in accordance with other *spa* types reported from different countries of the world such as t021, t030, t064, t632 have been reported from China, Australia, Canada, India, Sweden, Germany, Netherland, Norway, USA, UAE, Austria, and UK (*spa*.ridom.de/). In the current study, most of the *spa* types were reported for the first time from Pakistan. Our current findings suggest that certain *spa* types were restricted to a certain region, while some are circulating globally and this might be due to the organism's adaptation to diverse conditions such as geographical location, geographical diversity and climate.

Some *spa* types (n=4) were also shared by both MRSA and MSSA in the current study which indicates that methicillin-susceptible strains became resistant due to the

acquisition of *mec*(A) gene. One study has reported t030 and t008 from Asia and America in MRSA isolates (Asadollahi *et al.,* 2018), the same finding was also observed in the current study. In case of t008 which was reported in MSSA isolates in Europe (Asadollahi *et al.,* 2018), in the current study it was found in MRSA isolates (n=4/25), which may be due to the acquisition of *mec*(A) gene by MSSA strain. In Europe, t032 has been reported as the predominant one followed by t008, t032 from Germany and UK, while t008 from Italy and France and t002 from Sweden. Worldwide dominant *spa* types reported in various countries were t032, t008, t002, t044, t003, t067, t018, and t004 from Europe, t030, t037, t002, t437, t1081, t004, t001, t242 and t2460 from Asia, t008, t002, t242, t012, t084, t003, t311and t0149 from America, t037, t084, t064, t1257, t045, t012, t1443 and t314 from Africa while t011, t172, t037 and t202 was the prominent *spa* types in Australia (Asadollahi *et al.,* 2018b). In this study t008 (n=4/25), t030 (n=1/25) and t314 (n=2/25) were also reported which indicates that certain *spa* types circulate globally.

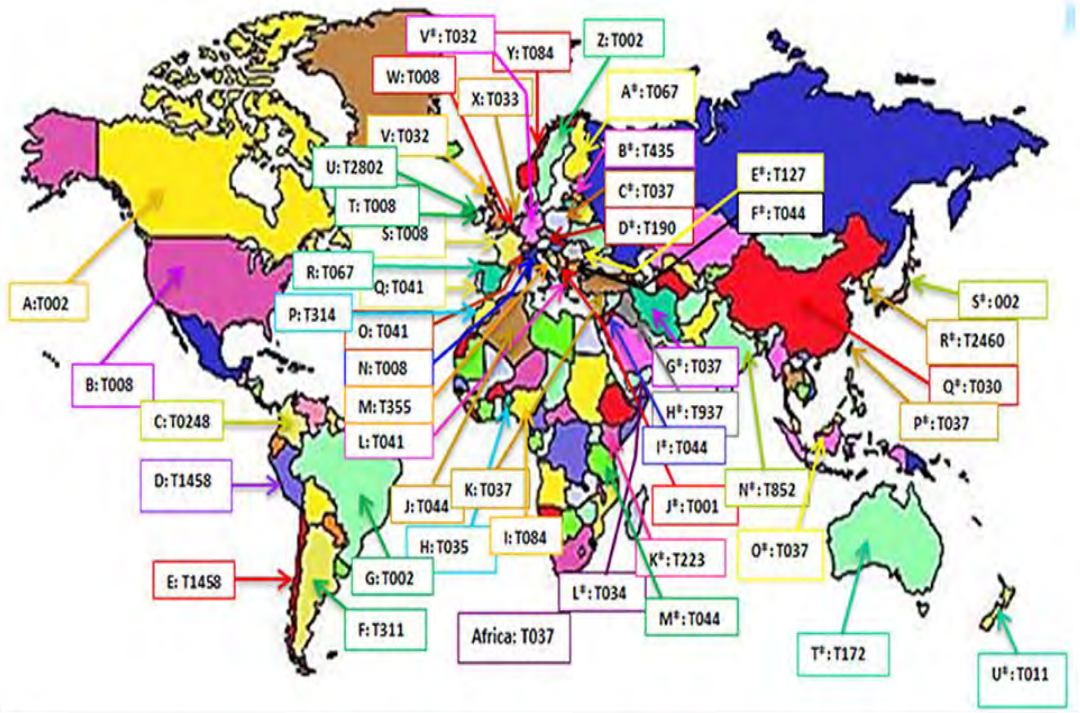


Figure 4.9: The most prevalent spa types across the world (Asadollahi *et al.,* 2018).

Some *spa* types were more frequent in some regions; such as t030 (338/397) was dominant in Turkish hospital, t044 (111/149), and t042 (109/149) in North Africa while t008 (94/102) in USA and Kenya (Omuse *et al.,* 2016). Besides that, t002 was most frequent one in 26 different European countries (Satta *et al.,* 2013). In Sudan (3/3) and Brazil (12/13), the most prevalent *spa* type was t037 whereas in Saudi Arabia and Scotland diverse *spa* types were noticed (Mazi *et al.,* 2015). Another study reported the distribution of different *spa* types as; t065, t084, and t012 (Sangvik *et al.,* 2011). In one study from Iran, t037 was the most dominant one while 8 isolates out of total of 90 (8.9%) remained non-typeable using *spa* type method (Goudarzi *et al.,* 2016), and as compared to this, the current study reported t657 (n=6/25) and t021 (n=6/25) as the most prevalent *spa* types in MRSA and MSSA isolates respectively and all were typeable.

Omuse *et al*, reported that t037 (n=13/32) was the most prominent one in MRSA while in MSSA *spa* types were quite diverse and only 2 MSSA isolates shared the same *spa* type, 2 new MRSA *spa* types, t13150 (n=1/32) and t13149 (n=1/32) and 3 new *spa* types in MSSA t13194, t13182 (n=1/32) and t13194 (n=1/32) were identified (Omuse *et al.,* 2016). In the current study, the most prevalent *spa* type in MRSA was t657 (n=6/25), while in MSSA it was t021 (n=6/25) and more diverse as compared to MRSA.

We found ST772 (n=3), ST1 (n=1) in MRSA isolates while ST1535 (n=1) in MSSA isolate, only two MRSA isolates were identified as the same clone. Previous study from Pakistan reported that out of total of 126 isolates the major sequence type ST8 (n=23) among 18 CA-MRSA presented by 12 isolates, while three isolates were ST239, and the remaining three were ST1 (PVL negative), ST1175, and ST217. ST8 and ST239 (n=17) were found in 11 and 14 HA-MRSA isolates respectively (Zafar *et al.,* 2011). Likewise, ST1 in our study differed from the previous report (Zafar *et al.,* 2011). ST1-V (t127,t321) in 4 out of a total of 103 isolates with PVL marker, showed resistance to gentamicin, tetracycline, fusidic acid, ciprofloxacin and tobramycin (Samar *et al.,* 2016), same findings were observed in the current study,

as the Sequence type ST1 with PVL marker, *spa* type t321 showed resistance to ciprofloxacin and tetracycline. A previous study from Pakistan reported the most frequently one ST in 60 hospital MRSA was ST239 (n=9) followed by ST113 (n=2), ST8 (n=3), and ST30 (n=1) (Shabir *et al.,* 2010).

In addition we investigated *mec*(C) positive MRSA that belonged to *spa* types t657 (n=2) and t127 (n=1) which are different from the *spa* types reported in a study from Austria where 6 *mec*(C) positive clinical MRSA out of 295 isolates were of *spa* types t843 (n=1), t1535 (n=2), t3256 (n=1), t5930 (n=2) (Kerschner *et al.,* 2015), and displayed susceptibility to non β-lactam drugs while our isolates were resistant. Other studies have reported *mec*(C) MRSA, where in reports from Germany, 10 out of total of 14 *mec*(C) clinical MRSA were of *spa* types t843 (n=6), t978 (n=1), t1535 (n=1), t1773 (n=1), t7189 (n=1), in which t843 was the dominant one (Kriegeskorte *et al.,* 2012), In total, 1,604 (collected during 2004 and 2005) and 1,603 (collected during 2010 and 2011) MRSA isolates were examined; one isolate from each sampling period harbored one *mec*(C) clinical MRSA of *spa* type t843 (Schaumburg *et al.,* 2012a) and 2 *mec*(C) (18/227) clinical MRSA were of *spa* type t843 with MICs of 3µg/ml and 8µg/ml (Sabat *et al.,* 2012). However, among human *mec*(C) MRSA the reported prevalence rates remains low, ranging from 0.08%-5.9% (11/12691,25/643, 12/6960, 21/1097) (Christiane *et al.,* 2011; Petersen *et al.,* 2013; Pichon *et al.,* 2012; Stegger *et al.,* 2012). A similar kind of observation was also noticed in the current study. In our findings, MRSA isolates of ST1(n=1, t127) and ST772(n=2, t657) carried the *mec*(C) gene, had PVL marker, showed resistance to ciprofloxacin, and tetracycline. In this study, low prevalence of *mec*(C) (n=6) gene was noticed and many isolates were negative both for *mec*(A) and *mec*(C) gene which could be due to the presence of *mec*(B) gene or *fem*(A) gene which is also responsible for methicillin resistance in staphylococci. This needs to be investigated further.

Five representative isolates of *mec*(A) (n=1) and *mec*(C) (n=3) carriage were also typed using multi-locus sequence typing. Combining MLST and *spa* typing, 3 genotypic patterns were formed: ST772-t657 (n=3), ST1-t127 (n=1) in MRSA, and

ST1535-t3214 (n=1) in MSSA. ST772-t657 and ST1 (n=1/3) have been reported previously (Syed *et al.,* 2018; Zafar *et al.,* 2011) whereas the MSSA with ST1535-t3214 (n=1) has not been previously reported from Pakistan. ST1535 with CC15, with a single variant of ST15 in MSSA (n=4), was reported from Saudi Arabia (Senok *et al.,* 2017). In our study, ST772 MRSA harboring SCC*mec* IV element with PVL marker and multidrug resistance phenotype is different from ST772 reported from Bangladesh which was methicillin-sensitive, and ST772 (n=5/128, n=13/55) from Malaysia and India as that carried SCC*mec* V element (D'Souza *et al.,* 2010; Neela *et al.,* 2009). Likewise, ST1 in our study differed from the previous report of ST1 (Zafar *et al.,* 2011) as it carried a PVL marker. The occurrence of ST772, ST1535, and ST1 in Pakistan reflects frequent traveling of people among India, Pakistan, and Saudi Arabia.

Chapter 5: Virulence markers and Pathogenicity

5.1 Introduction

Staphylococcus aureus (*S. aureus*) is an opportunistic human pathogen occurring both in community and hospital settings. It is the cause of severe clinical manifestations like pneumonia, toxic shock syndrome, scalded skin syndrome, bacteremia, endocarditis, relatively mild skin infection and food poisoning (Tong *et al.,* 2015). The ability of *S. aureus* to successfully invade, persist and cause infections within a vast range of hosts is due to the expression of a variety of virulence factors that promote bacterial adhesion to surfaces, acquisition of nutrients, and evasion from host immune system (Serruto *et al.,* 2010). The morbidity and mortality associated with *S. aureus* are augmented when virulent strains acquire antibiotic resistance (Aslam *et al.,* 2018). Multidrug-resistant strains (MDR) *S. aureus* have spread globally with increasing frequency, leading to high levels of mortality and morbidity, and presenting an alarming situation for healthcare professionals (Wilson, *et al.,* 2016). The virulence factors of *S. aureus* play a key role in pathogenesis of infections. The fibronectin-binding proteins (*fnBPs*) and clumping factors A and B (*clfA* and *clfB*) (Sabat *et al.,* 2006) and Panton-Valentine leukocidin (PVL), and staphylococcal enterotoxin genes (*sec, sea*, *seb*,) were detected through PCR in *S. aureus* isolates (Li *et al.,* 2019). In one study, t002 as well as t008 were the most commonly distributed *spa* types in *S. aureus* isolates worldwide (Asadollahi *et al.,* 2018). The various virulence factors of bacteria encourage damage of host tissue, bacterial evasion and their survival in circulatory system (Bhatty *et al.,* 2015; Thammavongsa *et al.,* 2015). The virulence factors of *S. aureus* include exopolysaccharides, exoenzymes, exotoxins, enterotoxins, surface proteins, immune evasion complex, biofilm, and exfoliative toxins. Among the virulence factors secreted by *S. aureus* are the superantigens known as enterotoxins (sea-sev) which function by massive activation of T-cells hence causing the food poisoning (Al-Ajealy *et al.,* 2017).

Sigma factor is part of core genome conserved among bacterial species which provides promotor specificity for RNA polymerase. Four sigma factors have been recoznized in *S. aureus*; Sig A is involved in housekeeping gene transcription, Sig B

is a stress response regulator, while Sig S has role in gene expression responsible for the survival and overall fitness of bacteria and Sig H is involved in competence, excision and prophage integration (Morikawa *et al.,* 2003). Sig B also regulates capsule and other several extracellular virulence markers through SpoVG (Meier *et al.,* 2007; Schulthess *et al.,* 2011), indicating a role for Sig B in virulence as a response to stress. Sig B also regulates capsule formation through Sar A which has role in biofilm formation (Beenken *et al.,* 2003). Other transcription regulator which is highly conserved known as CodY is also involved in the regulation of several virulence factors which contribute in *S. aureus* pathogenicity (Majerczyk *et al.,* 2008; Pohl *et al.,* 2009; Tu Quoc *et al.,* 2007). Another Sar A Homolog Rot is a repressor of toxin regulator, which positively regulates the Protein A in *S. aureus* (Oscarsson *et al.,* 2006). Another regulatory component essential for *S. aureus* bacterial survival is YycGF, involved in the formation of biofilm and crosslinking of peptidoglycan synthesis (Dubrac *et al.,* 2007). The two-component system ArlS-ArlR is involved in various types of cell activities, mainly involved in protein A, and some secreted proteins (lipase*,* *α*-toxin, coagulase, β-haemolysin) (Benedicte *et al.,* 2004). Adhesion proteins such as fibrinogen binding proteins (fnbB and fnbA) are involved in *S. aureus* attachment to the cell surface and contribute in tissue colonization (Mirzaee *et al.,* 2016). *S. aureus* also produces several toxins including staphylococcal enterotoxin (*sea*, *seb*, *sec*, *ser*, *seg*, and *sie*) which are heat stable and result in complication of food poisoning (Argudín, Mendoza, & Rodicio, 2010).

In this chapter, the frequency of virulence markers in methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA), the intracellular survival and pathogenicity of selected clinical *S. aureus* strains using *in vitro* RAW 264.7 cells, and *in vivo Galleria mellonella* (*G. mellonella*) infection model has been described.

5.2 Results

5.2.1 Hemolysis test

Hemolysis test was done for determining the phenotypic activity of *S. aureus* hemolysins. All the study isolates were proceeded for hemolytic activity (Figure 5.1). The majority of isolates were positive for alpha-hemolysis, followed by beta-hemolysis and gamma-hemolysis.

5.2.2 Phenotypic and genotypic detection of hemolysin in *S. aureus* isolates (200 MRSA and 50 MSSA)

Among MRSA, alpha-hemolysis was observed in 45% (n=90), beta-hemolysis in 28% (n=56) while gamma hemolysis in 27% (n=54) isolates. Among MSSA alpha-hemolysis was the most prevalent one (60%, n=30) followed by beta-hemolysis (28%, n=14), and gamma-hemolysis in 12% (n=6) isolates. Phenotypically positive isolates were screened for the detection of hemolysin genes including; alpha-hemolysin gene (*hla*), beta-hemolysin gene (*hlb*), and gamma-hemolysin gene (*hlg*) with the help of PCR, as shown in Figure 5.2.

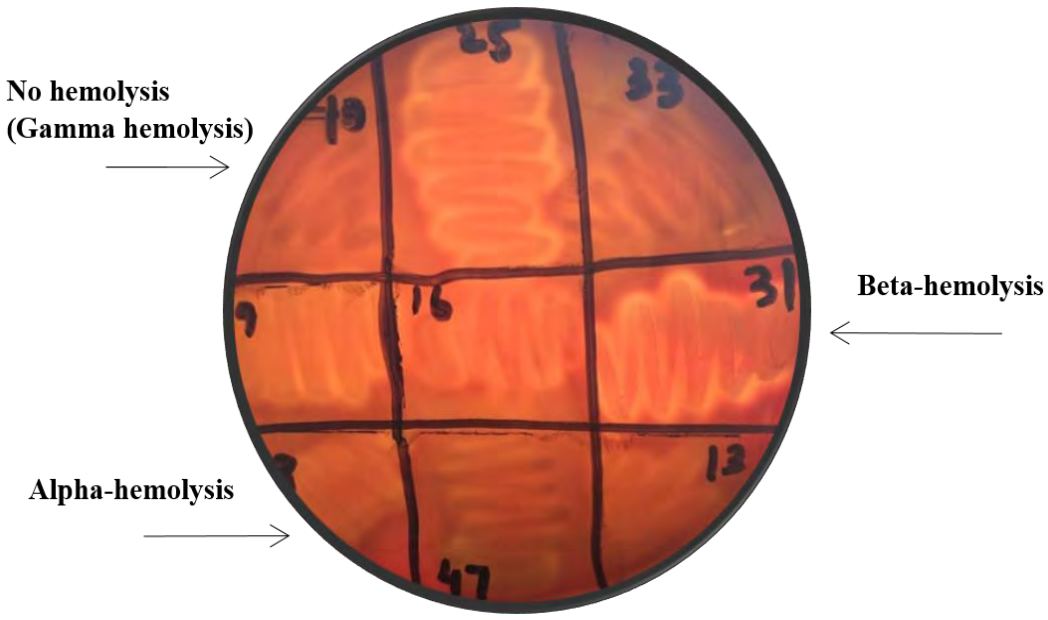


Figure 5.1: A representative sheep blood agar plate with *S. aureus* growth shows the different type of hemolysis, a clear zone termed beta-hemolysis, and partially used red blood cell alpha-hemolysis and lack of any change in the medium is termed as gamma hemolysis.

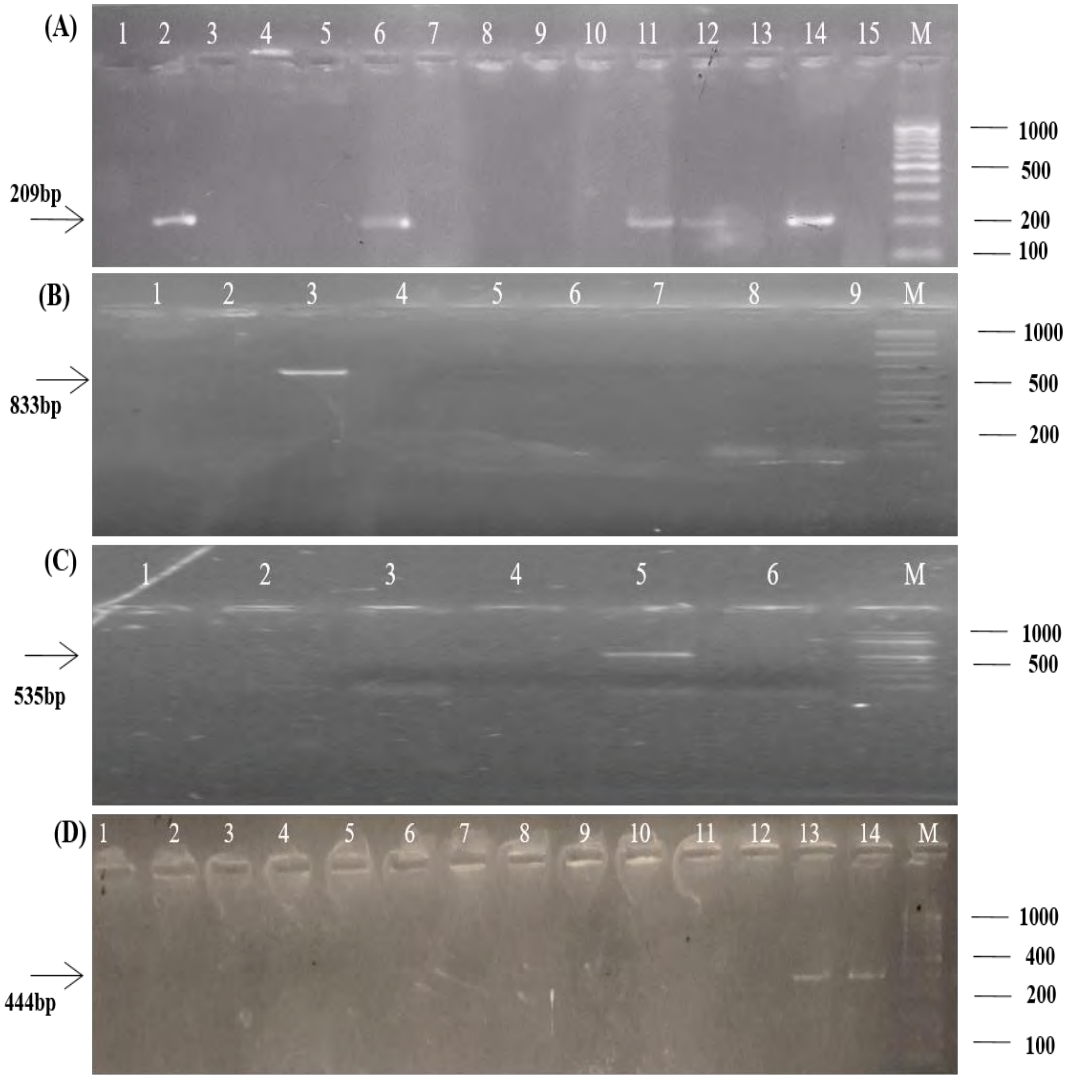


Figure 5.2: Representative gel images showing PCR amplification of the hemolysin gene. **(A)** Lane M shows the molecular weight marker of Gene Ruler 100bp (Thermo scientific). Lanes 6, 11, 12, 14 shows amplification of *hla* gene (209bp). Lanes 1, 3, 4, 5, 7, 8, 9, 10 and 13 showed no amplification, lane 15 shows negative control while lane 2 shows positive control for *hla* gene. **(B)** Lane M shows the molecular weight marker of Gene Ruler 100bp (Thermo scientific). Lanes 1, 2, 4, 6, 7, 8, 9 showed no amplification. Lanes 3 and 5 show the amplification of the *hlb* gene (833bp). **(C)** Lane M shows the molecular weight marker of Gene Ruler 100bp (Thermo scientific). Lanes 1, 2, 3, 4, 6 showed no amplification. Lane 5 shows the amplification of the *hlg* gene (535bp). **(D)** Lane M shows the molecular weight marker of Gene Ruler 100bp (Thermo scientific). Lanes 1-12 showed no amplification. Lane 13 shows the positive control and 14 show amplification of *hld* gene (444bp).

5.2.3 Frequency of hemolysin genes in clinical isolates

Phenotypically positive isolates for hemolysis were screened to find out the hemolysin genes, with the help of PCR. PCR was used to identify the presence and prevalence of following genes; *hla, hlb, hlg,* and *hld*. Among MRSA, *hla* gene was detected in 80% (n=72) of phenotypically positive isolates, followed by *hlb* in 65% (n=36), and 58% (n=31) for *hlg* gene, and delta-hemolysin gene (*hld*) was found in 3.2% (n=6) of the isolates. Among the MSSA, *hla* gene was detected in 80% (n=24) of phenotypically positive isolates, followed by *hlb* in 71.42% (n=10), and *hlg* gene in 50% (n=3). Overall alpha-hemolysin production was observed more as compared to beta, and gamma-hemolysins.

5.2.4 Frequency of Panton-Valentine leucocidin (PVL) toxin in clinical isolates

All *S. aureus* isolates were screened for PVL toxin. A total of 24% MRSA and 10% MSSA isolates were positive for PVL toxin.

5.3 Virulence factors and other regulatory system of *S. aureus*

Besides hemolysin, other virulence factors are also involved in host-pathogen interaction to establish *S. aureus* pathogenicity, such as alternative sigma factor (*sig*B), expression regulator (*cod* Y), fibronectin-binding protein A (*fnbA*), clumping factor A (*clfA*), fibronectin-binding protein B (*fnbB*), staphylococcal accessory regulator (Biofilm-related) (*sarA*) WalK/WalR (YycG/YycF) autolytic activity (*yycG*), the stage V sporulation protein G (*spoVG*), surface and secreted protein for bacterial aggregation (*spa*) and transcriptional regulator (*rot*) were also studied. Virulence genes detection in MRSA and MSSA isolates was done by multiplex PCR assay and represented in Figure 5.3.

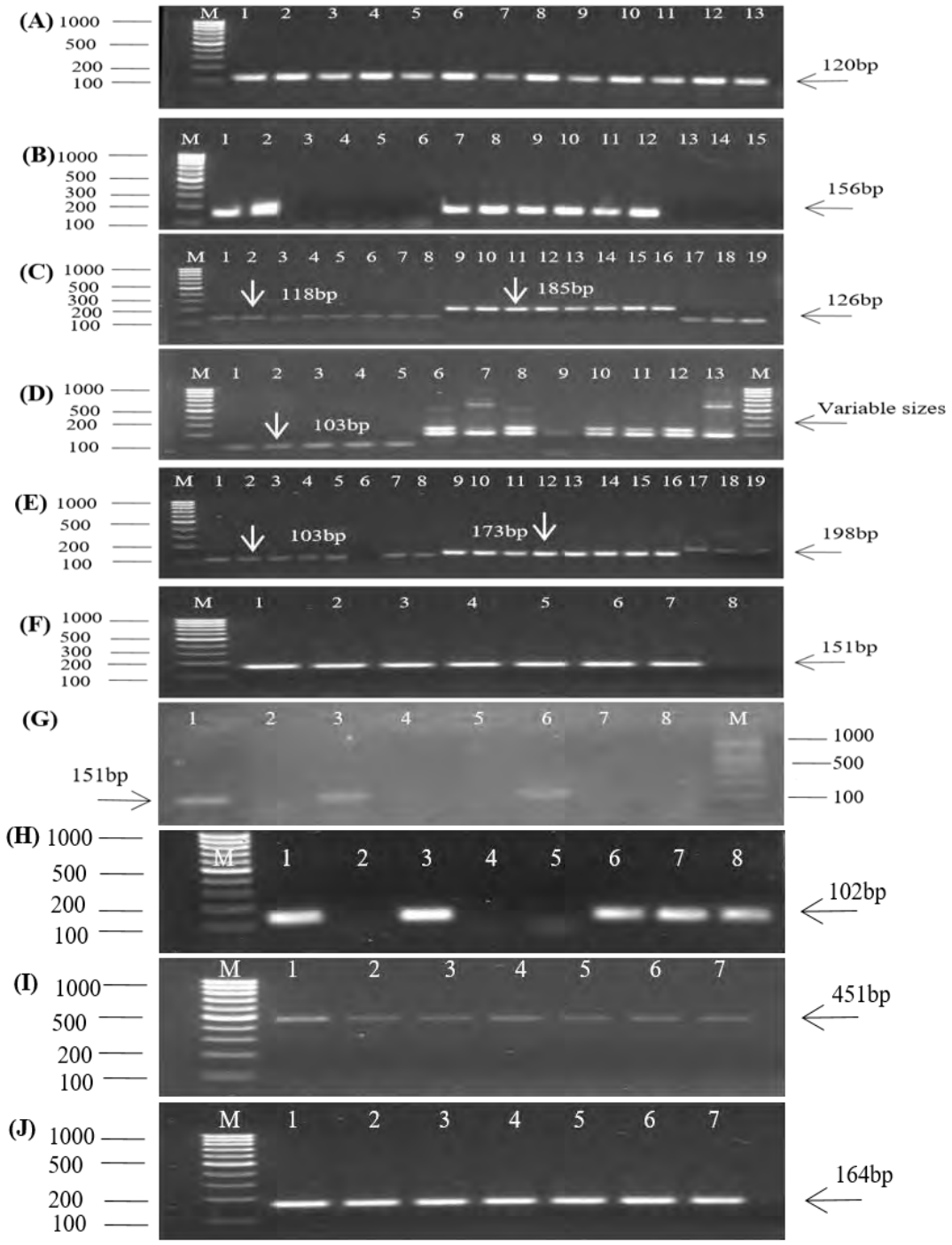


Figure 5.3: Representative gel images show PCR amplification of various virulence and regulatory markers (M) shows the molecular weight marker of 100bp (Thermo scientific). **(A)** Lane 13 shows positive control. Lanes 1-12 show the amplification of *cod*Y gene (120bp). **(B)** lane 3 and lane 12 shows negative control and positive control, respectively. Lanes 4, 5, 6, 13, 14 and 15 showed no amplification. Lanes 1, 2, and 7-11 show amplification of *sig*B gene (156bp). **(C)** Lane 8 shows positive control. Lanes 1-7 show the amplification of *fnb*B gene(118bp). Lane 16 shows

positive control. Lanes 9-15 show the amplification of *rot* gene (185bp). Lane 17 shows positive control, while18-19 show the amplification of *fnb*A gene(126bp). **(D)** Lanes 1-4 show the amplification of *sae* gene (103bp), lane 5 shows positive control and Lanes 6-13 show the amplification of *spo*v*G* gene of variable sizes. **(E)** Lane 5 shows positive control and lane 6 shows negative control, Lanes 1-4, 7 and 8 show the amplification of the *arls* gene (103bp). Lane 16 shows positive control, Lanes 9-15 show amplification of the *sar*A gene (173bp) and Lane 17 shows positive control while 18-19 show the amplification of *yycG* gene (198bp). **(F)** Lanes 1-6 show the amplification of the *clf*A gene (151bp), lane 7 shows the positive control, while lane 8 showed no amplification. **(G)** Lanes 1, 3 show the amplification of PVL toxin (151bp), lane 6 and lane 2 shows positive and negative control respectively, lanes 4, 5, 7, and 8 showed no amplification. **(H)** Lanes 1, 3, 6 and 7 show amplification of enterotoxin gene *sae* (102bp), lane 8 and lane 2 shows positive and negative control, respectively, lanes 4 and 5 showed no amplification.**(I)** Lanes 1-6 show the amplification of the *seb* gene (451bp) and lane 7 shows positive control**. (J)** Lanes 1-6 show the amplification of the *sec* gene (164bp) and lane 7 shows positive control.

5.4 Prevalence of virulence factors in MRSA and MSSA isolates

Various virulence factors were studied in the study isolates. The distribution of virulence factors was almost similar both in MRSA and MSSA. The most prevalent virulence factors in MRSA were *fnbA* (87%, n=174)*, codY* (86% n=172)*, fnbB* (86% n=172), *rot* (79%, n=158), *spoVG* (78%, n=156), and *yycG* (74%, n=148) while low frequencies of *sae* (10%, n=20), *seb* (5%, n=10), and *sec* (2%, n=4) were observed in MRSA isolates (Figure 5.4 (A)). In the case of MSSA, the most dominant virulence factors were *fnbA* (86%, n=43), *codY* (84%, n=42), and *fnbB* with (82%, n=41), followed by *rot* (76%, n=38), *spoVG* (70%, n=35), and *yycG* with (72%, n=36), while low frequencies of *sae* (6%, n=3), *seb* (4%, n=2), and *sec* (4%, n=2) were observed in MSSA isolates (Figure 5.4 (B)).

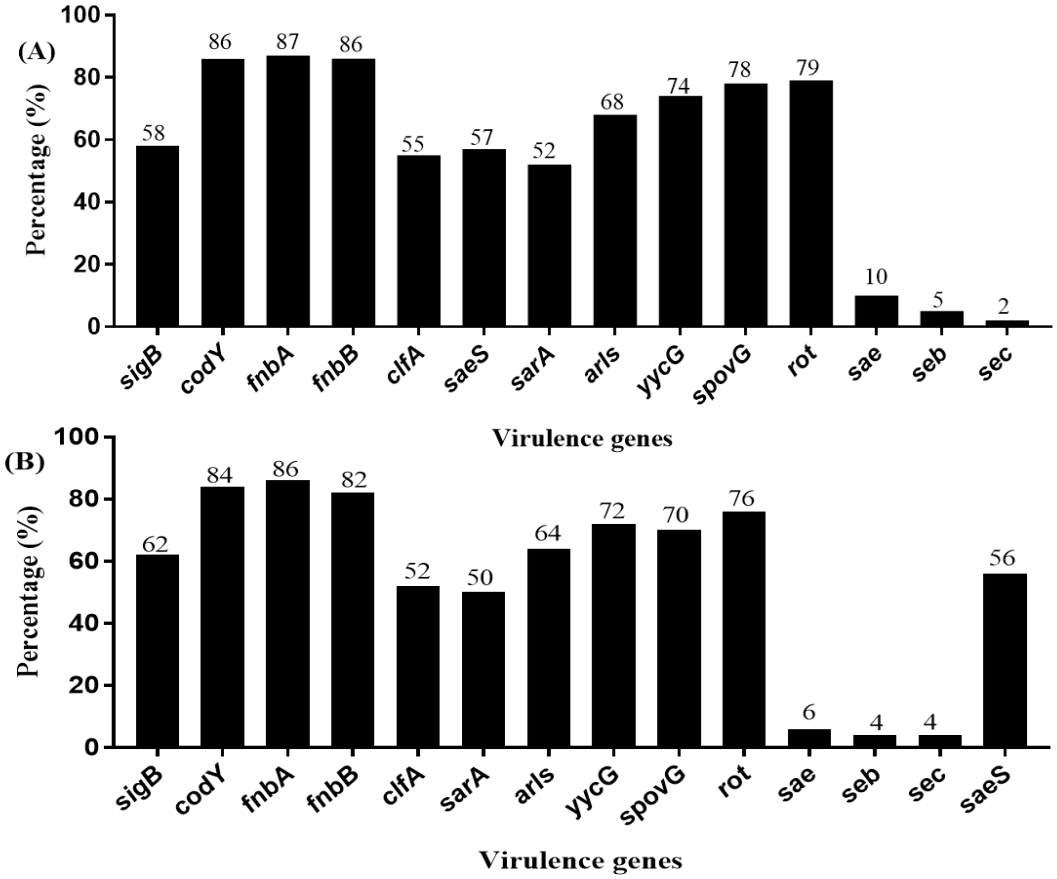


Figure 5.4: Distribution of virulence markers in *S. aureus* isolates. **(A)** virulence markers in MRSA isolates. **(B)** virulence markers in MSSA isolates.

5.5 Expression of Accessory gene regulator (*agr*) and intercellular adhesion (*ica*) gene

Agr is associated via autophagosome protection of bacterial cells that induces intracellular survival inside phagocytes, and mainly controls a variety of virulence factors in *S. aureus* strains. This experiment was performed to check the expression of *agr* and *ica* gene in HA-MRSA (SCC*mec* type III) and CA-MRSA (SCC*mec* type IV) without any stress, antibiotic treatment or temperature. Several *S. aureus* infections are link with the biofilms production, as well as endocarditis, osteomyelitis, septic arthritis, and infections closely associated with medical devices includings catheters, skeletal prostheses, and prosthetic heart valves. Biofilm formation not only facilitates the colonization of bacterial cell, but also contributes

in resistance to various antibiotics and the host immune system (Boles & Horswill, 2011; Fitzpatrick *et al.,* 2005; Götz, 2002; O'Gara, 2007; Otto, 2008). *Ica* locus significantly promotes biofilm formation in *S. aureus* isolates. No antibiotic or any other stress was given, expression was checked only in normal culture of *S. aureus* isolates. A decrease in expression of *agr* gene was observed in both the hospital acquired MRSA (HA-MRSA) and community acquired MRSA (CA-MRSA) as compared to control strain. However, the overall expression of *agr* in all three samples (control (*S. aureus*) + HA-MRSA and CA-MRSA) was high as compared to the expression of *ica* in all the three samples. Expression in an increase of *ica* genes was observed both in HA-MRSA and CA-MRSA.

5.5.1 Fold change increase and decrease in expression of *agr* and *ica*

Expression of *agr* in HA-MRSA and CA-MRSA decreased by 0.5-fold as compared to control. A significant increase of 2.5 fold was noticed in the expression of *ica* gene in HA-MRSA and CA-MRSA as compared to control as shown in Figure 5.5.

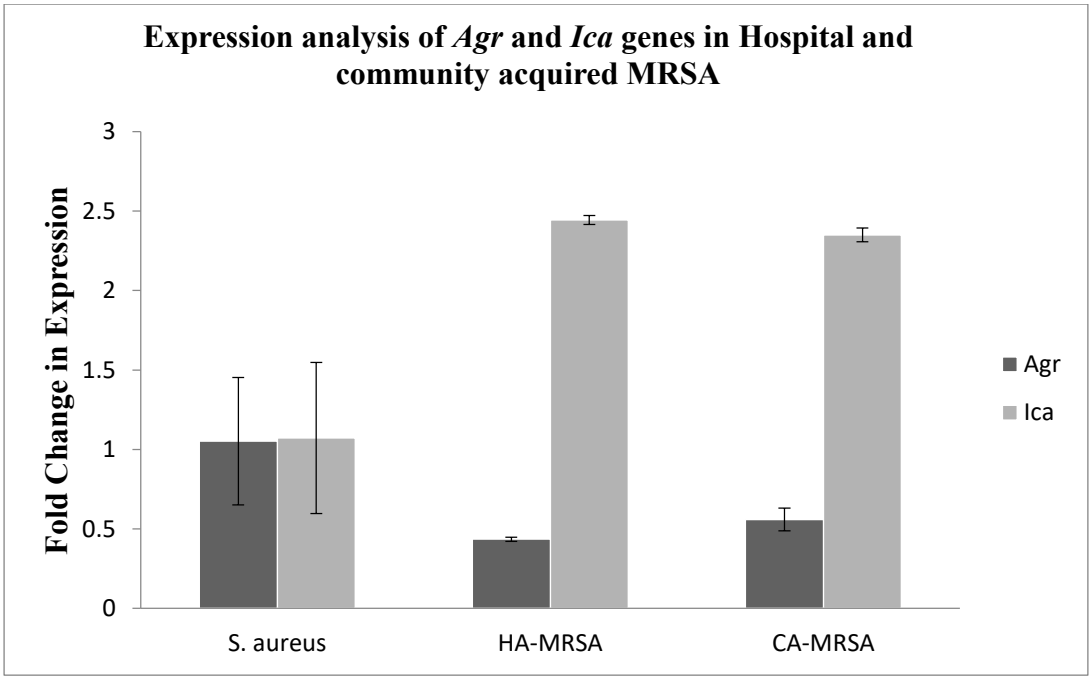


Figure 5.5: Expression of *agr* and *ica* genes in HA-MRSA and CA-MRSA clinical isolates. A considerable increase in expression of *ica* gene in HA-MRSA and CA-MRSA as compared to *S. aureus*

5.6 *In vitro* assay

For the evaluation of intracellular killing RAW 264.7 cell were used for representative isolates carrying *agr* group II, *agr* groups II, III, and *agr* mutant strain RN4220. After infection, the time interval of 2 and 20 hours, SDS with a final concentration of 0.02% was added to lyse macrophages. Cell lysates were serially diluted and CFUs were enumerated by plating on TSA plates using spotting techniques (Figure 5.6). No killing effect was observed by macrophages after 2 and 20 hours of infection.

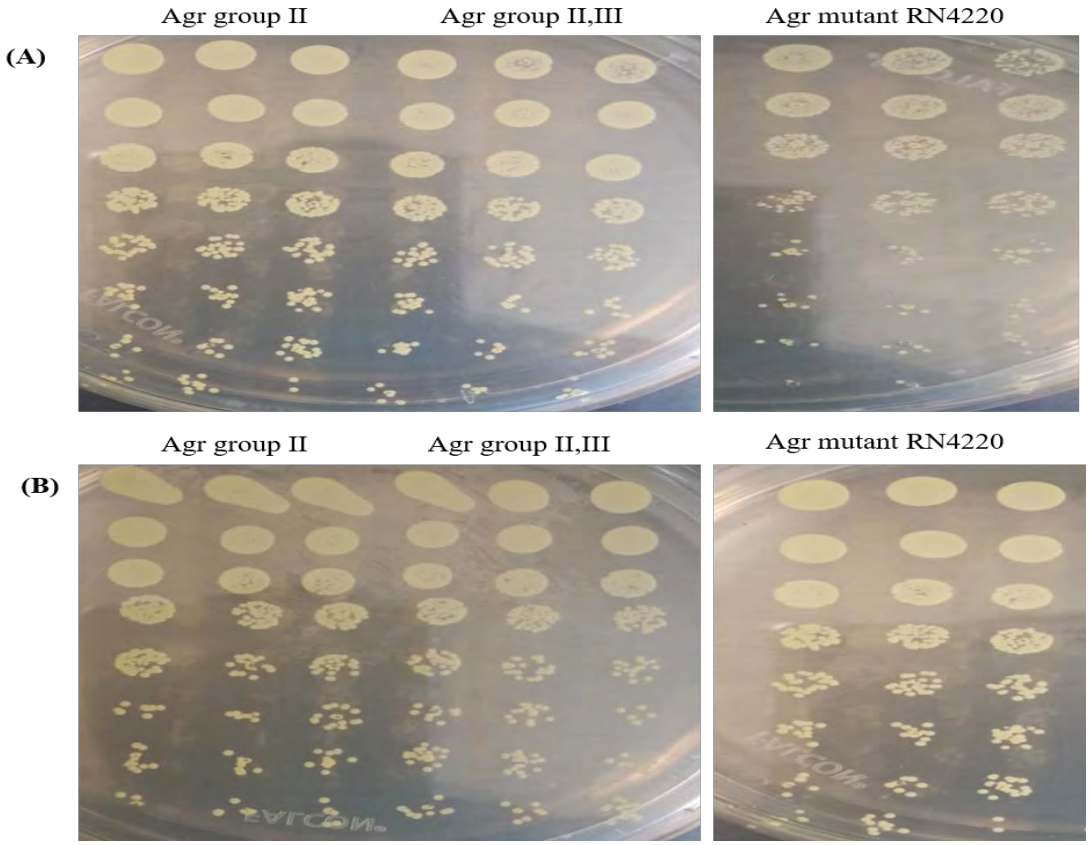


Figure 5.6: Spotting images show the intracellular survival of representative MRSA isolates inside the RAW 264.7 macrophages **(A)** shows the *agr* groups II, *agr* group II, III, and *agr* mutant strain RN4220 after 2 hours of infection **(B)** shows the *agr* groups II, *agr* group II, III, and *agr* mutant strain RN4220 after 20 hours of lysis.

5.7 Optimization of *S. aureus* dose in *G. mellonella* infection model and comparison with PBS

G. melonella infected by a clinical MRSA isolate resulted in the 100% death rate of the larvae, as compared to the control group. The 1.0×10^7 CFU was injected into larvae and 100% killing was observed after 24 hours of incubation, while PBS injected group served as control (Figure 5.7(A)). Comparison of MRSA with only PBS injected groupwas statistically significant (p=0.0001) (Figure 5.7 (B).

5.7.1 Effect of bacterial inoculum on the *G. mellonella* model

G. mellonella killing by *S. aureus* occurs in a dose-dependent manner, higher dose showed greater killing response. 100% killing was observed when the larvae were injected with 1.0×10^7 CFU/larvae in 16 larvae/groups compared to the lower dose (10^6 CFU) after 24 hours of incubation (Figure 5.7 (C).

5.7.2 Survival assay of MSSA, VISA, CA-MRSA, HA-MRSA and *Agr* Mutant RN4220strains

Five representative *S. aureus* isolates were chosen from this study for survival assay and hemocyte density measurement. Among these, 1 was MSSA with ST1535(CC15) with *spa* type 3214 from urine sample, 3 were MRSA carrying *mec*(A) gene, one strain was vancomycin intermediate *S. aureus* having MICs of 8mg/L (VISA, 8mg/L) from pus sample, one was CA-MRSA, staphylococcal cassette chromosome *mec* (SCC*mec*) type IV with ST772 (CC1) having *spa* type t657 from pus sample and one was HA-MRSA, SCC*mec* type III from blood and one was *agr* mutant RN4220 a control strain obtained from Dr. Mylonakis lab Rhode Island Hospital Brown University, USA. A representative MRSA isolate was injected into *G. mellonella* larvae, and the survival was observed during a 144 hours observation period. The representative MRSA isolates exhibited 100% killing rate after 24 hours of incubation as compared to the representative VISA strain (75%) and a representative community-acquired isolate (50%). However, a 100% of killing rate was observed when the wax moth larvae was injected by VISA and CA-MRSA isolates after 48 and 72 hours of incubation respectively. In contrast less killing rate (75%) was observed when the MSSA was injected with wax moth larvae and 90% survival was observed in wax moth larvae injected with *agr* mutant strain RN4220 (Figure 5.7(K)). The uninfected control groups (PBS) was maintained in each

experiment as shown in Kaplan-Meier survival curve (p= 0.00001; chi square test) which is highly significant (Figure 5.7).

5.7.3 Comparative survival assay of representative strains and their comparison with PBS

Comparison of VISA isolate with PBS, MRSA with MSSA, MRSA with *agr* mutant strain RN4220, CA-MRSA with *agr* mutant, CA-MRSA with MSSA, VISA with *agr* mutant strain, and VISA with MSSA, are shown in Figure 5.7 (D, E, F, G, H, I and J) respectively. All the comparative analysis were highly significant (*P*-value was Pr>chi2 = 0.0000).

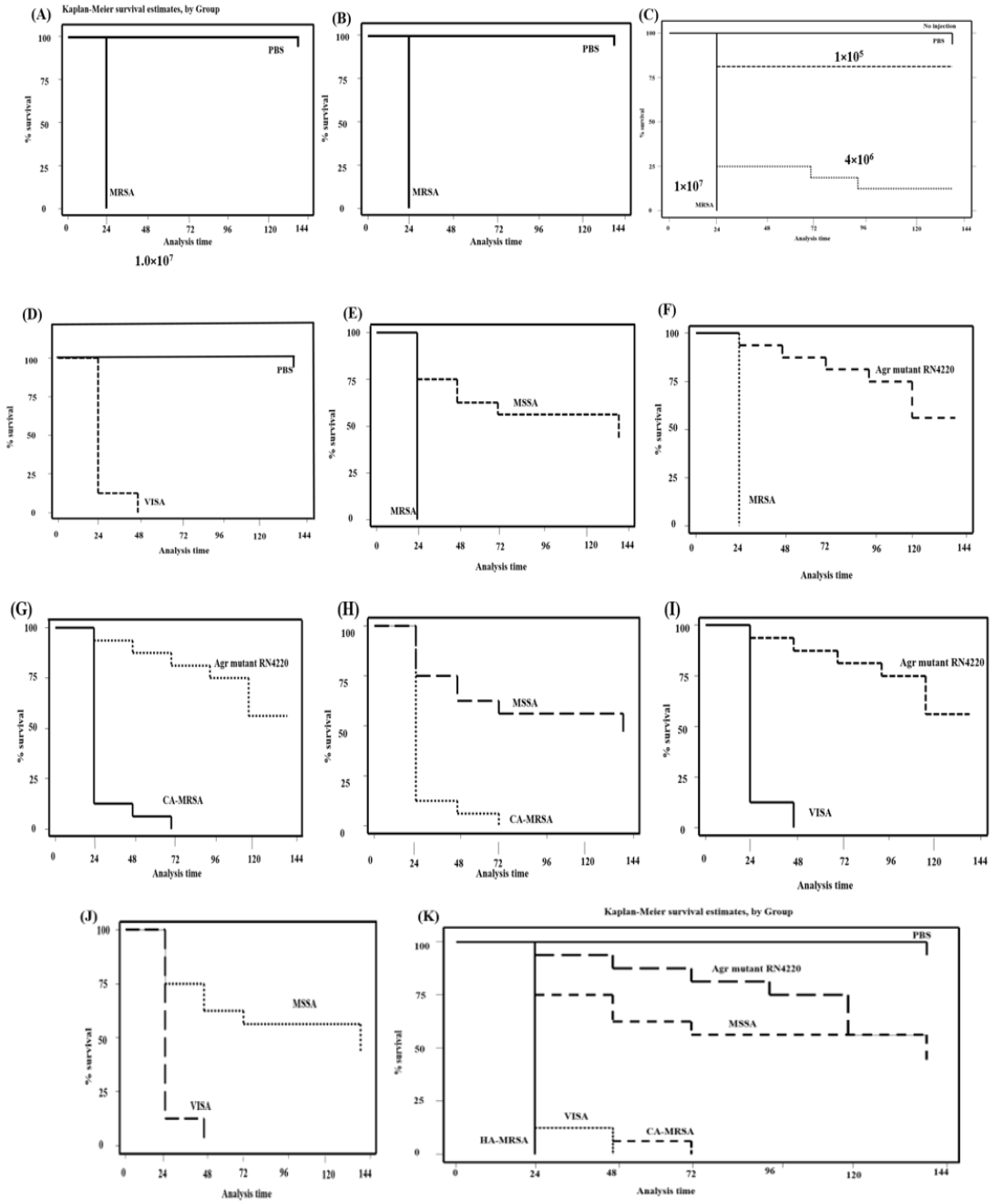


Figure 5.7: Kaplan-Meier plot of survival after infection with MRSA isolate shows the pattern of infection. *G. mellonella* were infected with 1.0×10^7 CFU/larvae with 16 larvae per group. In the control group, *G. mellonella* were injected with only PBS. Death of all *G. mellonella* were observed after 24 hours in case of MRSA isolate **(A)** Optimization of bacterial dose within 24 hours of infection **(B)** comparison of MRSA isolate with PBS **(C)** dose-dependent killing of MRSA isolates **(D)** comparison of VISA isolate with PBS **(E)** comparison of MRSA with MSSA **(F)** comparison of MRSA with RN4220 **(G)** comparison of CA-MRSA with RN4220 **(H)** comparison of CA-MRSA with MSSA **(I)** comparison of VISA with

RN4220 **(J)** comparison of VISA with MSSA **(K)** survival assay of representative isolates, HA-MRSA kills all the *G. mellonella* within 24 hours, followed by VISA, CA-MRSA, MSSA and *agr* mutant RN4220

5.8 Hemocyte Density Experiment

Pathogenesis analysis of each representative isolate in *G. mellonella* was done by hemocyte density experiment. *G. mellonella* was infected with representative clinical MRSA isolates, after 5 hours of incubation, hemolymph was collected, and hemocytes were counted using a hemocytometer. Hemocyte density decrease in CA-MRSA infected larvae indicated that the CA-MRSA is virulent. However, a hemocyte density decrease was also found in HA-MRSA, MSSA, VISA, and *agr* mutant RN4220 infected larvae but higher than the CA-MRSA infected larvae and was found significant (p= 0.001; student t-test). *G. mellonella* injected with PBS was used as a control group. Our local clinical CA-MRSA strain was found to be more virulent as compared to selected HA-MRSA, MSSA, VISA, and *agr* mutant RN4220 (Figure 5.8 A and B). The decrease in hemocytes indicates infection with higher virulence bacteria.

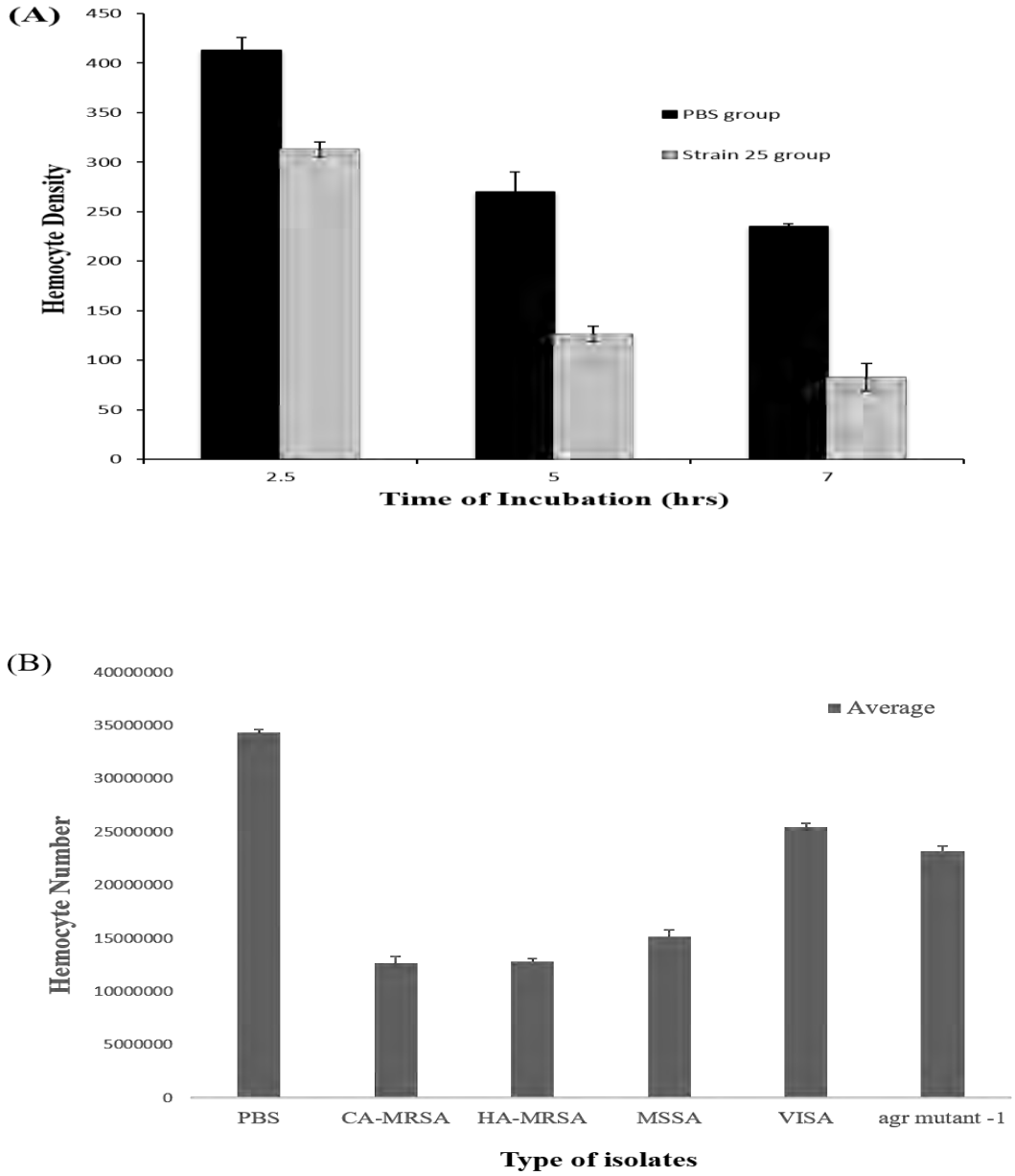


Figure 5.8: (A) shows the hemocyte density collected from the *G. mellonella* after 5 hours of infection, PBS is a controlled group, **(B)** CA-MRSA is more virulent than HA-MRSA, MSSA, VISA, and *agr* mutant RN4220 respectively.

5.3 Discussion

In this study, the prevalence of alpha-hemolysis, beta-hemolysis and gamma-hemolysis production in MRSA were observed as 45%, 28%, and 27% respectively, while genotypically 80%, 65%, 58% and 3.22% MRSA were positive for hemolysin genes *hla, hlb, hlg* and *hld* respectively, this agrees with the findings of a study from Pakistan, where alpha-toxin was the more dominant one in 40% *S. aureus* isolates whereas 25% were positive for beta-hemolysin (Haque *et al.,* 2017). In the current study, mostly the isolates were positive for alpha and beta-hemolysis, while a study from Germany reported that not all clones of *S. aureus* express alpha and beta hemolysin (Monecke *et al.,* 2014). The differences we observed in toxin production may be due to various factors such as geographical regions, source of the sample (animal, human, food), or the total number of isolates. The more prevalence of alpha-hemolysin in this study agrees with the findings of other studies, that reported alpha-hemolysin production as the dominant one in clinical *S. aureus* isolates (Grumann *et al.,* 2014; Haque *et al.,* 2017; Larsen *et al.,* 2002; Silva & Cardoso, 2000; Todar, 2005). The human monocytes and platelets are more sensitive to alpha-toxin (Löffler *et al.,* 2010).

In one study, the pore-forming alpha-toxin encoded by the *hla* gene was found in 95% of *S. aureus* isolates (Grumann *et al.,* 2014). Hassan *et al*, reported from Egypt that the prevalence of the *hla* gene was in 56.9% isolates, while the *hlb* gene was in 93% isolates (Hassan *et al.,* 2012). However, our study demonstrated the high prevalence of *hla* (80%), and low prevalence of *hlb* gene (65%). Nahar *et al*. reported from Bangladesh that 63.33% of isolates harbored *hla* gene, 75% of isolates were positive for *hld* gene, 15% of isolates were positive for *hlb* gene, and 11.67% were positive for *hlg* (Nahar *et al.,* 2017). One study from Egypt reported *hld* gene as the most prominent in different clinical specimens with prevalence of 88.2%, followed by *hla*, *hlg* and *hlb* with frequency of 30.5%, 81.1% and 64.7%, respectively (El-baz *et al.,* 2016). These reports are not similar with the current finding. As compared to this study, a report from Iraq described high prevalence of *hld* gene in 96.67% isolates, followed by *hla* (95%), *hlg* (90%) and *hlb* (79.16%), respectively (Saleem, 2009).

The variation in hemolysin genes could be due to geographical location, number of isolates, sample size, different source of samples, or the horizontal gene transfer of these virulence markers. Other studies reported that the prevalence of *hla* and *hlb* was 70, and 85% respectively from Iraq (Degaim *et al.,* 2015), 85% of *hla* and *hlb* from USA (Shukla *et al.,* 2010) and 100% frequency of *hla* gene from Uganda (Kateete *et al.,* 2011). Our study demonstrated that the low prevalence of hemolysin genes was due to the inclusion of both MRSA and MSSA strains in the analysis. However, other studies (Dunyach-Remy *et al.,* 2016; Mir *et al.,* 2019; Motamedi *et al.,* 2018) were more focused on MRSA. The variation in *hla* and *hlb* genes was observed and plausible reasons for these variations could be, geographical location, and due to existence of different genotypes. The high prevalence of *hla* and *hlb* (100%) was reported from China in the phenotypically positive isolates (Liu *et al.,* 2020), and South Africa (Schmidt *et al.,* 2017). Another study from China noticed *hla*, *hld* and *hlg* genes in almost all isolates (Xie *et al.,* 2018). The low occurance of these hemolysin genes in the phenotypically positive in this current study, could be due to the weak binding of primers, poor efficiency of Taq-polymerase enzymes or other factors affecting PCR reaction. In the current study, the coexistence of the *hla* and PVL was found in 3% isolates as observed previously by Rosatto *et al* (Rossato *et al.,* 2018).

Our study demonstrated that for gamma hemolysin only 27% of isolates were positive, and 58% were positive for the *hlg* gene. As compared to our findings, other studies described a low occurance of *hlg* gene with prevalence of 11.4% from China (Liu *et al.,* 2015), 10.7% from Iran (Mir *et al.,* 2019) and in 7.69% *S. aureus* isolates from Sudan (Elboshra *et al.,* 2020). In the current study, 3.22% of isolates were positive for *hld* gene. However, other studies reported high prevalence of *hld* gene in 94.5% of MRSA isolates from China (Liu *et al.,* 2015), in 89.8% from Iran (Mir *et al.,* 2019), and 96.67% from Iraq (Saleem, 2017). Delta hemolysin also has a key role in the staphylococcal virulence which is more frequently expressed in coagulase-negative staphylococci (CoNS) (Pinheiro *et al.,* 2015).

S. aureus produces a range of virulence factors, for example, leukocidin, staphylococcal enterotoxins (SEs), hemolysin, exfoliatin, toxic shock syndrome toxin 1 (TSST-1) that can assist in pathogenicity (Al-Tarazi *et al.,* 2009). As compared to our findings, previous study from Pakistan reported a high prevalence of *sea, seb* and *sec* genes i.e. 10.43%, 11.30% and 13.91% in MRSA isolates, respectively, while similar level of *sea*, *seb*, and *sec* with 3.82%, 3.27% and 4.91% was observed in case of MSSA (Taj *et al.,* 2014). Our findings contradict with the study from Iran where the enterotoxin genes in MRSA isolates were reported to have a prevalence of 15%, 48% and 0% for *seb, sea*, and *sec* genes respectively, while in MSSA it was 23%, 33% and 6% (Sabouni *et al.,* 2014). As compared to this study, another study from Iran described high prevalence of *sea* gene in 100% MRSA isolates (Rahimi & Shokoohizadeh, 2018). Another report from China described high prevalence of *sea*, *seb* and *sec* i.e. 21%, 65% and 68% in MRSA while 15%, 66% and 68% in MSSA isolates respectively (Kumburu *et al.,* 2018), which also contradicts with our study.

The variation in the prevalence of enterotoxin in various studies may be due to different geographical study location, sample source, incorporation of virulence factors within bacterial genome through bacteriophages. In this study, the presence of various virulence factors as determined by PCR for MRSA and MSSA essentially showed no difference. Current findings are in agreement with two previous studies (Ellis *et al.,* 2009; Kim *et al.,* 2006) but these findings do not coincide with a study in which virulence genes were diverse in MSSA as compared to MRSA (Jiménez *et al.,* 2011). As compared to this study, another study from Hungry described high virulence factors in MRSA isolates as compared to MSSA (Horvath *et al.,* 2020). One study from China reported that the prevalence of virulence markers in MRSA was high as compared to MSSA (Liu *et al.,* 2015). Whereas one study reported that MRSA isolates harbored more virulence makers than MSSA (Yu *et al.,* 2012). Virulence gene carriage may vary according to clinical-epidemiological factors and geographic locations, specific clone carry the virulence genes, environmental conditions, and sources of samples.

The most prevalent regulatory factors were *codY* (86% n=172), followed by *rot* (79%, n=158), *spoVG* (78%, n=156), and *yycG* (74%, n=148), *sae* (n=114, 57%), *sar* A (n=104, 52%), and *arls* (n=136, 68%). In the case of MSSA, the most dominant regulatory factors were *codY* (84%, n=42), followed by *rot* (76%, n=38), *spoVG* (70%, n=35), and *yycG* with (72%, n=36), *sae* (n=23, 56%), *sar* A (n=25, 50%), and *arls* (n=32, 68%). These are the regulatory factors present in all bacteria and essential for bacterial survival. Most of the studies (Ma *et al.,* 2012, Truong-Bolduc *et al.,* 2011, Bischoff *et al.,* 2016, Wozniak *et al.,* 2012, Mootz *et al.,* 2015, Dubrac *et al.,* 2007) have focused on their expression analysis through RT-PCR, but in the current study we screened these factors through multiplex PCR, the low prevalence of these regulatory factors could be due to the weak binding of primers, poor efficiency of Taq-polymerase enzymes or other factors affecting PCR reaction. Therefore, without further confirmatory experiments, the conclusions on presence or absence of these regulatory factors cannot be supported.

In this study, the prevalence of PVL was reported as 24% in MRSA and 10% in MSSA isolates. Our findings are in line with the results of a study from Pakistan that 21.62% MRSA and 8.33% MSSA were noticed as PVL positive (Haque *et al.,* 2017). However, as compared to this study, other studies from Pakistan have reported more prevalence of PVL i.e. 33.33% in MSSA (Khan *et al.,* 2018), 46% in MSSA (M. S. Iqbal *et al.,* 2018) and 40% MRSA isolates. As compared to this study, a report from Iran described low prevalence of PVL i.e. 5.7% of *S. aureus* isolates (Rahimi & Shokoohizadeh, 2018). As compared to our findings, one study from Iran reported low prevalence of PVL, 0.5% in *S. aureus* isolates (Mir *et al.,* 2019). Variation in the prevalence of PVL toxin within different cities from Pakistan could be due to different number of study isolates or acquisition of PVL toxin through phages in different *S. aureus* clones, which is worth investigating.

A study from Saudi Arabia reported that 54% of isolates were PVL carriers (Monecke *et al.,* 2012), while 14.6% PVL carriers were found in Kuwait hospital (Udo *et al.,* 2014). USA300 and USA400 class of MRSA strains, which are associated with community-acquired infections and harbor PVL toxin are believed

to be the main reason for causing soft tissue and skin infection in humans (Chua *et al.,* 2014). The PVL toxin is the hallmark of CA-MRSA and usually not present in nosocomial MRSA (Bukharie, 2010). Various studies have reported variation in the prevalence of PVL toxin such as 15% of *S. aureus* from Netherlands (Adedeji *et al.,* 2007), 10% in CA-MRSA from Iraq (Habeeb *et al.,* 2014), and about 8% of the MRSA strains from USA isolated from nasal carriage harbored PVL (Kuehnert *et al.,* 2006).

One study from China observed virulence factors of *S. aureus* with prevalence as; *fnb*A was found in 56% MSSA 78% MRSA isolates, while *fnb*B was present in 36% MSSA and 12% MRSA, 100% isolates were *hla* carrier*,* while *hlb* was found in 42% MSSA and 78% MRSA isolates (Li *et al.,* 2018), and these results are not in accordance with current study. As compared to the current study, one study from Iran reported the prevalence of *fnb*A, *fib*, *cna*, *ebs*, *eno*, *bbp*, and *fnb*B genes in 63%, 50%, 82%, 63%, 59%, 0%, and 6% isolates, respectively (Ghasemian *et al.,* 2015), which is not similar to the current study. Another study from Iran reported the prevalence of *fnb*A, *fnbB*, *clfB, clfA* genes in 7.2%, 43.6%, 86.3%, and 36.3% isolates, respectively (Soltani *et al.,* 2019). Variation in the presence of virulence factors could be due to different geographical location, acquiring through phages, and transfer through mobile genetic elements.

Gill *et al*, reported that the antibiotic-resistant strains are subjected to express less virulence due to the associated fitness cost (Gill *et al.,* 2005). This strongly depends upon the genetic makeup of the isolates and these may be affected by location as one clone may be prevalent in one region. However, we report that the antibiotic-resistant *S. aureus* express various virulence factors as observed by multiplex PCR. The MRSA strains collected from different geographical regions have been shown to hold different toxin genes profiles (Alfatemi *et al.,* 2014). Current findings are in agreement with two previous studies (Ellis *et al.,* 2009; Kim *et al.,* 2006). But these findings do not coincide with a study in which virulence genes were diverse in MSSA as compared to MRSA (Jiménez *et al.,* 2011). We found that the overall

expression of *agr* in all three samples (control+ HA-MRSA and CA-MRSA) was high as compared to the expression of *ica* in all three samples.

A significant increase of 2.5 fold was noticed in the expression of *ica* gene in HA-MRSA and CA-MRSA, supporting the study reported (Ma *et al.,* 2012, Qiu *et al.,* 2010). We found that the clinical strains with *agr* groups II and III can survive in mouse macrophages and macrophage induced killing was not observed after 20 hours incubation. We used *agr* mutant strain RN4220 as a control, supporting the study reported by Seral *et al*, (Seral, *et al.,* 2003). Another study reported that the MSSA strain ATCC25923, MRSA strain 43300 and MDR strain of *spa* type t034 can survive and proliferate inside the RAW 264.7 macrophages (Wang *et al.,* 2018). *S. aureus* were engulfed by macrophages and the other suggested reason of survival inside the macrophages was that the phagosomse did not mature in macrophages (O'Keeffe *et al.,* 2015).

Agr controls a variety of virulence factors in *S. aureus*. The *agr* mutant strains were reported to have attenuated virulence in mammalian models, and exhibited reduced virulence in *G. mellonella* (Peleg *et al.,* 2009), supporting our observation. In addition, we investigated the association between the *S. aureus* pathogenesis and larvae hemocytes. Commonly, larval hemocytes density and the microbial pathogenesis load are inversely related (Bergin et al,. 2003). One study, investigated the association between the larvae hemocytes in order to estimate the association of fungal strains with hemocytes density (Fuchs *et al.,* 2010), which also supports our observations since a decrease in hemocyte number was observed which indicates increased pathogenecity. We checked the infection assay of representative HA-MRSA, VISA, CA-MRSA, MSSA, and *agr* mutant RN4220 isolates to see the bacterial-induced wax moth larvae killing. The 100% killing was observed by HA-MRSA within 24 hours, as compared to VISA, and CA-MRSA isolates, while less killing was by MSSA and *agr* mutant RN4220. The same findings were observed in a previous report (Desbois & Coote, 2011; Peleg *et al.,* 2009). Hemocyte density experiment showed that CA-MRSA was more virulent as compared to HA-MRSA, MSSA, VISA, and *agr* mutant RN4220 strain.

Chapter 6: General Discussion

Antimicrobial resistance (AMR) is one of the serious global health problems. Multidrug-resistant (MDR) *S. aureus* strains have spread globally with increasing frequency, subsequently high levels of morbidity and mortality, and presenting an alarming situation for healthcare professionals (Wilson *et al.,* 2016). In general, the carriage of *S. aureus* was noticed in 33% general population however it causes a wide range of skin and soft tissue infections, joints, bone, and infections related with indwelling catheters or prosthetic devices (Tong *et al.,* 2015). In the United States, the Centre of Disease Control and Prevention (CDC) estimates that the carriage rate of MRSA is approximately 2% (Kavanagh, 2019). In all WHO regions, the overall ratio of *S. aureus* isolates that were MRSA is reported to be >20% and even >80% in some reports (WHO, 2014). Pakistan has developed a national action plan to combat AMR due to ESKAPE (*Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter, Pseudomonas aeruginosa,* and *Enterobacter/E. coli* species) pathogens but still there is not an efficient national surveillance network and global reporting is done through the WHO Global Antimicrobial Surveillance System (GLASS) (Letter, 2011).

According to the one AMR surveillance study of Lahore, Pakistan, *S. aureus* was the most prevalent pathogen in adults, followed by *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Ching *et al.,* 2019). Complicated infections due to antibiotic-resistant strains in Pakistan are due to poor hygiene and lack of infection control programs. It is need of the hour to highlight the presence of MDR pathogens in healthcare settings and introduce protocols for the handling of such infections. The current study describes the molecular epidemiology and typing of clinical *S. aureus* strains from a tertiary care hospital in Islamabad and presents information of circulating strains which has not been reported earlier from Pakistan. Among the novel findings, we report clinical MRSA carrying *mec*(C) alone and in combination with *mec*(A), SCC*mec* VI element and various *spa* types along with NEW *spa* type and sequence type ST1535.

This study noticed a high prevalence of MDR *S. aureus* showing resistance to routinely used antibiotics leaving limited therapeutic options for treating complicated life-threatening infections. In the current study, 95% of isolates were sensitive to vancomycin with 5% intermediate and 0% resistant which is first line treatment for MRSA, but increasing use of vancomycin and linezolid in Pakistan have lead towards resistance, as previously reported from Pakistan (Azhar *et al.,* 2017). It is important to note that most of the healthcare settings in the rural areas of Pakistan do not have access to the antimicrobial susceptibility testing to properly screen the disease pathogen for routinely used antibiotics and hence the problem of AMR is increasing due to the misuse of antibiotics. In comparison to antistaphylococcal penicillins, cefazolin has shown a good activity and a decrease in *S. aureus* toxicity in the case of MSSA bacteremia (Loubet *et al.,* 2018; Shi *et al.,* 2018).

Antimicrobial stewardship should be developed to minimize AMR which should be done by the support of clinical microbiologists, clinicians, medical practitioners, and pharmacists, while proper policies should be applied to stop the unnecessory use of antibiotics. In Pakistan, MRSA accounts for a considerable proportion of nosocomial infections and a number of studies have highlighted the increasing clinical significance of MRSA (Bukhari *et al.,* 2011), (Ashiq & Tareen, 1989; Brohi & Noor, 2017; Hussain *et al.,* 2019; Naeem *et al.,* 2013; Siddiqui *et al.,* 2017; Ullah *et al.,* 2016). This shows a persistent increment in the flow of this bacterium in clinical settings. However, most of these studies have investigated resistance by phenotypic methods, and very limited data is available on molecular typing of the clinical isolates.

Furthermore, *mec*(A) negative isolates are mostly not investigated for alternative resistance traits. In this study, we found 3% *mec*(A) negative isolates carrying *mec*(C) gene, while one MRSA harbored both *mec*(A) and *mec*(C) genes along with resistance to various antibiotics such as tetracycline, ciprofloxacin, and cefoxitin. A similar observation was also noticed by Peterson *et al*. in the UK (Paterson *et al.,* 2014). To the best of our knowledge, this is the first report of clinical MRSA strains

from Pakistan carrying both *mec*(C) only and in combination with the *mec*(A) gene. In Pakistan, there should be proper protocols for *mec*(A) negative isolates to further screen them for the presence of *mec*(C), *mec*(B), *fem*(A) using molecular assays and/or commercial PBP2a detection assays.

In MRSA, *mec*(C) gene is localized on SCC*mec* element known as type SCC*mec* XI (García-Álvarez *et al.,* 2011; Shore *et al.,* 2011). We found *mec*(C) gene in 3% MRSA isolate. Other studies from Germany, Denmark and UK also reported low prevalence of *mec*(C) MRSA ranging from 0.08 to 5.9% (Cuny *et al.,* 2011; Petersen *et al.,* 2013; Pichon *et al.,* 2012; Stegger *et al.,* 2012). In Pakistan, no data is available about MRSA carrying *mec*(C) gene in clinical isolates. MRSA harboring *mec*(C) gene from various sample sources in this study indicates its transmission from the community to the hospital environment as a result of *mec*(C) gene on mobile genetic element.

MDR-MRSA and MDR-MSSA in hospitals and other healthcare units are a great concern. Treatments against multidrug resistant *S. aureus* (MDRSA) have become worrying and less successful (Chambers & DeLeo, 2009). In this study, the prevalence of MDR-MRSA was 83% while 20% of isolates were identified as MDR-MSSA. One study from Lahore, Pakistan reported that among MSSA, 37.84% isolates were MDR, while in the case of MRSA all isolates (100%) were found to be MDR (Taj *et al.,* 2016). Other studies from various countries of the world have reported different levels from our neighbouring countries, 73% MDR-MRSA from India (Ahmad *et al.,* 2014), 100% MDR-MRSA from China (Wang *et al.,* 2012), 93% MDR-MRSA and 61% MDRSA (Rahimi *et al.,* 2013), and 78% MDR-MRSA from Iran (Moosavian, Dehkordi, & Hashemzadeh, 2020), 83.8% MDRSA from Afghanistan (Naimi *et al.,* 2017), 40.1% from Nepal (Tiwari *et al.,* 2009) and 51.8% from Vietnam (Son *et al.,* 2019), 93% MDR-MRSA and 31% MDRSA from Jordan (Al-Zoubi *et al.,* 2015), 47% MDR-MRSA from Saudi Arabia (Albarrag *et al.,* 2020) and 78.5% MDR-MRSA from Kuwait (Alfouzan *et al.,* 2019), 30.4% from Italy (Mascaro *et al.,* 2019), 92.9% MDR-MRSA from Poland (Kot *et al.,* 2020), 59.3% from Brazil (Chamon *et al.,* 2017), 50.5% MDRSA from Ethiopia (Dilnessa

& Bitew, 2016), 19% from Ghana (Saba *et al.,* 2017), and 23.9% MDRSA from Northeast Ohio (Kadariya *et al.,* 2019) and 13.13% MDR-MRSA from USA (Duncan *et al.,* 2020). These differences could be due to the misuse of antibiotics, different immune status of patients, different geographical location and sample size, variable hospital managements, number of samples and severity of infections.

Globally, vancomycin is the drug of choice in complicated MRSA infections with the promising exception of linezolid (Wunderink *et al.,* 2012). In this study, MIC profiles of the study isolates revealed that 10 (5%) MRSA isolates had intermediate resistance to vancomycin (MICs in the range of 4-8mg/L) which is similar to a previous study conducted in Pakistan which described 8.9% vancomycin-intermediate *S. aureus* (VISA) isolates using E-test method (Ullah *et al.,* 2016). Due to disproportionate use of vancomycin in Pakistan for complicated MRSA infection, a high level of vancomycin resistance at 13% was recoded in MRSA using the E-test method (Azhar *et al.,* 2017). Reason for increase in vancomycin resistance could be suboptimal use of vancomycin as well as the possible transfer of vancomycin resistance genes from other vancomycin resistant organisms including *Enterococcus*. Specifically, our physician should be worried about resistance to vancomycin so that they can prescribe vancomycin appropriately to prevent therapeutic failure.

In the present study, 10 different *spa* types were found in MRSA with t657 (24%) as the dominant *spa* type while 15 *spa* types were found in MSSA with t021 (24%) with the highest prevalence. Limited work on *spa* typing of clinical *S. aureus* has been done in Pakistan. One study has reported 8 different *spa* types in clinical *S. aureus* where t021 was in only one isolate (Yasrab Arfat, 2013) whereas it is the dominant *spa* type in MSSA in this work. Another study recently reported t657 from table eggs (Syed *et al.,* 2018). The current study advances information of *spa* types of local clinical *S. aureus* isolates as out of 21 *spa* types in this study, 17 are being reported first time from Pakistan.

Certain *spa* types are confined to specific geographic areas while some are widely circulated around the world. The polymorphic X region of *spa* protein and the type

of protein A have possible associations with the organisms' adaptations to diverse conditions such as altered host populations, geographical diversity and the weather condition (Asadollahi *et al.,* 2018). Frequent migration from one continent to another gives a reasonable justification concerning why some *spa* types are shared among certain continents. The most predominant *spa* types in Europe were t002, t032, and t008; t002 and t037 in Asia; t020 in Australia; t008, t242, and t002 in USA and t064 in Africa. t030 *spa* type was most dominant in Asia mainly located in China and Iran (Asadollahi *et al.,* 2018). One study has reported t030 and t008 from Asia and America in MRSA isolates (Asadollahi *et al.,* 2018), the same finding was also observed in the current study. In case of t008 which was reported in MSSA isolates in Europe (Asadollahi *et al.,* 2018), in the current study it was found in MRSA isolates, which may be due to the acquisition of *mec*(A) gene by MSSA strain. Presence of t030 and t064 in current study and in previous study (Asadollahi *et al.,* 2018b), showed that these *spa* types circulate worldwide.

SCC*mec* III was the most prevalent type in our study, similar findings were observed from China (Wang *et al.,* 2012), Pakistan (Arfat *et al.,* 2013; Arshad *et al.,* 2015; Asghar, 2014; Mirani *et al.,* 2017; Muhamad Sohail & Zakia Latif, 2018), Iran (Bahrami *et al.,* 2019; Fasihi *et al.,* 2017; Ghaznavi-Rad *et al.,* 2018; Taherirad *et al.,* 2016), reporting SCC*mec* III as the most dominant in MRSA isolates.

We found all SCC*mec* types to be associated with multidrug resistance phenotype which is similar to the previous study (Ito *et al.,* 2003). Furthermore, in the current study SCC*mec* III showed resistance to up to 8 classes of antibiotics. One study from Iran reported that SCC*mec* type III isolates were mostly resistant to six antimicrobial agents (Taherirad *et al.,* 2016). Other researchers also reported a similar kind of observation (Deurenberg *et al.,* 2007). The reason behind this is that the SCC*mec* type III is the longest and can combine with other genetic elements, including pT181 plasmid and Tn554 transposon, thus adding additional resistance traits for other antimicrobial agents. Our findings showed the prevalence of SCC*mec* NT in 7.1% MRSA isolates, which is similar to the previous studies (Abdollahi *et al.,* 2012; Oliveira & de Lencastre, 2002; Zeinali *et al.,* 2011). One study from Iran

also reported SCC*mec* NT in 4.8% isolates (Taherirad *et al.,* 2016). The reason for NT could be as a result of other SCC*mec* types (e.g., VII-XI SCC*mec*) and other unknown types that were not screened in the present study.

In this study, as the dominant Sequence type was ST772 with type IV element and having *spa* type t657 carried several antibiotic-resistant determinants such as *tet*M, *mec*(C), *gyr*(A), *gyr*(B), *aacph*D1, *mec*(A), *bla*(Z), *rpo*(B) and *fos*(A) genes, and same findings were observed from Iran (Mohammadi *et al.,* 2014). ST1-PVL positive MSSA was obtained from pus in Malaysia (Suhaili *et al.,* 2016), and Nepal (Pokhrel *et al.,* 2016). In the current study, ST1-PVL was found in MRSA isolate from a tissue specimen, which may be due to the acquisition of *mec*(A) gene by MSSA strain.

In this study, the predominant sequence type ST772, ST1, and ST1535 were collected from different wards and different type of specimens. An effective infection control strategy and proper training of healthcare workers is needed to further control the spreading of these specific clones in the hospital settings. There should be strict policies to screen the healthcare workers for MRSA carriage in the hospital environment. In this study, 3 genotypic patterns were formed: ST772-t657, ST1-t127 in MRSA and ST1535-t3214 in MSSA. ST772-t657 and ST1 have been previously reported from Pakistan (Syed *et al.,* 2018; Zafar *et al.,* 2011) whereas the MSSA with ST1535-t3214 has not been previously reported from Pakistan. ST1535 with CC15, with a single variant of ST15 in MSSA, was reported from Saudi Arabia (Senok *et al.,* 2017). One study has reported CC772-MRSA-V, ST239-MRSA, CC8-MRSA-IV and CC6-MRSA-IV as common circulating strains and showed epidemiological linkage with the Middle Eastern/Arabian Gulf region (Jamil *et al.,* 2018). ST772 is characterized as an MDR clone (Magiorakos *et al.,* 2012) and resistance to fluoroquinolones, aminoglycosides, and macrolides are common (Blomfeldt *et al.,* 2016; Samar *et al.,* 2016; Brennan *et al.,* 2016; Nadig *et al.,* 2012; Rajan *et al.,* 2015). A similar kind of observation was noticed in the current study. ST772 in this study had PVL marker and virulence genes such as hemolysin gene *hla*, enterotoxin gene (*sea* and *sec*) and adhesions (*fnbA*, *fnbB*), and similar kind of

observation was noticed from a study in Kathmandu, Nepal, that reported ST722 with PVL marker carrying *hla*, *hld* and *hlg*, enterotoxin genes (*sea*, *sei*, *seo*, *sem*, *sec*, *sel*, *sen*, *seg*) and adhesions *fnbA*, *ebps* and *sdrE* genes (Pokhrel *et al.,* 2016).

The clinical outcome of *S. aureus* infections is influenced by their virulence factors and antimicrobial resistance. One study from China reported that the prevalence of virulence markers in MRSA was high as compared to MSSA (Liu *et al.,* 2015). Whereas one study reported that MRSA isolates harbored more virulence makers than MSSA (Yu *et al.,* 2012). Gill *et al*, reported that the antibiotic-resistant strains were observed to express lesser virulence due to the associated fitness cost (Gill *et al.,* 2005). However, we reported that the antibiotic-resistant *S. aureus* expressed various virulence factors confirmed by multiplex PCR. The MRSA strains collected from different geographical regions have been shown to hold different toxin gene profiles (Alfatemi *et al.,* 2014). Current findings are in congruent with two previous studies (Ellis *et al.,* 2009; Kim *et al.,* 2006) but these findings do not coincide with a study in which virulence factors were diverse in MSSA than MRSA (Jiménez *et al.,* 2011). The clinical outcome of *S. aureus* infections is influenced by their virulence factors and antimicrobial resistance pattern (Liu *et al.,* 2015). We observed a high prevalence of both antibiotic resistance markers and virulence genes, this combination is particularly worrisome as it may lead difficulty in treating complicated infections.

We found that the clinical strains with *agr* groups II and III can survive in the mouse macrophages and macrophage induced killing was observed after 20 hours incubation. We used *agr* mutant strain RN4220 as a control, supporting the study reported by Seral *et al*, (Cristina Seral *et al.,* 2003). Another study reported that the MSSA strain ATCC25923, MRSA strain 43300 and MDR strain with *spa* type t034 can survive and multiply inside the RAW 264.7 macrophages (Wang *et al.,* 2018). *S. aureus* engulfment by macrophages and the survival inside macrophages suggests that the phagosome did not mature in macrophages (O'Keeffe *et al.,* 2015).

In *S. aureus, agr* controls a variety of virulence factors. The *agr* mutant strains are reported to have attenuated virulence in mammalian models, and exhibit reduced

virulence in *G. mellonella* (Peleg *et al.,* 2009), supporting our observation that the *agr* mutant RN4220 strain was less virulent as compared to other representative isolates. In our study, as compared to *agr* mutant RN4220 greater killing was observed by HA-MRSA, VISA, CA-MRSA, and MSSA isolates, and similar findings have been observed previously (Desbois & Coote, 2011). HA-MRSA was observed to be more pathogenic in *G. mellonella* as compared to other representative isolates. In case of hemocyte density experiment, CA-MRSA and HA-MRSA both showed comparable virulence. This may be due to the possible movement of MRSA between hospital and community. Higher virulence of MRSA isolates is of concern as it may result in higher infection rate. More studies with larger sample inclusions are required to look into exact rates of infection and virulence of *S. aureus* isolates.

In addition, we investigated the association between the *S. aureus* pathogenesis and larvae hemocytes. Commonly, larval hemocytes density and the microbial pathogenesis load are inversely related (Bergin *et al.,* 2003). One study investigated the association between the larvae hemocytes in order to estimate fungal strains association with the hemocyte density (Fuchs *et al.,* 2010) supporting our observation. The decrease in hemocytes results in virulence of bacterial infection. We found decrease in hemocytes in CA-MRSA, as compared to other representative isolates which revealed that CA-MRSA was more virulent than HA-MRSA, MSSA, VISA, and *agr* mutant strain RN4220.

Chapter 7: Concluding Remarks

7.1: Summary of work presented in this thesis

In the current study *S. aureus* isolates were isolated from various specimen, patients of both genders of varying ages and from various hospital wards. The predominant clinical source of *S. aureus* isolates including MSSA and MRSA was found to be pus, followed by blood and urine. The overall prevalence of MRSA was 65%, while 35% were MSSA. Among MRSA isolates, high levels of susceptibility were found against linezolid, quinupristin/dalfopristin and chloramphenicol while highest resistance was recorded against ciprofloxacin and gentamicin. Among MSSA isolates highest resistance was found against ciprofloxacin and tetracycline while MSSA isolates were sensitive to linezolid, cefoxitin, quinupristin/dalfoprestin and chloramphenicol. None of the isolates was resistant to vancomycin while 10 isolates exhibited intermediate resistance. The prevalence of antibiotic resistance genes (ARGs) in MRSA was: 80% *dfr*(B), 87% *tet*K, 81% *tet*M, 54% *mec*(A), 3% *mec*(C), 100% *bla*(Z), 95% *gyr*(A), 33% *gyr*(B), 9% *grl*(A), 6% *grl*(B), 80% *rpo*(B), 75% *aacphD1*, 13% *erm*(A), 81% *fos*(A), 0% *fos*(B), 56% *vat*(C), 29% *vat*(B), 4% *vat*(A), and 100% *erm*(C). The prevalence of ARGs in MSSA was: 75% *dfr*(B), 31% *tet*K, 0% *tet*M, 52% *gyr*(A), 0% *gyr*(B), 0% *grl*(A), 0% *grl*(B), 67% *rpo*(B), 75% *aacphD1*, 75% *erm*(A), 50% *vat*(C), 2% *vat*(B),0% *vat*(A) and 100% isolates for *erm*(C). To the best of our knowledge, this is the first report from Pakistan of clinical MRSA strains carrying *mec*(C) alone and in combination with *mec*(A) gene. In this study, *agr* I (22%) followed by *agr* III (20%) was the most predominant group in MRSA while in MSSA it was *agr* III (16%) followed by *agr* II (8%) whereas *agr* IV was absent in MRSA isolates while present in 4% MSSA. In MRSA, 42% were found to be positive for HVR whereas 58% isolates did not carry HVR. The most dominant was type 6 followed by type 5, type 7, type 3 and type 4. Current findings regarding the SCC*mec* types, revealed that the prevalent *mec* types in this region were III, IV, VI, II and I found in 86%, 40%, 23%, 23%, and in 14% isolates, respectively, whereas type V was not detected. The *spa* type t657 in MRSA isolates was found most common while t021 was in MSSA. Most of the *spa* types were reported first time from Pakistani isolates. Out of total 21 *spa* types, 15 *spa*

types were observed in MSSA while 10 in MRSA. The same *spa* types were also found in both MSSA and MRSA isolates such as; t657, t632, t5598, and t345. In MSSA isolates 1 NEW *spa* type was identified. We found only ST772 and ST1 in MRSA isolates while ST1535 in MSSA isolate. Among multi-locus sequence types, ST1535 is first time reported from Pakistan. Clonal relatedness of selected isolates showed that 2 isolates were of same clone (100%), 2 isolates had 85% similarity and one isolate had 75% similarity index. The alpha-hemolysin (45%, 60%) was more dominant as compared to beta-hemolysin (28%, 28%) and gamma-hemolysin (27%, 12%) in MRSA, and MSSA, respectively. Among MRSA, alpha-hemolysin gene (*hla*) was detected in 80% of phenotypically positive isolates, followed by 65% for beta-hemolysin (*hlb*) and 58% were positive for gamma-hemolysin gene (*hlg*), and *hld* gene was found in 3.2% of isolates. Virulence factors in MRSA isolates were recorded as 58%, 86%, 87%, 86%, 55%, 57%, 52%, 68%, 74%, 78%, 10%, 5%, 2%, 79% and 24%, for *sigB, codY, fnbA, fnbB, clfA, saeS, sarA, arlS, yycG, spoVG, sea, seb, sec, rot,* and PVL toxin genes, respectively. The percent prevalence were 62%, 84%, 86%, 82%, 52%, 56%, 50%, 64%, 72%, 70%, 6%, 4%, 4%, 76% and 10%, for *sigB, codY, fnbA, fnbB, clfA, saeS, sarA, arlS, yycG, spoVG, sea, seb, sec, rot* and PVL genes respectively in MSSA isolates. Macrophages induced intracellular killing effect was not observed after 20 hours of incubation for *agr* groups II, III, and *agr* mutant strainRN4220. Survival assay in *G. mellonella* infection model showed that HA-MRSA was more virulent than VISA, CA-MRSA, and MSSA. Hemocyte density experiment demonstrated that the CA-MRSA provoked a more pronounced immune response.

7.2 Recommendations

This study will be helpful to provide a good strategy to consider the large study area (adding more cities from Pakistan) for the screening of MDR-MRSA and MDR-MSSA. *mec*(A) negative MRSA must be screened for *mec*(C) gene in clinical isolates from hospitals of Pakistan. A comparative study can be carried out to consider their geographical location, which is municipally and culturally different from one another. Such types of studies will contribute us a comprehensive understanding of antibiotic resistance patterns and will support in planning a good antibiotic prescription strategy.

As the increase in antibiotic resistance is emerging, there should be a national antimicrobial surveillance policy to fight against ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens especially in the case of MRSA. All hospitals, clinics and microbiology labs should take part in the implementations of such policies and they should share their findings with healthcare officials and policy makers. This will facilitate the clinicians and medical practitioners to prescribe the proper drugs for treatment.

For incoming patients, the hospital environment must be made healthy and clean. Cleanliness of hospital wards and the environment with proper disinfection should be mandatory. The fomites like bed frame, floor surfaces, bedsides, toilets, tables, seats, chair, door handles, etc. should be appropriately cleaned with disinfecting techniques to discourage the growth of infectious agents so the patients could leave the hospital healthy, without conveying of any nosocomial contaminations. Antibiotic usage should be observed routinely, followed infection control and prevention policies. Easy availability and purchase of antibiotics in pharmacies without a doctor prescription carefully controlled. Uncontrolled use of antibiotics needs to be restricted. Health workers should make the public aware of the risk factors of antimicrobial agents, their side effects and knowledge about the increase in antibiotic resistance.

The clinician must recommend antibiotic susceptibility profiles against different antibiotics and then prescribe antibiotics for infection-causing bacteria. Despite the use of the last choice of antibiotics, we should use antibiotics in combination, which show synergistic effect, and this combination therapy may overcome the problem of antibiotic resistance and be helpful in complicated infections.

The hospital administration should plan strategies to ensure that proper screening of resistant pathogens in hospital laboratories becomes a constant practice and the data thus generated should be coordinated at national level to device strategies regarding the therapy and management protocols of such pathogens.

7.3 Future prospects

It might be of great value to identify the resistance mechanisms of *S. aureus* against linezolid, vancomycin and other last-resort antibiotics in the local hospital settings. The presence of *mec*(C) in our study warrants that a wider screening be carried out of all MRSA for the occurrence of *mec*(A), *mec*(C) and *mec*(A)+*mec*(C) genes in hospital laboratories to find out the presence and prevalence of *mec*(C) MRSA in other hospital setups. It would be good to screen the nursing staff for the carriage of MDR-MRSA and study their microbial flora for the presence of MRSA, as it will help provide data of particular resistant strain circulating in the community. Further investigations are required for screening of other SCC*mec* types such as VII-XII in local isolates in this region. Further screening of *spa* types is required in our local circulating clinical isolates. Insufficient data is available on the molecular typing and epidemiology of MRSA in clinical isolates and we suggest detailed molecular epidemiological investigation in local hospital settings. A comprehensive study which shows the link of antibiotic resistance with virulence marker in hospital and community-associated samples from the same geographical region will be of interest, to know about the particular MDR strain circulation both in community and hospital set up and also the emergence of community-associated bacteria in the hospital setting, and their way of transmission from community to hospital and vice versa.

7.4 Advances made in this study

In the current study, we identified first clinical MRSA carrying *mec*(C) gene alone and in combination with *mec*(A). The study reports SCC*mec* type VI which has not been reported previously from Pakistan. PVL gene was found associated not only with SCC*mec* types IV and VI but also with SCC*mec* type II, and III. Many of the *spa* types were reported first time from Pakistani isolates. In MSSA isolates 1 NEW *spa* type was identified. ST1535 was first time reported from our local strains. Our local clinical CA-MRSA strain was found to be more virulent as compared to selected HA-MRSA, MSSA, VISA, and *agr* mutant RN4220.

Chapter 8 References

Abbas, A., Nirwan, P., & Srivastava, P. J. C. A. I. (2015). Prevalence and antibiogram of hospital acquired-methicillin resistant Staphylococcus aureus and community acquired-methicillin resistant Staphylococcus aureus at a tertiary care hospital National Institute of Medical Sciences. *2*(1), 13.

Abdollahi, A., Koohpayeh, S., Najafipoor, S., Mansoori, Y., Abdollahi, S., & Jaafari, S. (2012). Evaluation of drug Resistance and Staphylococcal cassette chromosome (SCCmec) types among methicillin-Resistant Staphylococcus aureus (MRSA). *Alborz University Medical Journal, 1*(1), 47-52.

Abdulrahman, M. A., & Taher, A. I. (2018). Prevalence of Methicillin Resistant Staphylococcus Aureus Among Food Handlers in Duhok City. *Science Journal of University of Zakho, 6*(4), 140-145.

Aboshkiwa, M., Rowland, G., & Coleman, G. (1995). Nucleotide sequence of the Staphylococcus aureus RNA polymerase rpoB gene and comparison of its predicted amino acid sequence with those of other bacteria. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression, 1262*(1), 73-78.

Abraham, L., & Bamberger, D. M. (2020). Staphylococcus aureus Bacteremia: contemporary management. *Missouri Medicine, 117*(4), 341.

Adedeji, A., Weller, T., & Gray, J. (2007). MRSA in children presenting to hospitals in Birmingham, UK. *Journal of Hospital Infection, 65*(1), 29-34.

Adwan, K., Jarrar, N., Abu-Hijleh, A., Adwan, G., Awwad, E., & Salameh, Y. (2013). Molecular analysis and susceptibility patterns of methicillin-resistant Staphylococcus aureus strains causing community-and health care-associated infections in the northern region of Palestine. *American journal of infection control, 41*(3), 195-198.

Afroz, S., Kobayashi, N., Nagashima, S., Alam, M. M., Hossain, A., Rahman, M. A., Khan, M. (2008). Genetic characterization of Staphylococcus aureus isolates carrying Panton-Valentine leukocidin genes in Bangladesh. *Japanese Journal of infectious disease, 61*(5), 393-396.

Aghazadeh, M., Rahbar, M., Monnavar, M. K., & Moghadam, F. S. (2009). Sensitivity pattern of methicillin resistant and methicillin sensitive Staphylococcus aureus isolates, against several antibiotics including tigecycline in Iran: A hospital based study. *Pakistan journal of medical sciences, 25*(3), 443-446.

Ahmad, B., Khan, F., Ahmed, J., Cha, S. B., Shin, M., Bashir, S., & Yoo, H. S. (2014). Antibiotic resistance pattern and molecular epidemiology of methicillin-resistant Staphylococcus aureus colonization in burns unit of a tertiary care hospital in Peshawar, Pakistan. *Tropical Journal of Pharmaceutical Research, 13*(12), 2091-2099.

Akhtar, N. (2010). Staphylococcal nasal carriage of health care workers. *Journal of the College of Physicians and Surgeons Pakistan, 20*(7), 439-443.

Akhter, R., Khan, K. M. A., & Hasan, F. (2009). Isolation and antimicrobial susceptibility pattern of methicillin-resistant and methicillin sensitive Staphylococcus aureus. *Journal of Surgery Pakistan, 14*(4), 161-164.

Akpaka, P. E., Kissoon, S., Swanston, W. H., & Monteil, M. (2006). Prevalence and antimicrobial susceptibility pattern of methicillin resistant Staphylococcus

aureus isolates from Trinidad & Tobago. *Annals of Clinical Microbiology and Antimicrobials, 5*(1), 16.

Aktas, Z., Aridogan, A., Kayacan, C. B., & Aydin, D. (2007). Resistance to macrolide, lincosamide and streptogramin antibiotics in staphylococci isolated in Istanbul, Turkey. *The Journal of Microbiology, 45*(4), 286-290.

Aktas, Z., Aridogan, A., Kayacan, C. B., & Aydin, D. J. (2007). Resistance to macrolide, lincosamide and streptogramin antibiotics in staphylococci isolated in Istanbul, Turkey. *The Journal of Microbiology, 45*(4), 286-290.

Al-Ajealy, B. A., Al-Shukri, M. S., & Al-Jumaily, H. S. (2017). Detection of newly defined superantigenic toxin genes and coagulase gene polymorphism in Staphylococcus aureus isolates. *Reviews in Medical Microbiology, 28*(4), 158.

Al-Khulaifi, M. M., Amin, A. N. M., & Al Salamah, A. A. (2009). Phage typing, PCR amplification for mecA gene, and antibiotic resistance patterns as epidemiologic markers in nosocomial outbreaks of methicillin resistant Staphylococcus aureus. *Saudi journal of biological sciences, 16*(1), 37.

Al-Talib, H., Yean, C. Y., Al-Khateeb, A., Hassan, H., Singh, K.-K. B., Al-Jashamy, K., & Ravichandran, M. (2009). A pentaplex PCR assay for the rapid detection of methicillin-resistant Staphylococcus aureus and Panton-Valentine Leucocidin. *BMC microbiology, 9*(1), 113.

Al-Tarazi, Y. H., Albetar, M. A., & Alaboudi, A. R. (2009). Biotyping and enterotoxigenicity of Staphylococci isolated from fresh and frozen meat marketed in Jordan. *Food research international, 42*(3), 374-379.

Al-Zoubi, M. S., Al-Tayyar, I. A., Hussein, E., Al Jabali, A., & Khudairat, S. (2015). Antimicrobial susceptibility pattern of Staphylococcus aureus isolated from clinical specimens in Northern area of Jordan. *Iranian journal of microbiology, 7*(5), 265.

Albarrag, A., Shami, A., Almutairi, A., Alsudairi, S., Aldakeel, S., & Al-Amodi, A. (2020). Prevalence and Molecular Genetics of Methicillin-Resistant Staphylococcus aureus Colonization in Nursing Homes in Saudi Arabia. *Canadian Journal of Infectious Diseases and Medical Microbiology, 2020*.

Alfatemi, S. M. H., Motamedifar, M., Hadi, N., & Saraie, H. S. E. (2014). Analysis of virulence genes among methicillin resistant Staphylococcus aureus (MRSA) strains. *Jundishapur Journal of Microbiology, 7*(6).

Alfouzan, W., Al Fadhli, M., Abdo, N., Alali, W., & Dhar, R. (2019). Surgical site infection following cesarean section in a general hospital in Kuwait: trends and risk factors. *Epidemiology & Infection, 147*.

Allignet, J., Aubert, S., Morvan, A., & el Solh, N. (1996). Distribution of genes encoding resistance to streptogramin A and related compounds among staphylococci resistant to these antibiotics. *Antimicrobial agents chemotherapy, 40*(11), 2523-2528.

Allignet, J., Liassine, N., & El Solh, N. (1998). Characterization of a Staphylococcal Plasmid Related to pUB110 and Carrying Two Novel Genes, vatC andvgbB, Encoding Resistance to Streptogramins A and B and Similar Antibiotics. *Antimicrobial agents and chemotherapy, 42*(7), 1794-1798.

Allington, D. R., & Rivey, M. P. (2001). Quinupristin/dalfopristin: a therapeutic review. *Clinical therapeutics, 23*(1), 24-44.

Alonzo III, F., Benson, M. A., Chen, J., Novick, R. P., Shopsin, B., & Torres, V. J. (2012). Staphylococcus aureus leucocidin ED contributes to systemic infection by targeting neutrophils and promoting bacterial growth in vivo. *Molecular microbiology, 83*(2), 423-435.

Anderson, S. D., & Gums, J. G. (2008). Ceftobiprole: an extended-spectrum anti–methicillin-resistant Staphylococcus aureus cephalosporin. *Annals of Pharmacotherapy, 42*(6), 806-816.

Andersson, H. (2012). *MRSA and other resistant bacteria: prevalence, patient and staff experiences, wounds and infection control*: Inst för kliniska vetenskaper, Danderyds sjukhus/Dept of Clinical Sciences.

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *Journal of antimicrobial chemotherapy, 48*(1), 5-16.

Anupurba, S., Sen, M., Nath, G., Sharma, B., Gulati, A., & Mohapatra, T. (2003). Prevalence of methicillin resistant Staphylococcus aureus in a tertiary referral hospital in eastern Uttar Pradesh. *Indian journal of medical microbiology, 21*(1), 49.

Aqel, A. A., Alzoubi, H. M., Vickers, A., Pichon, B., & Kearns, A. M. (2015). Molecular epidemiology of nasal isolates of methicillin-resistant Staphylococcus aureus from Jordan. *Journal of infection and public health, 8*(1), 90-97.

Ardic, N., Ozyurt, M., Sareyyupoglu, B., & Haznedaroglu, T. (2005). Investigation of erythromycin and tetracycline resistance genes in methicillin-resistant staphylococci. *International journal of antimicrobial agents, 26*(3), 213-218.

Ardic, N., Sareyyupoglu, B., Ozyurt, M., Haznedaroglu, T., & Ilga, U. (2006). Investigation of aminoglycoside modifying enzyme genes in methicillin-resistant staphylococci. *Microbiological research, 161*(1), 49-54.

Arfat, Y. (2013). *Genotyping of methicillin resistant Staphylococcus aureus (MRSA) from local hospital of Rawalpindi/Islamabad, Pakistan.* Quaid-i-Azam University Islamabad, Pakistan,

Arfat, Y., Johnson, M., Malik, S., Morrissey, J., & Bayliss, C. (2013). Epidemiology of methicillin resistant Staphylococcus aureus (MRSA) isolates from Pakistan. *African Journal of Microbiology Research, 7*(7), 568-576.

Argudín, M. Á., Mendoza, M. C., & Rodicio, M. R. (2010). Food poisoning and Staphylococcus aureus enterotoxins. *Toxins, 2*(7), 1751-1773.

Arshad, F., Javed, I., Mushtaq, S., & Anwer, S. (2015). Emergence of Vancomycin Intermediate Staphylococcus aureus (VISA) in a Tertiary Care Hospital in Lahore: An Alarming Situation. *International Journal of Pathology, 13*(3), 89-94.

Arshad, M. U., Akhtar, R. W., & Irum, S. (2015). Prevalence of MRSA containing SCCmec types isolated from a tertiary care hospital, Rawalpindi. *Journal of infection and Molecular biology, 3*(4), 81-85.

Arvidson, S., & Tegmark, K. (2001). Regulation of virulence determinants in Staphylococcus aureus. *International journal of medical microbiology, 291*(2), 159-170.

Asadollahi, P., Farahani, N. N., Mirzaii, M., Khoramrooz, S. S., Van Belkum, A., Asadollahi, K., Darban-Sarokhalil, D. (2018). Distribution of the most prevalent spa types among clinical isolates of methicillin-resistant and-susceptible Staphylococcus aureus around the world: a review. *Frontiers in microbiology, 9*, 163.

Asadollahi, P., Farahani, N. N., Mirzaii, M., Khoramrooz, S. S., Van Belkum, A., Asadollahi, K., Darban-Sarokhalil, D. (2018). Distribution of the most prevalent spa types among clinical isolates of methicillin-resistant and-susceptible Staphylococcus aureus around the world: a review. *Frontiers in microbiology, 9*, 163.

Asghar, A. H. (2014). Molecular characterization of methicillin-resistant Staphylococcus aureus isolated from tertiary care hospitals. *Pakistan journal of medical sciences, 30*(4), 698.

Ashiq, B., & Tareen, A. K. (1989). Methicillin resistant Staphylococcus aureus in a teaching hospital of Karachi-a laboratory study. *Journal of Pakistan Medical Association, 39*, 6-9.

Askarian, F., Van Sorge, N. M., Sangvik, M., Beasley, F. C., Henriksen, J. R., Sollid, J. U., Johannessen, M. (2014). A Staphylococcus aureus TIR domain protein virulence factor blocks TLR2-mediated NF-κB signaling. *Journal of innate immunity, 6*(4), 485-498.

Aslam, B., Wang, W., Arshad, M. I., Khurshid, M., Muzammil, S., Rasool, M. H., Qamar, M. U. (2018). Antibiotic resistance: a rundown of a global crisis. *Infection drug Resistance Updates, 11*, 1645.

Atshan, S. S., Shamsudin, M. N., Karunanidhi, A., van Belkum, A., Lung, L. T. T., Sekawi, Z., Ali, A. M. (2013). Quantitative PCR analysis of genes expressed during biofilm development of methicillin resistant Staphylococcus aureus (MRSA). *Infection, genetics evolution, 18*, 106-112.

Aubry-Damon, H., Soussy, C.-J., & Courvalin, P. (1998). Characterization of Mutations in therpoB Gene That Confer Rifampin Resistance inStaphylococcus aureus. *Antimicrobial agents and chemotherapy, 42*(10), 2590-2594.

Ayed, S. B., Boubaker, I. B.-B., Ennigrou, S., & Redjeb, S. B. (2008). Accessory gene regulator (agr) typing of Staphylococcus aureus isolated from human infections. *Archives de l'Institut Pasteur de Tunis, 85*(1-4), 3.

Ayed, S. B., Boubaker, I. B.-B., Samir, E., & Redjeb, S. B. (2006). Prevalence of agr specificity groups among methicilin resistant Staphylococcus aureus circulating at Charles Nicolle hospital of Tunis. *Pathologie Biologie, 54*(8-9), 435-438.

Azhar, A., Rasool, S., Haque, A., Shan, S., Saeed, M., Ehsan, B., & Haque, A. (2017). Detection of high levels of resistance to linezolid and vancomycin in Staphylococcus aureus. *Journal of medical microbiology, 66*(9), 1328-1331.

Aziz, H. W., Al-Dulaimi, T. H., Al-Marzoqi, A. H., & Ahmed, N. K. (2014). Phenotypic detection of resistance in Staphylococcus aureus isolates: Detection of (mecA and femA) gene in methicillin resistant Staphylococcus aureus (MRSA) by Polymerase Chain Reaction. *Journal of Natural Sciences Research, 4*(1), 112-118.

Ba, X., Harrison, E. M., Edwards, G. F., Holden, M. T., Larsen, A. R., Petersen, A., Paterson, G. K. (2013). Novel mutations in penicillin-binding protein genes in clinical Staphylococcus aureus isolates that are methicillin resistant on susceptibility testing, but lack the mec gene. *Journal of antimicrobial chemotherapy, 69*(3), 594-597.

Baddour, M. M., Abuelkheir, M. M., & Fatani, A. J. (2006). Trends in antibiotic susceptibility patterns and epidemiology of MRSA isolates from several hospitals in Riyadh, Saudi Arabia. *Annals of Clinical Microbiology and Antimicrobials, 5*(1), 30.

Baggett, H. C., Hennessy, T. W., Leman, R., Hamlin, C., Bruden, D., Reasonover, A., Butler, J. C. (2003). An outbreak of community-onset methicillin-resistant Staphylococcus aureus skin infections in southwestern Alaska. *Infection Control & Hospital Epidemiology, 24*(6), 397-402.

Bahrami, N., Motamedi, H., Tofighi, S. E. R., & Akhoond, M. R. (2019). SCCmec typing and Panton-valentine leukocidin occurrence in methicillin resistant Staphylococcus aureus (MRSA) isolates from clinical samples of Ahvaz, southwest of Iran. *Microbiologia Medica, 34*(2).

Bannerman, T. L., Hancock, G. A., Tenover, F. C., & Miller, J. M. (1995). Pulsed-field gel electrophoresis as a replacement for bacteriophage typing of Staphylococcus aureus. *Journal of clinical microbiology, 33*(3), 551-555.

Bano, S., Tunio, S., Mal, S., & Jatt, A. (2015). Frequency of Methicillin Resistant Staphylococcus aureus Among Isolates of Wound Infections from Hyderabad. *Sindh University Research Journal-SURJ (Science Series), 44*(4).

Baragundi, M. C., Kulkarni, R. B., & Sataraddi, J. V. (2013). The prevalence of inducible and constitutive clindamycin resistance among the nasal isolates of staphylococci. *Journal of clinical and diagnostic research: JCDR, 7*(8), 1620.

Barcia-Macay, M. (2006). lemaire S, Mingeot-leclercq MP, tulkens PM, Van Bambeke F. evaluation of the extracellular and intracellular activities (human tHP-1 macrophages) of telavancin versus vancomycin against methicillin-susceptible, methicillin-resistant, vancomycin-intermediate and vancomycin-resistant Staphylococcus aureus. *Antimicrobial chemotherapy*, 58-56.

Barrett, F. F., McGehee Jr, R. F., & Finland, M. (1968). Methicillin-resistant Staphylococcus aureus at Boston City Hospital: bacteriologic and epidemiologic observations. *New England journal of medicine, 279*(9), 441-448.

Basset, P., Prod'hom, G., Senn, L., Greub, G., & Blanc, D. (2013). Very low prevalence of meticillin-resistant Staphylococcus aureus carrying the mecC gene in western Switzerland. *Journal of Hospital Infection, 83*(3), 257-259.

Bauer, A., Kirby, W., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American journal of clinical pathology, 45*(4), 493.

Bauer, A., Kirby, W., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American journal of clinical pathology, 45*(4_ts), 493-496.

Bdour, S. (2012). Heterogeneity of aminoglycoside resistance genes profile in clinical Staphylococcus aureus isolates. *African Journal of Microbiology Research, 6*(24), 5259-5265.

Beenken, K. E., Blevins, J. S., & Smeltzer, M. S. (2003). Mutation of sarA in Staphylococcus aureus limits biofilm formation. *Infection and immunity, 71*(7), 4206-4211.

Berger-Bächi, B., & Rohrer, S. (2002). Factors influencing methicillin resistance in staphylococci. *Archives of microbiology, 178*(3), 165-171.

Bergin, D., Brennan, M., & Kavanagh, K. (2003). Fluctuations in haemocyte density and microbial load may be used as indicators of fungal pathogenicity in larvae of Galleria mellonella. *Microbes and Infection, 5*(15), 1389-1395.

Berglund, C., Ito, T., Ikeda, M., Ma, X. X., Söderquist, B., & Hiramatsu, K. (2008). Novel type of staphylococcal cassette chromosome mec in a methicillin-resistant Staphylococcus aureus strain isolated in Sweden. *Antimicrobial agents and chemotherapy, 52*(10), 3512-3516.

Bhatta, D. R., Cavaco, L. M., Nath, G., Gaur, A., Gokhale, S., & Bhatta, D. R. (2015). Threat of multidrug resistant Staphylococcus aureus in Western Nepal. *Asian Pacific Journal of Tropical Disease, 5*(8), 617-621.

Bhatta, D. R., Cavaco, L. M., Nath, G., Kumar, K., Gaur, A., Gokhale, S., & Bhatta, D. R. (2016). Association of Panton Valentine Leukocidin (PVL) genes with methicillin resistant Staphylococcus aureus (MRSA) in Western Nepal: a matter of concern for community infections (a hospital based prospective study). *BMC Infectious diseases, 16*(1), 199.

Bhatty, M., Cruz, M. R., Frank, K. L., Laverde Gomez, J. A., Andrade, F., Garsin, D. A., Christie, P. J. (2015). E nterococcus faecalis pCF 10-encoded surface proteins PrgA, PrgB (aggregation substance) and PrgC contribute to plasmid transfer, biofilm formation and virulence. *Molecular microbiology, 95*(4), 660-677.

Bhutia, K. O., & Singh, T. (2012). The prevalence and risk factors which are associated with Staphylococcus aureus and methicillin resistant S. aureus which harboured the Panton Valentine Leukocidin gene in Sikkim. *Clinical Diagnostic Research, 6*, 393-399.

Bhutia, K. O., Singh, T., Adhikari, L., & Biswas, S. (2015). Molecular characterization of community-& hospital-acquired methicillin-resistant & methicillin-sensitive Staphylococcus aureus isolates in Sikkim. *The Indian journal of medical research, 142*(3), 330.

Bhutia, K. O., Singh, T., Adhikari, L., & Biswas, S. (2015). Molecular characterization of community-& hospital-acquired methicillin-resistant & methicillin-sensitive Staphylococcus aureus isolates in Sikkim. *Indian Journal of Medical Research, 142*(3), 330.

Bischoff, M., Brelle, S., Minatelli, S., & Molle, V. (2016). Stk1-mediated phosphorylation stimulates the DNA-binding properties of the Staphylococcus aureus SpoVG transcriptional factor. *Biochemical biophysical research communications, 473*(4), 1223-1228.

Blomfeldt, A., Eskesen, A., Aamot, H., Leegaard, T., & Bjørnholt, J. (2016). Population-based epidemiology of Staphylococcus aureus bloodstream infection: clonal complex 30 genotype is associated with mortality.

European journal of clinical microbiology & infectious diseases, 35(5), 803-813.

Bodley, J. W., Zieve, F. J., Lin, L., & Zieve, S. T. (1969). Formation of the ribosome-G factor-GDP complex in the presence of fusidic acid. *Biochemical and biophysical research communications, 37*(3), 437-443.

Bojang, E., Jafali, J., Perreten, V., Hart, J., Harding-Esch, E. M., Sillah, A., Roca, A. (2017). Short-term increase in prevalence of nasopharyngeal carriage of macrolide-resistant Staphylococcus aureus following mass drug administration with azithromycin for trachoma control. *BMC microbiology, 17*(1), 75.

Boles, B. R., & Horswill, A. R. (2011). Staphylococcal biofilm disassembly. *Trends in microbiology, 19*(9), 449-455.

Boman, H. G., & Hultmark, D. (1987). Cell-free immunity in insects. *Annual Reviews in Microbiology, 41*(1), 103-126.

Bondi Jr, A., & Dietz, C. C. (1945). Penicillin resistant staphylococci. *Proceedings of the Society for Experimental Biology and Medicine, 60*(1), 55-58.

Booth, M. C., Pence, L. M., Mahasreshti, P., Callegan, M. C., & Gilmore, M. S. (2001). Clonal associations among Staphylococcus aureus isolates from various sites of infection. *Infection and immunity, 69*(1), 345-352.

Boswihi, S. S., Udo, E. E., & Al-Sweih, N. (2016). Shifts in the clonal distribution of methicillin-resistant Staphylococcus aureus in Kuwait hospitals: 1992-2010. *PloS one, 11*(9).

Boswihi, S. S., Udo, E. E., & Al-Sweih, N. (2016). Shifts in the clonal distribution of methicillin-resistant Staphylococcus aureus in Kuwait hospitals: 1992-2010. *PloS one, 11*(9), e0162744.

Boyle-Vavra, S., & Daum, R. S. (2007). Community-acquired methicillin-resistant Staphylococcus aureus: the role of Panton–Valentine leukocidin. *Laboratory investigation, 87*(1), 3.

Boyle-Vavra, S., Ereshefsky, B., Wang, C.-C., & Daum, R. S. (2005). Successful multiresistant community-associated methicillin-resistant Staphylococcus aureus lineage from Taipei, Taiwan, that carries either the novel staphylococcal chromosome cassette mec (SCCmec) type VT or SCCmec type IV. *Journal of clinical microbiology, 43*(9), 4719-4730.

Bozdogan, B., Ednie, L., Credito, K., Kosowska, K., & Appelbaum, P. C. (2004). Derivatives of a vancomycin-resistant Staphylococcus aureus strain isolated at Hershey Medical Center. *Antimicrobial agents and chemotherapy, 48*(12), 4762-4765.

Brakstad, O. G., Aasbakk, K., & Maeland, J. A. (1992). Detection of Staphylococcus aureus by polymerase chain reaction amplification of the nuc gene. *Journal of clinical microbiology, 30*(7), 1654-1660.

Brauner, J., Hallin, M., Deplano, A., De Mendonça, R., Nonhoff, C., De Ryck, R., . . . Denis, O. (2013). Community-acquired methicillin-resistant Staphylococcus aureus clones circulating in Belgium from 2005 to 2009: changing epidemiology. *European journal of clinical microbiology & infectious diseases, 32*(5), 613-620.

Brennan, G. I., Abbott, Y., Burns, A., Leonard, F., McManus, B. A., O'Connell, B., . . . Shore, A. C. (2016). The emergence and spread of multiple livestock-

associated clonal complex 398 methicillin-resistant and methicillin-susceptible Staphylococcus aureus strains among animals and humans in the Republic of Ireland, 2010–2014. *PloS one, 11*(2), e0149396.

Brohi, N. A., & Noor, A. A. (2017). Frequency of the Occurrence of Methicilin Resistant Staphylococcus aureus (MRSA) Infections in Hyderabad, Pakistan. *Pakistan Journal of Analytical Environmental Chemistry, 18*(1), 84-90.

Brown, S. E., Howard, A., Kasprzak, A. B., Gordon, K. H., & East, P. D. (2008). The discovery and analysis of a diverged family of novel antifungal moricin-like peptides in the wax moth Galleria mellonella. *Insect biochemistry and molecular biology, 38*(2), 201-212.

Brown, S. E., Howard, A., Kasprzak, A. B., Gordon, K. H., & East, P. D. (2009). A peptidomics study reveals the impressive antimicrobial peptide arsenal of the wax moth Galleria mellonella. *Insect biochemistry and molecular biology, 39*(11), 792-800.

Browne, N., Heelan, M., & Kavanagh, K. (2013). An analysis of the structural and functional similarities of insect hemocytes and mammalian phagocytes. *Virulence, 4*(7), 597-603.

Bryskier, A. (2005). Agents against methicillin-resistant Staphylococcus aureus. In *Antimicrobial Agents* (pp. 1183-1238): American Society of Microbiology.

Bukhari, M., Iqbal, N., Naeem, S., Qureshi, G., Naveed, I., Iqbal, A., & Khatoon, N. (2004). A laboratory study of susceptibility of methicillin resistant Staphylococcus aureus (MRSA). *Pakistan journal of medical sciences, 20*(3), 229-233.

Bukhari, S. Z., Ahmed, S., & Zia, N. (2011). Antimicrobial susceptibility pattern of Staphylococcus aureus on clinical isolates and efficacy of laboratory tests to diagnose MRSA: a multi-centre study. *J Ayub Med Coll Abbottabad, 23*(1), 139-142.

Bukhari, S. Z., Ahmed, S., & Zia, N. (2011). Antimicrobial susceptibility pattern of Staphylococcus aureus on clinical isolates and efficacy of laboratory tests to diagnose MRSA: a multi-centre study. *Journal of Ayub Medical College Abbottabad, 23*(1), 139-142.

Bukhari, S. Z. H. (2006a). *Trends in Antimicrobial Resistance and Occurrence of Methicillin-resistant Staphylococcus aureus (MRSA): Molecular basis and Clinical implications.* Quaid-i-Azam University, Islamabad,

Bukhari, S. Z. H. (2006b). *Trends in antimicrobial resistance and occurrence of methicillin resistant Staphylococcus aureus (MRSA): molecular basis and clinical implications.* Quaid-i-Azam University Islamabad, Pakistan,

Bukharie, H. A. (2010). A review of community-acquired methicillin-resistant Staphylococcus aureus for primary care physicians. *Journal of family and community medicine, 17*(3), 117.

Caiazza, N. C., & O'toole, G. (2003). Alpha-toxin is required for biofilm formation by Staphylococcus aureus. *Journal of bacteriology, 185*(10), 3214-3217.

CDC. (2017). Centers for Disease Control and Prevention. Centers for Disease Control and Prevention (CDC), from Centers for Disease Control and Prevention. CDC Wonder. http://wonder.cdc.gov/

Cetin, E. S., Gunes, H., Kaya, S., Aridogan, B. C., & Demirci, M. (2008). Macrolide-lincosamide-streptogramin B resistance phenotypes in clinical

staphylococcal isolates. *Internation Journal of Antimicrobial agents, 31*(4), 364-368. doi:10.1016/j.ijantimicag.2007.11.014

Chambers, H. (1988). Methicillin-resistant staphylococci. *Clinical Microbiology Reviews, 1*(2), 173-186.

Chambers, H. F., & DeLeo, F. R. (2009). Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nature Reviews Microbiology, 7*(9), 629-641.

Chamon, R. C., Ribeiro, S. d. S., Costa, T. M. d., Nouér, S. A., & Santos, K. R. N. d. (2017). Complete substitution of the Brazilian endemic clone by other methicillin-resistant Staphylococcus aureus lineages in two public hospitals in Rio de Janeiro, Brazil. *Brazilian Journal of Infectious Diseases, 21*(2), 185-189.

Champion, O. L., Cooper, I. A., James, S. L., Ford, D., Karlyshev, A., Wren, B. W., Titball, R. W. (2009). Galleria mellonella as an alternative infection model for Yersinia pseudotuberculosis. *Microbiology, 155*(5), 1516-1522.

Chavadi, M., Narasanna, R., Chavan, A., & Oli, A. K. (2018). Prevalence of Methicillin Resistant and Virulence Determinants in Clinical Isolates of Staphylococcus aureus. *The Open Infectious Diseases Journal, 10*(1).

Chen, C., Xu, X., Qu, T., Yu, Y., Ying, C., Liu, Q., Li, G. (2014). Prevalence of the fosfomycin-resistance determinant, fosB3, in Enterococcus faecium clinical isolates from China. *Journal of medical microbiology, 63*(11), 1484-1489.

Ching, C., Nizamuddin, S., Rasheed, F., Seager, R., Litvak, F., Sultan, F., & Zaman, M. H. (2019). Antimicrobial resistance trends from a hospital and diagnostic facility in Lahore, Pakistan: A five-year retrospective analysis (2014-2018). *medRxiv*, 19012617.

Choi, J., Whitten, M., Cho, M., Lee, K., Kim, M., Ratcliffe, N., & Lee, B. (2002). Calreticulin enriched as an early-stage encapsulation protein in wax moth Galleria mellonella larvae. *Developmental & Comparative Immunology, 26*(4), 335-343.

Choi, S. M., Kim, S.-H., Kim, H.-J., Lee, D.-G., Choi, J.-H., Yoo, J.-H., Kang, M.-W. (2003). Multiplex PCR for the detection of genes encoding aminoglycoside modifying enzymes and methicillin resistance among Staphylococcus species. *Journal of Korean medical science, 18*(5), 631.

Chua, K. Y., Howden, B. P., Jiang, J.-H., Stinear, T., & Peleg, A. Y. (2014). Population genetics and the evolution of virulence in Staphylococcus aureus. *Infection, Genetics and Evolution, 21*, 554-562.

Cicconi-Hogan, K., Belomestnykh, N., Gamroth, M., Ruegg, P., Tikofsky, L., & Schukken, Y. (2014). Prevalence of methicillin resistance in coagulase-negative staphylococci and Staphylococcus aureus isolated from bulk milk on organic and conventional dairy farms in the United States. *Journal of Dairy Science, 97*(5), 2959-2964.

Cohen, A. L., Calfee, D., Fridkin, S. K., Huang, S. S., Jernigan, J. A., Lautenbach, E., Weinstein, R. A. (2008). Recommendations for metrics for multidrug-resistant organisms in healthcare settings: SHEA/HICPAC position paper. *Infection Control Hospital Epidemiology, 29*(10), 901-913.

Cohen, M. L. (1992). Epidemiology of drug resistance: implications for a post—antimicrobial era. *Science, 257*(5073), 1050-1055.

Coia, J., Duckworth, G., Edwards, D., Farrington, M., Fry, C., Humphreys, H., Tucker, D. (2006). Hospital Infection Society; Infection Control Nurses Association. Guidelines for the control and prevention of meticillin-resistant Staphylococcus aureus (MRSA) in healthcare facilities. *Journal of Hospital Infection, 63*(Suppl 1), S1-44.

Commission, J. (1958). Opinion 17. Conservation of the Generic name Staphylococcus Rosenbach, Designation of Staphylococcus aure us Rosenbach as the Nomenclatural Type of the Genus Staphylococcus aure us Rosenbach, and Designation of the Neotype culture of Staphylococcus aure us Rosenbach. *International Bulletin of Bacteriological Nomenclature and Taxonomy, 8*(3-4), 153-154.

Conceicao, T., Diamantino, F., Coelho, C., de Lencastre, H., & Aires-de-Sousa, M. (2013). Contamination of public buses with MRSA in Lisbon, Portugal: a possible transmission route of major MRSA clones within the community. *PloS one, 8*(11).

Cookson, B., Aparicio, P., Deplano, A., Struelens, M., Goering, R., & Marples, R. (1996). Inter-centre comparison of pulsed-field gel electrophoresis for the typing of methicillin-resistant Staphylococcus aureus. *Journal of medical microbiology, 44*(3), 179-184.

Coombs, G. W., Monecke, S., Ehricht, R., Slickers, P., Pearson, J. C., Tan, H.-L., . . . O'Brien, F. G. (2010). Differentiation of clonal complex 59 community-associated methicillin-resistant Staphylococcus aureus in Western Australia. *Antimicrobial agents chemotherapy, 54*(5), 1914-1921.

Cooper, J. E., & Feil, E. J. (2004). Multilocus sequence typing–what is resolved? *Trends in microbiology, 12*(8), 373-377.

Cotter, G., Doyle, S., & Kavanagh, K. (2000). Development of an insect model for the in vivo pathogenicity testing of yeasts. *FEMS Immunology & Medical Microbiology, 27*(2), 163-169.

Coughlan, A., Scanlon, K., Mahon, B. P., & Towler, M. (2010). Zinc and silver glass polyalkenoate cements: An evaluation of their antibacterial nature. *Bio-medical materials engineering, 20*(2), 99-106.

Courvalin, P. (2006). Vancomycin resistance in gram-positive cocci. *Clinical Infectious Diseases, 42*(Supplement_1), S25-S34.

Crisóstomo, M. I., Westh, H., Tomasz, A., Chung, M., Oliveira, D. C., & de Lencastre, H. (2001). The evolution of methicillin resistance in Staphylococcus aureus: similarity of genetic backgrounds in historically early methicillin-susceptible and-resistant isolates and contemporary epidemic clones. *Proceedings of the National Academy of Sciences, 98*(17), 9865-9870.

Critchley, I. A., Draghi, D. C., Sahm, D. F., Thornsberry, C., Jones, M. E., & Karlowsky, J. A. (2003). Activity of daptomycin against susceptible and multidrug-resistant Gram-positive pathogens collected in the SECURE study (Europe) during 2000–2001. *Journal of antimicrobial chemotherapy, 51*(3), 639-649.

Crossley, K., Jefferson, K., Archer, G., & Fowler, V. (2009). Staphylococci in human disease. *Staphylococci in human disease.*(Ed. 2).

Culebras, E., & Martínez, J. L. (1999). Aminoglycoside resistance mediated by the bifunctional enzyme 6′-N-aminoglycoside acetyltransferase-2 ″-O-aminoglycoside phosphotransferase. *Frontiers in Biosciences, 4*, D1-D8.

Cuny, C., Layer, F., Strommenger, B., & Witte, W. (2011). Rare occurrence of methicillin-resistant Staphylococcus aureus CC130 with a novel mecA homologue in humans in Germany. *PloS one, 6*(9), e24360.

Cuny, C., Layer, F., Strommenger, B., & Witte, W. (2011). Rare occurrence of methicillin-resistant Staphylococcus aureus CC130 with a novel mecA homologue in humans in Germany. *PloS one, 6*(9).

D'Souza, N., Rodrigues, C., & Mehta, A. (2010). Molecular characterization of methicillin-resistant Staphylococcus aureus with emergence of epidemic clones of sequence type (ST) 22 and ST 772 in Mumbai, India. *Journal of clinical microbiology, 48*(5), 1806-1811.

Dahl, K. H., & Sundsfjord, A. (2003). Transferable vanB2 Tn 5382-containing elements in fecal streptococcal strains from veal calves. *Antimicrobial agents and chemotherapy, 47*(8), 2579-2583.

Dantes, R., Mu, Y., Belflower, R., Aragon, D., Dumyati, G., Harrison, L., Petit, S. (2013). Emerging infections program–active bacterial core surveillance MRSA surveillance investigators. National burden of invasive methicillin-resistant Staphylococcus aureus infections, United States, 2011. *JAMA Intern Med, 173*(21), 1970-1978.

David, M. Z., & Daum, R. S. (2010). Community-associated methicillin-resistant Staphylococcus aureus: epidemiology and clinical consequences of an emerging epidemic. *Clinical Microbiology Reviews, 23*(3), 616-687.

De, L. F., & Wolf Miller, F. (2008). Epidemiology, surveillance and control of methicilillin resistant Staphylococcus aureua. *An overview. Asian Pac J Trop Med, 1*, 50-55.

de Sousa, M. A. (2004). Bridges from hospitals to the laboratory: genetic portraits of methicillin-resistant Staphylococcus aureus clones. *FEMS Immunology & Medical Microbiology, 40*(2), 101-111.

Degaim, Z. D., Shani, W. S., & Hamim, S. S. (2015). Virulence factors of Methicillin Resistant Staphylococcus aureus (MRSA) isolated from burn patients. *Internation Journal of Current Microbiology and Applied Sciences, 4*(7), 898-906.

Degaim, Z. D., Shani, W. S., & Hamim, S. S. (2015). Virulence factors of Methicillin Resistant Staphylococcus aureus (MRSA) isolated from burn patients. *International Journal of Current Microbiology and Applied Sciences, 4*(7), 898-906.

DeLeo, F. R., Otto, M., Kreiswirth, B. N., & Chambers, H. F. (2010). Community-associated meticillin-resistant Staphylococcus aureus. *The Lancet, 375*(9725), 1557-1568.

Deplano, A., Vandendriessche, S., Nonhoff, C., & Denis, O. (2014). Genetic diversity among methicillin-resistant Staphylococcus aureus isolates carrying the mecC gene in Belgium. *Journal of antimicrobial chemotherapy, 69*(6), 1457-1460.

Desbois, A. P., & Coote, P. J. (2011). Wax moth larva (Galleria mellonella): an in vivo model for assessing the efficacy of antistaphylococcal agents. *Journal of antimicrobial chemotherapy, 66*(8), 1785-1790.

Deurenberg, R., Kalenic, S., Friedrich, A., Van Tiel, F., & Stobberingh, E. (2007). Molecular epidemiology of methicillin-resistant Staphylococcus aureus. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology, 1*, 766-777.

Deurenberg, R. H., & Stobberingh, E. E. (2008). The evolution of Staphylococcus aureus. *Infection, genetics evolution, 8*(6), 747-763.

Deurenberg, R. H., Vink, C., Oudhuis, G. J., Mooij, J. E., Driessen, C., Coppens, G., Wagenvoort, H. (2005). Different clonal complexes of methicillin-resistant Staphylococcus aureus are disseminated in the Euregio Meuse-Rhine region. *Antimicrobial agents and chemotherapy, 49*(10), 4263-4271.

Devriese, L., Van Damme, L., & Fameree, L. (1972). Methicillin (cloxacillin)-resistant Staphylococcus aureus strains isolated from bovine mastitis cases. *Zentralblatt für Veterinärmedizin Reihe B, 19*(7), 598-605.

Diep, B. A., & Otto, M. (2008). The role of virulence determinants in community-associated MRSA pathogenesis. *Trends in microbiology, 16*(8), 361-369.

Dilnessa, T., & Bitew, A. (2016). Antimicrobial susceptibility pattern of Staphylococcus aureus with emphasize on methicilin resistance with patients postoperative and wound infections at Yekatit 12 Hospital Medical College in Ethiopia. *American Journal of Clinical and Experimental Medicine, 4*(1), 7-12.

Ding, Z., Zhang, H., Tang, W., Tong, C., Li, R., Chen, L., Cui, Y. (2012). Methylase genes-mediated erythromycin resistance in Staphylococcus aureus from bovine mastitis in China. *Israel Journal of Veterinary Medicine, 67*, 170-179.

Dinges, M. M., Orwin, P. M., & Schlievert, P. M. (2000). Exotoxins of Staphylococcus aureus. *Clinical Microbiology Reviews, 13*(1), 16-34.

Drancourt, M., Stein, A., Argenson, J., Roiron, R., Groulier, P., & Raoult, D. (1997). Oral treatment of Staphylococcus spp. infected orthopaedic implants with fusidic acid or ofloxacin in combination with rifampicin. *The Journal of antimicrobial chemotherapy, 39*(2), 235-240.

Dubrac, S., Boneca, I. G., Poupel, O., & Msadek, T. (2007). New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in Staphylococcus aureus. *Journal of bacteriology, 189*(22), 8257-8269.

Dufour, P., Gillet, Y., Bes, M., Lina, G., Vandenesch, F., Floret, D., Richet, H. (2002). Community-acquired methicillin-resistant Staphylococcus aureus infections in France: emergence of a single clone that produces Panton-Valentine leukocidin. *Clinical Infectious Diseases, 35*(7), 819-824.

Dumitrescu, O., & Lina, G. (2012). Current outbreak of Staphylococcus aureus. *Clinical microbiology and infection, 18*(8), 723-724.

DuMont, A. L., Nygaard, T. K., Watkins, R. L., Smith, A., Kozhaya, L., Kreiswirth, B. N., . . . Torres, V. J. (2011). Characterization of a new cytotoxin that contributes to Staphylococcus aureus pathogenesis. *Molecular microbiology, 79*(3), 814-825.

Duncan, L. R., Smith, C. J., Flamm, R. K., & Mendes, R. E. (2020). Regional analysis of telavancin and comparator antimicrobial activity against multidrug-resistant Staphylococcus aureus collected in the USA 2014–2016. *Journal of global antimicrobial resistance, 20*, 118-123.

Dunyach-Remy, C., Ngba Essebe, C., Sotto, A., & Lavigne, J.-P. (2016). Staphylococcus aureus toxins and diabetic foot ulcers: role in pathogenesis and interest in diagnosis. *Toxins, 8*(7), 209.

Ebrahim-Saraie, H. S., Motamedifar, M., Sarvari, J., & Alfatemi, S. M. H. (2015). Emergence of SCCmec type I obtained from clinical samples in Shiraz teaching hospitals, South-West of Iran. *Jundishapur Journal of Microbiology, 8*(6).

Ehsan, M. M., Memon, Z., Ismail, M. O., & Fatima, G. (2013). Identification and antibiotic susceptibility pattern of coagulase-negative staphylococci in various clinical specimens. *Pakistan journal of medical sciences*.

El-baz, R., Rizk, D. E., Barwa, R., & Hassan, R. (2016). Virulence factors profile of Staphylococcus aureus isolated from different clinical sources. *Journal of Microbiology, 43*, 126-144.

Elboshra, M. M. E., Hamedelnil, Y. F., Moglad, E. H., & Altayb, H. N. (2020). Prevalence and characterization of virulence genes among methicillin-resistant Staphylococcus aureus isolated from Sudanese patients in Khartoum state. *New Microbes New Infect, 38*, 100784. doi:10.1016/j.nmni.2020.100784

Elie-Turenne, M.-C., Fernandes, H., Mediavilla, J. R., Rosenthal, M., Mathema, B., Singh, A., Kreiswirth, B. N. (2010). Prevalence and characteristics of Staphylococcus aureus colonization among healthcare professionals in an urban teaching hospital. *Infection Control & Hospital Epidemiology, 31*(06), 574-580.

Ellis, M. W., Griffith, M. E., Jorgensen, J. H., Hospenthal, D. R., Mende, K., & Patterson, J. E. (2009). Presence and molecular epidemiology of virulence factors in methicillin-resistant Staphylococcus aureus strains colonizing and infecting soldiers. *Journal of clinical microbiology, 47*(4), 940-945.

Elston, D. M. (2007). Community-acquired methicillin-resistant Staphylococcus aureus. *Journal of the American Academy of Dermatology, 56*(1), 1-16.

Emaneini, M., Bigverdi, R., Kalantar, D., Soroush, S., Jabalameli, F., Khoshgnab, B. N., Taherikalani, M. (2013). Distribution of genes encoding tetracycline resistance and aminoglycoside modifying enzymes in Staphylococcus aureus strains isolated from a burn center. *Annals of burns and fire disasters, 26*(2), 76.

Enright, M. C., Day, N. P., Davies, C. E., Peacock, S. J., & Spratt, B. G. (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus. *Journal of clinical microbiology, 38*(3), 1008-1015.

Enright, M. C., Day, N. P., Davies, C. E., Peacock, S. J., & Spratt, B. G. (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones ofStaphylococcus aureus. *Journal of clinical microbiology, 38*(3), 1008-1015.

Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H., & Spratt, B. G. (2002). The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA). *Proceedings of the National Academy of Sciences, 99*(11), 7687-7692.

Etienne, J., Gerbaud, G., Fleurette, J., & Courvalin, P. (1991). Characterization of staphylococcal plasmids hybridizing with the fosfomycin resistance gene fosB. *FEMS microbiology letters, 84*(1), 119-122.

Evans, B. A., & Rozen, D. (2012). A Streptococcus pneumoniae infection model in larvae of the wax moth Galleria mellonella. *European journal of clinical microbiology & infectious diseases, 31*(10), 2653-2660.

Evans, F. G. (1957). *Stress and strain in bones*: Blackwell.

Falagas, M. E., Karageorgopoulos, D. E., Leptidis, J., & Korbila, I. P. (2013). MRSA in Africa: filling the global map of antimicrobial resistance. *PloS one, 8*(7), e68024.

Falagas, M. E., Kastoris, A. C., Karageorgopoulos, D. E., & Rafailidis, P. I. (2009). Fosfomycin for the treatment of infections caused by multidrug-resistant non-fermenting Gram-negative bacilli: a systematic review of microbiological, animal and clinical studies. *International journal of antimicrobial agents, 34*(2), 111-120.

Fasih, N., Irfan, S., Zafar, A., Khan, E., & Hasan, R. (2010). Inducible clindamycin resistance due to expression of erm genes in Staphylococcus aureus: report from a tertiary care hospital Karachi, Pakistan. *Journal of the Pakistan Medical Association, 60*(9), 750.

Fasih, N., Irfan, S., Zafar, A., Khan, E., & Hasan, R. (2010). Inducible clindamycin resistance due to expression of erm genes in Staphylococcus aureus: report from a tertiary care Hospital Karachi, Pakistan. *Journal of Pakistan Medical Association, 60*(9), 750-753.

Fasihi, Y., Kiaei, S., & Kalantar-Neyestanaki, D. (2017). Characterization of SCCmec and spa types of methicillin-resistant Staphylococcus aureus isolates from health-care and community-acquired infections in Kerman, Iran. *Journal of Epidemiology and Global Health, 7*(4), 263-267.

Fatholahzadeh, B., Emaneini, M., Feizabadi, M. M., Sedaghat, H., Aligholi, M., Taherikalani, M., & Jabalameli, F. (2009). Characterisation of genes encoding aminoglycoside-modifying enzymes among meticillin-resistant Staphylococcus aureus isolated from two hospitals in Tehran, Iran. *International journal of antimicrobial agents, 33*(3), 264-265.

Fatima, A., Sajid, I., Riaz, S., & Saeed, M. (2019). Detection of methicillin resistant staphylococcus aureus using mec a, ribotyping and antibiogram profile of Pakistani clinical isolates. *The Professional Medical Journal, 26*(06), 993-999.

Fayyaz, M., Mirza, I. A., Ahmed, Z., Abbasi, S. A., Hussain, A., & Ali, S. (2013). In vitro susceptibility of chloramphenicol against methicillin-resistant Staphylococcus aureus. *Journal of the College of Physicians and Surgeons Pakistan, 23*(9), 637-640.

Ferreira, F. A., Souza, R. R., de Sousa Moraes, B., de Amorim Ferreira, A. M., Américo, M. A., Fracalanzza, S. E. L., Figueiredo, A. M. S. (2013). Impact of agr dysfunction on virulence profiles and infections associated with a

novel methicillin-resistant Staphylococcus aureus (MRSA) variant of the lineage ST1-SCCmec IV. *BMC microbiology, 13*(1), 93.

Fischer, J., Lee, J., Peters, G., & Kahl, B. (2014). Acapsular clinical Staphylococcus aureus isolates lack agr function. *Clinical Microbiology Infection, 20*(7), O414-O417.

Fishovitz, J., Hermoso, J. A., Chang, M., & Mobashery, S. (2014). Penicillin-binding protein 2a of methicillin-resistant Staphylococcus aureus. *IUBMB life, 66*(8), 572-577.

Fitzpatrick, F., Humphreys, H., & O'Gara, J. P. (2005). Evidence for icaADBC-independent biofilm development mechanism in methicillin-resistant Staphylococcus aureus clinical isolates. *Journal of clinical microbiology, 43*(4), 1973-1976.

Foster, T. (1983). Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiological reviews, 47*(3), 361.

Foster, T. J. (2017). Antibiotic resistance in Staphylococcus aureus. Current status and future prospects. *FEMS microbiology reviews, 41*(3), 430-449.

Foucault, M.-L., Courvalin, P., & Grillot-Courvalin, C. (2009). Fitness cost of VanA-type vancomycin resistance in methicillin-resistant Staphylococcus aureus. *Antimicrobial agents and chemotherapy, 53*(6), 2354-2359.

Fournier, B., & Klier, A. (2004). Protein A gene expression is regulated by DNA supercoiling which is modified by the ArlS–ArlR two-component system of Staphylococcus aureus. *Microbiology, 150*(11), 3807-3819.

Fournier, B., Klier, A., & Rapoport, G. (2001). The two-component system ArlS–ArlR is a regulator of virulence gene expression in Staphylococcus aureus. *Molecular microbiology, 41*(1), 247-261.

Fowler Jr, V. G., Boucher, H. W., Corey, G. R., Abrutyn, E., Karchmer, A. W., Rupp, M. E., Vigliani, G. A. (2006). Daptomycin versus standard therapy for bacteremia and endocarditis caused by Staphylococcus aureus. *New England journal of medicine, 355*(7), 653-665.

Fowler Jr, V. G., Sakoulas, G., McIntyre, L. M., Meka, V. G., Arbeit, R. D., Cabell, C. H., Ralph Corey, G. (2004). Persistent bacteremia due to methicillin-resistant Staphylococcus aureus infection is associated with agr dysfunction and low-level in vitro resistance to thrombin-induced platelet microbicidal protein. *The Journal of infectious diseases, 190*(6), 1140-1149.

Fraunholz, M., & Sinha, B. (2012). Intracellular Staphylococcus aureus: live-in and let die. *Frontiers in cellular and infection microbiology, 2*, 43.

Fu, Z., Liu, Y., Chen, C., Guo, Y., Ma, Y., Yang, Y., Wang, M. (2016). Characterization of fosfomycin resistance gene, fosB, in methicillin-resistant Staphylococcus aureus isolates. *PloS one, 11*(5), e0154829.

Fuchs, B. B., O'Brien, E., El Khoury, J. B., & Mylonakis, E. (2010). Methods for using Galleria mellonella as a model host to study fungal pathogenesis. *Virulence, 1*(6), 475-482.

García-Álvarez, L., Holden, M. T., Lindsay, H., Webb, C. R., Brown, D. F., Curran, M. D., Teale, C. (2011). Meticillin-resistant Staphylococcus aureus with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study. *The Lancet infectious diseases, 11*(8), 595-603.

García-García, J. A., Santos-Morano, J., Castro, C., Bayoll-Serradilla, E., Martín-Ponce, M. L., Vergara-López, S., Martín-Mazuelos, E. (2011). Prevalencia y factores asociados a la colonización por Staphylococcus aureus resistente a meticilina en centros de larga estancia en el sur de España. *Enfermedades infecciosas y microbiologia clinica, 29*(6), 405-410.

Garcia, P., Arca, P., & Suarez, J. E. (1995). Product of fosC, a gene from Pseudomonas syringae, mediates fosfomycin resistance by using ATP as cosubstrate. *Antimicrobial agents and chemotherapy, 39*(7), 1569-1573.

Garoy, E. Y., Gebreab, Y. B., Achila, O. O., Tekeste, D. G., Kesete, R., Ghirmay, R., Tesfu, T. (2019). Methicillin-resistant Staphylococcus aureus (MRSA): prevalence and antimicrobial sensitivity pattern among patients—a multicenter study in Asmara, Eritrea. *Canadian Journal of Infectious Diseases Medical Microbiology, 2019*.

Garzoni, C., Francois, P., Huyghe, A., Couzinet, S., Tapparel, C., Charbonnier, Y., Vaudaux, P. (2007). A global view of Staphylococcus aureus whole genome expression upon internalization in human epithelial cells. *BMC genomics, 8*(1), 171.

Geha, D. J., Uhl, J. R., Gustaferro, C. A., & Persing, D. H. (1994). Multiplex PCR for identification of methicillin-resistant staphylococci in the clinical laboratory. *Journal of clinical microbiology, 32*(7), 1768-1772.

Ghasemian, A., Najar Peerayeh, S., Bakhshi, B., & Mirzaee, M. (2015). Several virulence factors of multidrug-resistant Staphylococcus aureus isolates from hospitalized patients in Tehran. *International Journal of Enteric Pathogens, 3*(2), 1-6.

Ghaznavi-Rad, E., Fard-Mousavi, N., Shahsavari, A., Japoni-Nejad, A., & Van Belkum, A. (2018). Distribution of staphylococcal cassette chromosome mec types among methicillin-resistant coagulase negative staphylococci in central Iran. *Iranian journal of microbiology, 10*(1), 7.

Ghias, W., Sharif, M., Yazdani, F. A., & Rabbani, M. (2016). Isolation and identification of Methicillin and Vancomycin Resistance Staphylococcus aureus From Pus Samples of Injured Skin Patients in Lahore, Pakistan. *Biomedical Letters, 2*(2), 103-112.

Gibreel, T. M., & Upton, M. (2013). Synthetic epidermicin NI01 can protect Galleria mellonella larvae from infection with Staphylococcus aureus. *Journal of antimicrobial chemotherapy, 68*(10), 2269-2273.

Gill, S. R., Fouts, D. E., Archer, G. L., Mongodin, E. F., DeBoy, R. T., Ravel, J., . . . Beanan, M. (2005). Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant Staphylococcus aureus strain and a biofilm-producing methicillin-resistant Staphylococcus epidermidis strain. *Journal of bacteriology, 187*(7), 2426-2438.

Gilot, P., Lina, G., Cochard, T., & Poutrel, B. (2002). Analysis of the genetic variability of genes encoding the RNA III-activating components Agr and TRAP in a population of Staphylococcus aureus strains isolated from cows with mastitis. *Journal of clinical microbiology, 40*(11), 4060-4067.

Gindonis, V., Taponen, S., Myllyniemi, A.-L., Pyörälä, S., Nykäsenoja, S., Salmenlinna, S., Rantala, M. (2013). Occurrence and characterization of

methicillin-resistant staphylococci from bovine mastitis milk samples in Finland. *Acta veterinaria scandinavica, 55*(1), 61.

Glasner, C., Pluister, G., Westh, H., Arends, J. P., Empel, J., Giles, E., Matussek, A. (2015). Staphylococcus aureus spa type t437: identification of the most dominant community-associated clone from Asia across Europe. *Clinical microbiology and infection, 21*(2), 163. e161-163. e168.

Goering, R. V., Shawar, R. M., Scangarella, N. E., O'Hara, F. P., Amrine-Madsen, H., West, J. M., Miller, L. A. (2008). Molecular epidemiology of methicillin-resistant and methicillin-susceptible Staphylococcus aureus isolates from global clinical trials. *Journal of clinical microbiology, 46*(9), 2842-2847.

Gomes-Fernandes, M., Laabei, M., Pagan, N., Hidalgo, J., Molinos, S., Hernandez, R. V., Prat, C. (2017). Accessory gene regulator (Agr) functionality in Staphylococcus aureus derived from lower respiratory tract infections. *PloS one, 12*(4).

Goswami, N. N., Trivedi, H. R., Goswami, A. P. P., Patel, T. K., & Tripathi, C. (2011). Antibiotic sensitivity profile of bacterial pathogens in postoperative wound infections at a tertiary care hospital in Gujarat, India. *Journal of pharmacology & pharmacotherapeutics, 2*(3), 158.

Götz, F. (2002). Staphylococcus and biofilms. *Molecular microbiology, 43*(6), 1367-1378.

Goudarzi, M., Fazeli, M., Goudarzi, H., Azad, M., & Seyedjavadi, S. S. (2016). Spa typing of Staphylococcus aureus strains isolated from clinical specimens of patients with nosocomial infections in Tehran, Iran. *Jundishapur Journal of Microbiology, 9*(7).

Goudarzi, M., Goudarzi, H., Sá Figueiredo, A. M., Udo, E. E., Fazeli, M., Asadzadeh, M., & Seyedjavadi, S. S. (2016). Molecular characterization of methicillin resistant Staphylococcus aureus strains isolated from intensive care units in Iran: ST22-SCC mec IV/t790 emerges as the major clone. *PloS one, 11*(5), e0155529.

Goudarzi, M., Seyedjavadi, S. S., Nasiri, M. J., Goudarzi, H., Nia, R. S., & Dabiri, H. (2017). Molecular characteristics of methicillin-resistant Staphylococcus aureus (MRSA) strains isolated from patients with bacteremia based on MLST, SCCmec, spa, and agr locus types analysis. *Microbial pathogenesis, 104*, 328-335.

Gould, I. (2006). Costs of hospital-acquired methicillin-resistant Staphylococcus aureus (MRSA) and its control. *International journal of antimicrobial agents, 28*(5), 379-384.

Gravet, A., Colin, D., Keller, D., Giradot, R., Monteil, H., & Prevost, G. (1998). Characterization of a novel structural member, LukE-LukD, of the bi-component staphylococcal leucotoxins family. *FEBS letters, 436*(2), 202-208.

Greenland, K., Rijnders, M. I., Mulders, M., Haenen, A., Spalburg, E., van de Kassteele, J., . . . Stobberingh, E. (2011). Low prevalence of methicillin-resistant Staphylococcus aureus in Dutch nursing homes. *Journal of the American Geriatrics Society, 59*(4), 768-769.

Greenwood, R. C., & Gentry, D. R. (2002). The effect of antibiotic treatment on the intracellular nucleotide pools of Staphylococcus aureus. *FEMS microbiology letters, 208*(2), 203-206.

Gresham, H. D., Lowrance, J. H., Caver, T. E., Wilson, B. S., Cheung, A. L., & Lindberg, F. P. (2000). Survival of Staphylococcus aureus inside neutrophils contributes to infection. *The Journal of Immunology, 164*(7), 3713-3722.

Grosz, M., Kolter, J., Paprotka, K., Winkler, A. C., Schäfer, D., Chatterjee, S. S., Otto, M. (2014). Cytoplasmic replication of S taphylococcus aureus upon phagosomal escape triggered by phenol-soluble modulin α. *Cellular microbiology, 16*(4), 451-465.

Grumann, D., Nübel, U., & Bröker, B. M. (2014). Staphylococcus aureus toxins–their functions and genetics. *Infection, Genetics and Evolution, 21*, 583-592.

Grundmann, H., Aires-de-Sousa, M., Boyce, J., & Tiemersma, E. (2006). Emergence and resurgence of meticillin-resistant Staphylococcus aureus as a public-health threat. *The Lancet, 368*(9538), 874-885.

Guo, Y., Zhu, D., Hu, F., Wang, F., Yu, Y., & Yang, Q. (2013). CHINET 2010 surveillance and analysis of antibiotic resistance in Staphylococcus spp. in China. *Chinese Journal of Infection and chemotherapy, 13*, 86-92.

Gurung, R. R., Maharjan, P., & Chhetri, G. G. (2020). Antibiotic resistance pattern of Staphylococcus aureus with reference to MRSA isolates from pediatric patients. *Future Science OA*(0), FSO464.

Habeeb, A., Hussein, N. R., Assafi, M. S., & Al-Dabbagh, S. A. (2014). Methicillin resistant Staphylococcus aureus nasal colonization among secondary school students at Duhok City-Iraq. *Journal of Microbiology and Infectious Diseases, 4*(2).

Habeeb, A., Hussein, N. R., Assafi, M. S., Al-Dabbagh, S. A. J. J. o. M., & Diseases, I. (2014). Methicillin resistant Staphylococcus aureus nasal colonization among secondary school students at Duhok City-Iraq. *4*(02), 59-63.

Hakim, S., Arshed, S., Iqbal, M., & Javaid, S. (2007). Vancomycin sensitivity of Staphylococcus aureus isolates from hospital patients in Karachi, Pakistan. *Libyan Journal of Medicine, 2*(4), 176-179.

Hallin, M., Denis, O., Deplano, A., De Mendonça, R., De Ryck, R., Rottiers, S., & Struelens, M. (2007). Genetic relatedness between methicillin-susceptible and methicillin-resistant Staphylococcus aureus: results of a national survey. *Journal of antimicrobial chemotherapy, 59*(3), 465-472.

Ham, J.-S., Lee, S.-G., Jeong, S.-G., Oh, M.-H., Kim, D.-H., Lee, T., Kim, H. (2010). Powerful usage of phylogenetically diverse Staphylococcus aureus control strains for detecting multidrug resistance genes in transcriptomics studies. *Molecules and cells, 30*(1), 71-76.

Hamza, T., & Li, B. (2014). Differential responses of osteoblasts and macrophages upon Staphylococcus aureus infection. *BMC microbiology, 14*(1), 207.

Hamzah, C., Mardziah, A., Yeo, C. C., Puah, S. M., Chua, K. H., & Chew, C. H. (2019). Staphylococcus aureus Infections in Malaysia: A Review of Antimicrobial Resistance and Characteristics of the Clinical Isolates, 1990–2017. *Antibiotics, 8*(3), 128.

Hannan, A., Javed, F., Saleem, S., Tahira, K., & Jahan, S. (2015). Frequency of Staphylococcal Cassette Chromosome mec Type IV and Type V in Clinical Isolates of Methicillin Resistant Staphylococcus aureus. *Open Journal of Medical Microbiology, 5*(02), 69.

Hanssen, A.-M., & Ericson Sollid, J. U. (2006). SCC mec in staphylococci: genes on the move. *FEMS Immunology & Medical Microbiology, 46*(1), 8-20.

Haque, A., Haque, A., Saeed, M., Azhar, A., Rasool, S., Shan, S., Nisar, Z. (2017). Rapid screening of pyogenic Staphylococcus aureus for confirmation of genus and species, methicillin resistance and virulence factors by using two novel multiplex PCR. *Pakistan journal of medical sciences, 33*(5), 1095.

Harms, J. M., Schlünzen, F., Fucini, P., Bartels, H., & Yonath, A. (2004). Alterations at the peptidyl transferase centre of the ribosome induced by the synergistic action of the streptogramins dalfopristin and quinupristin. *BMC biology, 2*(1), 4.

Harmsen, D., Claus, H., Witte, W., Rothgänger, J., Claus, H., Turnwald, D., & Vogel, U. (2003). Typing of methicillin-resistant Staphylococcus aureus in a university hospital setting by using novel software for spa repeat determination and database management. *Journal of clinical microbiology, 41*(12), 5442-5448.

Hasan, R., Acharjee, M., & Noor, R. (2016). Prevalence of vancomycin resistant Staphylococcus aureus (VRSA) in methicillin resistant S. aureus (MRSA) strains isolated from burn wound infections. *Tzu Chi Medical Journal, 28*(2), 49-53.

Hashemizadeh, Z., Hadi, N., Mohebi, S., Kalantar-Neyestanaki, D., & Bazargani, A. (2019). Characterization of SCCmec, spa types and Multi Drug Resistant of methicillin-resistant Staphylococcus aureus isolates among inpatients and outpatients in a referral hospital in Shiraz, Iran. *BMC research notes, 12*(1), 614.

Hassan, R., El-Naggar, W., Habib, E., & El-Bargisy, R. (2012). Comparative studies on Staphylococcus aureus isolates associated with infections in diabetic and non-diabetic patients. *Egyptian Journal of Microbiology, 21*(2), 71-78.

Hauschild, T., Sacha, P., Wieczorek, P., Zalewska, M., Kaczyńska, K., & Tryniszewska, E. (2008). Aminoglycosides resistance in clinical isolates of Staphylococcus aureus from a University Hospital in Bialystok, Poland. *Folia histochemica et cytobiologica, 46*(2), 225-228.

Havaei, S., Moghadam, S. O., Pourmand, M. R., & Faghri, J. (2010). Prevalence of genes encoding bi-component leukocidins among clinical isolates of methicillin resistant Staphylococcus aureus. *Iranian journal of public health, 39*(1), 8.

Havaei, S. A., Ghanbari, F., Rastegari, A. A., Azimian, A., Khademi, F., Hosseini, N., Shahin, M. (2014). Molecular typing of hospital-acquired Staphylococcus aureus isolated from Isfahan, Iran. *International scholarly research notices, 2014*.

Hawraa, W., Al-Dulaimi, T., & Al-Marzoqi, A. (2014). Phenotypic detection of resistance in Staphylococcus aureus isolates: detection of (mec A and fem A) gene in methicillin resistant Staphylococcus aureus (MRSA) by polymerase chain reaction. *Journal of Natural Sciences Research, 4*, 112-118.

Healy, C. M., Hulten, K. G., Palazzi, D. L., Campbell, J. R., & Baker, C. J. (2004). Emergence of new strains of methicillin-resistant Staphylococcus aureus in a neonatal intensive care unit. *Clinical Infectious Diseases, 39*(10), 1460-1466.

Hefzy, E. M., Hassan, G. M., & Abd El Reheem, F. (2016). Detection of Panton-Valentine leukocidin-positive methicillin-resistant Staphylococcus aureus nasal carriage among Egyptian health care workers. *Surgical infections, 17*(3), 369-375.

Herold, B. C., Immergluck, L. C., Maranan, M. C., Lauderdale, D. S., Gaskin, R. E., Boyle-Vavra, S., Daum, R. S. (1998). Community-acquired methicillin-resistant Staphylococcus aureus in children with no identified predisposing risk. *Jama, 279*(8), 593-598.

Heudorf, U., Gustav, C., Mischler, D., & Schulze, J. (2014). Healthcare associated infections (HAI), antibiotic use and prevalence of multidrug-resistant bacteria (MDRO) in residents of long-term care facilities: the Frankfurt HALT plus MDRO project 2012. *Bundesgesundheitsblatt, Gesundheitsforschung, Gesundheitsschutz, 57*(4), 414.

Ho, C.-M., Ho, M.-W., Lee, C.-Y., Tien, N., & Lu, J.-J. (2012). Clonal spreading of methicillin-resistant SCCmec Staphylococcus aureus with specific spa and dru types in central Taiwan. *European Journal of Clinical Microbiology and Infectious Diseases, 31*(4), 499-504.

Ho, P.-L., Chuang, S.-K., Choi, Y.-F., Lee, R. A., Lit, A. C., Ng, T.-K., Cindy, W. (2008). Community-associated methicillin-resistant and methicillin-sensitive Staphylococcus aureus: skin and soft tissue infections in Hong Kong. *Diagnostic microbiology and infectious disease, 61*(3), 245-250.

Hooper, D. C. (2002). Fluoroquinolone resistance among Gram-positive cocci. *The Lancet infectious diseases, 2*(9), 530-538.

Hooper, D. C., & Jacoby, G. A. (2015). Mechanisms of drug resistance: quinolone resistance. *Annals of the New York academy of sciences, 1354*(1), 12-31.

Horst, S. A., Hoerr, V., Beineke, A., Kreis, C., Tuchscherr, L., Kalinka, J., Fuchs, T. (2012). A novel mouse model of Staphylococcus aureus chronic osteomyelitis that closely mimics the human infection: an integrated view of disease pathogenesis. *The American journal of pathology, 181*(4), 1206-1214.

Huang, H., Flynn, N. M., King, J. H., Monchaud, C., Morita, M., & Cohen, S. H. (2006). Comparisons of community-associated methicillin-resistant Staphylococcus aureus (MRSA) and hospital-associated MSRA infections in Sacramento, California. *Journal of clinical microbiology, 44*(7), 2423-2427.

Huang, J., Chen, L., Wu, Z., & Wang, L. (2016). Retrospective analysis of genome sequences revealed the wide dissemination of optrA in Gram-positive bacteria. *Journal of antimicrobial chemotherapy, 72*(2), 614-616.

Hucker, G., & Breed, R. (1948). Genus micrococcus. *Bergey's Manual of Determinatiue Bacteriology*.

Humphreys, H. (2008). Can we do better in controlling and preventing methicillin-resistant Staphylococcus aureus (MRSA) in the intensive care unit (ICU)? *European Journal of Clinical Microbiology and Infectious Diseases, 27*(6), 409-413.

Huseby, M., Shi, K., Brown, C. K., Digre, J., Mengistu, F., Seo, K. S., Earhart, C. A. (2007). Structure and biological activities of beta toxin from Staphylococcus aureus. *Journal of bacteriology, 189*(23), 8719-8726.

Hussain, M., Basit, A., Khan, A., Rahim, K., Javed, A., Junaid, A., Hussain, T. (2013). Antimicrobial sensitivity pattern of methicillin resistant Staphylococcus aureus isolated from hospitals of Kohat district. *Pakistan Journal of Infection and Molecular biology, 1*(1), 13-16.

Hussain, M. S., Naqvi, A., & Sharaz, M. (2019). Methicillin Resistant Staphylococcus Aureus (Mrsa). *The Professional Medical Journal, 26*(01), 122-127.

Hussain, S., Shams, R., Ahmad, K., Perveen, R., & Riaz, B. (2005). Prevalence of methicillin resistant Staphylococcus aureus (MRSA) in surgical site infections in a tertiary care hospital. *Journal Pathology, 3*(2), 81-85.

Hussein, N. R., Assafi, M. S., & Ijaz, T. (2017). Methicillin-resistant Staphylococcus aureus nasal colonisation amongst healthcare workers in Kurdistan Region, Iraq. *Journal of global antimicrobial resistance, 9*, 78-81.

Huygens, F., Nimmo, G. R., Schooneveldt, J., Munckhof, W. J., & Giffard, P. M. (2002). Genotyping of methicillin-resistant Staphylococcus aureus by assaying for the presence of variable elements associated with mecA. *Journal of clinical microbiology, 40*(8), 3093-3097.

Idrees, F., Jabeen, K., Khan, M. S., & Zafar, A. (2009). Antimicrobial resistance profile of methicillin resistant staphylococcal aureus from skin and soft tissue isolates. *Journal of the Pakistan Medical Association, 59*(5), 266.

Indrawattana, N., Sungkhachat, O., Sookrung, N., Chongsa-Nguan, M., Tungtrongchitr, A., Voravuthikunchai, S., Chaicumpa, W. (2013). Staphylococcus aureus clinical isolates: antibiotic susceptibility, molecular characteristics, and ability to form biofilm. *BioMed research international, 2013*.

Iqbal, K., Khan, M. I., & Satti, L. (2011). Microbiology of chronic suppurative otitis media: experience at Dera Ismail Khan. *Gomal Journal of Medical Sciences, 9*(2).

Iqbal, M. S., Saleem, Y., Ansari, F., Qamar, M. U., Mazhar, S., Hassan, A., Syed, Q. (2018). Staphylococcus aureus carrying lukS/F Panton-Valentine Leukocidin (PVL) toxin genes in hospitals of Lahore city. *The Journal of Infection in Developing Countries, 12*(09), 720-725.

Ira, P., Sujatha, S., & Chandra, P. S. (2013). Virulence factors in clinical and commensal isolates of Enterococcus species. *Indian Journal of Pathology and Microbiology, 56*(1), 24.

Ito, T., Hiramatsu, K., Tomasz, A., De Lencastre, H., Perreten, V., Holden, M. T., Skov, R. L. (2012). Guidelines for reporting novel mecA gene homologues. *Antimicrobial agents and chemotherapy, 56*(10), 4997-4999.

Ito, T., Katayama, Y., Asada, K., Mori, N., Tsutsumimoto, K., Tiensasitorn, C., & Hiramatsu, K. (2001). Structural comparison of three types of staphylococcal cassette chromosome mec integrated in the chromosome in methicillin-resistant Staphylococcus aureus. *Antimicrobial agents and chemotherapy, 45*(5), 1323-1336.

Ito, T., Ma, X. X., Takeuchi, F., Okuma, K., Yuzawa, H., & Hiramatsu, K. (2004). Novel type V staphylococcal cassette chromosome mec driven by a novel cassette chromosome recombinase, ccrC. *Antimicrobial agents and chemotherapy, 48*(7), 2637-2651.

Ito, T., Okuma, K., Ma, X. X., Yuzawa, H., & Hiramatsu, K. (2003). Insights on antibiotic resistance of Staphylococcus aureus from its whole genome: genomic island SCC. *Drug Resistance Updates, 6*(1), 41-52.

IWG-SCC. (2009). Classification of staphylococcal cassette chromosome mec (SCCmec): guidelines for reporting novel SCCmec elements. *Antimicrobial agents and chemotherapy, 53*(12), 4961-4967.

Jain, S., Chowdhury, R., Datta, M., Chowdhury, G., & Mukhopadhyay, A. K. (2019). Characterization of the clonal profile of methicillin resistant Staphylococcus aureus isolated from patients with early post-operative orthopedic implant based infections. *Annals of clinical microbiology and antimicrobials, 18*(1), 1-7.

Jamil, B., Gawlik, D., Syed, M. A., Shah, A. A., Abbasi, S. A., Müller, E., Monecke, S. (2018). Hospital-acquired methicillin-resistant Staphylococcus aureus (MRSA) from Pakistan: molecular characterisation by microarray technology. *European journal of clinical microbiology & infectious diseases, 37*(4), 691-700.

Jans, B., Schoevaerdts, D., Huang, T.-D., Berhin, C., Latour, K., Bogaerts, P., Glupczynski, Y. (2013). Epidemiology of multidrug-resistant microorganisms among nursing home residents in Belgium. *PloS one, 8*(5), e64908.

Jarraud, S., Mougel, C., Thioulouse, J., Lina, G., Meugnier, H., Forey, F., Vandenesch, F. J. I. (2002). Relationships between Staphylococcus aureus genetic background, virulence factors, agr groups (alleles), and human disease. *Infection and immunity, 70*(2), 631-641.

Javdan, S., Narimani, T., Abadi, M. S. S., & Gholipour, A. (2019). Agr typing of Staphylococcus aureus species isolated from clinical samples in training hospitals of Isfahan and Shahrekord. *BMC research notes, 12*(1), 363.

Jayamani, E., Tharmalingam, N., Rajamuthiah, R., Coleman, J. J., Kim, W., Okoli, I., Ausubel, F. M. (2017). Characterization of a Francisella tularensis-Caenorhabditis elegans pathosystem for the evaluation of therapeutic compounds. *Antimicrobial agents and chemotherapy, 61*(9), e00310-00317.

Jensen, L. B., Frimodt-Møller, N., & Aarestrup, F. M. (1999). Presence of erm gene classes in gram-positive bacteria of animal and human origin in Denmark. *FEMS microbiology letters, 170*(1), 151-158.

Jensen, S. O., & Lyon, B. R. (2009). Genetics of antimicrobial resistance in Staphylococcus aureus. *Future microbiology, 4*(5), 565-582.

Jevons, M. P. (1961). "Celbenin"-resistant staphylococci. *British medical journal, 1*(5219), 124.

Ji, M., Lee, M., Noh, S., & Kim, M. N. (2010). Evaluation of the Performance of the MicroScan Pos Breakpoint Combo Panel Type 28 for Susceptibility Testing of Staphylococcus aureus: Low-range Minimum Inhibitory Concentration of Vancomycin, Cefoxitin Screening, and Inducible Clindamycin Resistance Detec. *The Korean journal of laboratory medicine, 30*(6), 637-646.

Jiang, S., Gilpin, M. E., Attia, M., Ting, Y.-L., & Berti, P. J. (2011). Lyme disease enolpyruvyl-UDP-GlcNAc synthase: fosfomycin-resistant MurA from Borrelia burgdorferi, a fosfomycin-sensitive mutant, and the catalytic role of the active site Asp. *Biochemistry, 50*(12), 2205-2212.

Jiménez, J. N., Ocampo, A. M., Vanegas, J. M., Rodríguez, E. A., Garcés, C. G., Patiño, L. A., Correa, M. M. (2011). Characterisation of virulence genes in methicillin susceptible and resistant Staphylococcus aureus isolates from a paediatric population in a university hospital of Medellin, Colombia. *Memórias do Instituto Oswaldo Cruz, 106*(8), 980-985.

Jones, C. H., Tuckman, M., Howe, A. Y., Orlowski, M., Mullen, S., Chan, K., & Bradford, P. A. (2006). Diagnostic PCR analysis of the occurrence of methicillin and tetracycline resistance genes among Staphylococcus aureus isolates from phase 3 clinical trials of tigecycline for complicated skin and skin structure infections. *Antimicrobial agents and chemotherapy, 50*(2), 505-510.

Jubrail, J., Morris, P., Bewley, M. A., Stoneham, S., Johnston, S. A., Foster, S. J., Dockrell, D. H. (2016). Inability to sustain intraphagolysosomal killing of Staphylococcus aureus predisposes to bacterial persistence in macrophages. *Cellular microbiology, 18*(1), 80-96.

Junior, J. C., Sabino, C. P., Tan, X., Junqueira, J. C., Wang, Y., Fuchs, B. B., Mylonakis, E. (2013). Selective photoinactivation of Candida albicans in the non-vertebrate host infection model Galleria mellonella. *BMC microbiology, 13*(1), 217.

Juyal, D., Shamanth, A., Pal, S., Sharma, M. K., Prakash, R., & Sharma, N. (2013). The prevalence of inducible clindamycin resistance among Staphylococci in a tertiary care hospital–a study from the Garhwal Hills of Uttarakhand, India. *Journal of clinical and diagnostic research: JCDR, 7*(1), 61.

Kadariya, J., Thapaliya, D., Bhatta, S., Mahatara, R. L., Bempah, S., Dhakal, N., & Smith, T. C. (2019). Multidrug-resistant Staphylococcus aureus Colonization in Healthy Adults Is more Common in Bhutanese Refugees in Nepal than Those Resettled in Ohio. *BioMed research international, 2019*.

Kahlmeter, G., Brown, D. F., Goldstein, F. W., MacGowan, A. P., Mouton, J. W., Österlund, A.,Vatopoulos, A. (2003). European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. *Journal of antimicrobial chemotherapy, 52*(2), 145-148.

Kaito, C., Kurokawa, K., Hossain, M. S., Akimitsu, N., & Sekimizu, K. (2002). Isolation and characterization of temperature-sensitive mutants of the Staphylococcus aureus dnaC gene. *FEMS microbiology letters, 210*(1), 157-164.

Kaleem, F., Usman, J., Hassan, A., Omair, M., & Uddin, R. (2010). Sensitivity pattern of methicillin resistant Staphylococcus aureus isolatedfrom patients admitted in a tertiary care hospital of Pakistan. *Iranian journal of microbiology*, 3.

Kateete, D. P., Namazzi, S., Okee, M., Okeng, A., Baluku, H., Musisi, N. L., Najjuka, F. C. (2011). High prevalence of methicillin resistant Staphylococcus aureus in the surgical units of Mulago hospital in Kampala, Uganda. *BMC research notes, 4*(1), 1-5.

Kavanagh, K., & Reeves, E. P. (2004). Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens. *FEMS microbiology reviews, 28*(1), 101-112.

Kavanagh, K. T. (2019). Control of MSSA and MRSA in the United States: protocols, policies, risk adjustment and excuses. *Antimicrobial Resistance & Infection Control, 8*(1), 1-8.

Kazakova, S. V., Hageman, J. C., Matava, M., Srinivasan, A., Phelan, L., Garfinkel, B., Jensen, B. (2005). A clone of methicillin-resistant Staphylococcus aureus among professional football players. *New England journal of medicine, 352*(5), 468-475.

Kelesidis, T., Humphries, R., Uslan, D. Z., & Pegues, D. A. (2011). Daptomycin nonsusceptible enterococci: an emerging challenge for clinicians. *Clinical Infectious Diseases, 52*(2), 228-234.

Kerschner, H., Harrison, E. M., Hartl, R., Holmes, M. A., & Apfalter, P. (2015). First report of mecC MRSA in human samples from Austria: molecular characteristics and clinical data. *New microbes and new infections, 3*, 4-9.

Khan, F., Ahmad, B., Ahmed, J., Yoo, H. S., & Bashir, S. (2018). Investigation of Staphylococcus aureus, prevailing in the environment of Khyber Teaching Hospital, Peshawar, Pakistan. *Pakistan journal of pharmaceutical sciences, 31*(2).

Khan, S., Rasheed, F., & Zahra, R. (2014). Genetic polymorphism of agr locus and antibiotic resistance of Staphylococcus aureus at two hospitals in Pakistan. *Pakistan journal of pharmaceutical sciences, 30*(1), 172.

Khan, T. M., Kok, Y. L., Bukhsh, A., Lee, L.-H., Chan, K.-G., & Goh, B.-H. (2018). Incidence of methicillin resistant Staphylococcus aureus (MRSA) in burn intensive care unit: a systematic review. *Germs, 8*(3), 113.

Khandavilli, S., Wilson, P., Cookson, B., Cepeda, J., Bellingan, G., & Brown, J. (2009). Utility of spa typing for investigating the local epidemiology of MRSA on a UK intensive care ward. *Journal of Hospital Infection, 71*(1), 29-35.

Khatib, R., Johnson, L., Sharma, M., Fakih, M., Ganga, R., & Riederer, K. (2009). Persistent Staphylococcus aureus bacteremia: incidence and outcome trends over time. *Scandinavian journal of infectious diseases, 41*(1), 4-9.

Khatoon, N., Bukhari, H., Riaz, J., Sheikh, A., Iqbal, A., & Naeem, S. (2002). Prevalence of methicillin resistant staphylococcus aureus (MRSA) infection laboratory study at Mayo Hospital Lahore. *Biomedica, 18*(2), 49-52.

Khattak, S. U., Bacha, N., Lutfullah, G., Bakht, J., Ali, S., Ali, J., & Khan, A. A. (2015). Study of the genetic traits associated with antibiotic resistance in Staphylococcus aureus isolated from skin wards of Khyber Pakhtunkhwa, Pakistan. *Asian Pacific Journal of Tropical Disease, 5*(5), 393-398.

Kheder, S. I., Ali, N. A., & Fathelrahman, A. I. (2012). Prevalence and antimicrobial susceptibility pattern of methicillin resistance staphylococcus in a sudanese surgical ward. *Pharmacology & Pharmacy, 3*(01), 103.

Khoramrooz, S. S., Dolatabad, S. A., Dolatabad, F. M., Marashifard, M., Mirzaii, M., Dabiri, H., Darban-Sarokhalil, D. (2017). Detection of tetracycline resistance genes, aminoglycoside modifying enzymes, and coagulase gene

typing of clinical isolates of Staphylococcus aureus in the Southwest of Iran. *Iranian Journal of Basic Medical Sciences, 20*(8), 912.

Khorvash, F., Mostafavizadeh, K., & Mobasherizadeh, S. (2008). Frequency of mecA gene and borderline oxacillin resistant Staphylococcus aureus in nosocomial acquired methicillin resistance Staphylococcus aureus infections. *Pakistan Journal of Biological Sciences, 11*(9), 1282-1285.

Khosravi, A. D., Hoveizavi, H., & Farshadzadeh, Z. (2012). The prevalence of genes encoding leukocidins in Staphylococcus aureus strains resistant and sensitive to methicillin isolated from burn patients in Taleghani Hospital, Ahvaz, Iran. *Burns, 38*(2), 247-251.

Kim, C., Milheiriço, C., Gardete, S., Holmes, M. A., Holden, M. T., de Lencastre, H., & Tomasz, A. (2012). Properties of a novel PBP2A protein homolog from Staphylococcus aureus strain LGA251 and its contribution to the β-lactam-resistant phenotype. *Journal of Biological Chemistry, 287*(44), 36854-36863.

Kim, H. B., Jang, H.-C., Nam, H. J., Lee, Y. S., Kim, B. S., Park, W. B., Oh, M.d. (2004). In vitro activities of 28 antimicrobial agents against Staphylococcus aureus isolates from tertiary-care hospitals in Korea: a nationwide survey. *Antimicrobial agents and chemotherapy, 48*(4), 1124-1127.

Kim, J.-S., Song, W., Kim, H.-S., Cho, H. C., Lee, K. M., Choi, M.-S., & Kim, E.-C. (2006). Association between the methicillin resistance of clinical isolates of Staphylococcus aureus, their staphylococcal cassette chromosome mec (SCCmec) subtype classification, and their toxin gene profiles. *Diagnostic microbiology and infectious disease, 56*(3), 289-295.

Kirby, W. M. (1944). Extraction of a highly potent penicillin inactivator from penicillin resistant staphylococci. *Science, 99*(2579), 452-453.

Kleerebezem, M., Quadri, L. E., Kuipers, O. P., & De Vos, W. M. (1997). Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Molecular microbiology, 24*(5), 895-904.

Kluytmans-VandenBergh, M., & Kluytmans, J. (2006). Community-acquired methicillin-resistant Staphylococcus aureus: current perspectives. *Clinical microbiology and infection, 12*, 9-15.

Ko, K. S., Lee, J. Y., Suh, J. Y., Oh, W. S., Peck, K. R., Lee, N. Y., & Song, J.-H. (2005). Distribution of major genotypes among methicillin-resistant Staphylococcus aureus clones in Asian countries. *Journal of clinical microbiology, 43*(1), 421-426.

Kondo, Y., Ito, T., Ma, X. X., Watanabe, S., Kreiswirth, B. N., Etienne, J., & Hiramatsu, K. (2007). Combination of multiplex PCRs for staphylococcal cassette chromosome mec type assignment: rapid identification system for mec, ccr, and major differences in junkyard regions. *Antimicrobial agents and chemotherapy, 51*(1), 264-274.

Konkel, M. E., & Tilly, K. (2000). Temperature-regulated expression of bacterial virulence genes. *Microbes and Infection, 2*(2), 157-166.

Kot, B., Wierzchowska, K., Piechota, M., & Grużewska, A. (2020). Antimicrobial resistance patterns in methicillin-resistant Staphylococcus aureus from patients hospitalized during 2015–2017 in hospitals in Poland. *Medical Principles and Practice, 29*(1), 61-68.

Kriegeskorte, A., Ballhausen, B., Idelevich, E. A., Köck, R., Friedrich, A. W., Karch, H., Becker, K. (2012). Human MRSA isolates with novel genetic homolog, Germany. *Emerging infectious diseases, 18*(6), 1016.

Kristich, C. J., Rice, L. B., & Arias, C. A. (2014). Enterococcal infection treatment and antibiotic resistance. *Enterococci: from commensals to leading causes of drug resistant infection [Internet]*.

Kubica, M., Guzik, K., Koziel, J., Zarebski, M., Richter, W., Gajkowska, B., Shaw, L. (2008). A potential new pathway for Staphylococcus aureus dissemination: the silent survival of S. aureus phagocytosed by human monocyte-derived macrophages. *PloS one, 3*(1), e1409.

Kuehnert, M. J., Kruszon-Moran, D., Hill, H. A., McQuillan, G., McAllister, S. K., Fosheim, G., Fridkin, S. K. (2006). Prevalence of Staphylococcus aureus nasal colonization in the United States, 2001–2002. *Journal of Infectious Diseases, 193*(2), 172-179.

Kumar, S., Joseph, N., Easow, J., Singh, R., Umadevi, S., Pramodhini, S., Kumari, G. (2012). Prevalence and current antibiogram of staphylococci isolated from various clinical specimens in a tertiary care hospital in Pondicherry. *International Jounal of Microbiology, 10*(1), 1937-8289.

Kumburu, H., Sonda, T., Leetcharoenphon, P., van Zwetselaar, M., Lukjancenko, O., Alifrangis, M., Aarestrup, F. M. (2017). *Hospital epidemiology of methicillin-resistant Staphylococcus aureus (MRSA) in a tertiary care hospital in Moshi Tanzania as determined by whole genome sequencing.* Paper presented at the 10th European Congress on Tropical Medicine and International Health.

Kumburu, H. H., Sonda, T., Leekitcharoenphon, P., van Zwetselaar, M., Lukjancenko, O., Alifrangis, M., Aarestrup, F. M. (2018). Hospital epidemiology of methicillin-resistant Staphylococcus aureus in a tertiary Care Hospital in Moshi, Tanzania, as determined by whole genome sequencing. *BioMed research international, 2018*.

Kwan, T., Liu, J., DuBow, M., Gros, P., & Pelletier, J. (2005). The complete genomes and proteomes of 27 Staphylococcus aureus bacteriophages. *Proceedings of the National Academy of Sciences, 102*(14), 5174-5179.

La Rosa, S. L., Casey, P. G., Hill, C., Diep, D. B., Nes, I. F., & Brede, D. A. (2013). In vivo assessment of growth and virulence gene expression during commensal and pathogenic lifestyles of luxABCDE-tagged Enterococcus faecalis strains in murine gastrointestinal and intravenous infection models. *Applied Enviromental Microbiology, 79*(13), 3986-3997.

Lacey, R. (1975). Antibiotic resistance plasmids of Staphylococcus aureus and their clinical importance. *Bacteriological reviews, 39*(1), 1.

Lacey, R., & Chopra, I. (1974). Genetic studies of a multi-resistant strain of Staphylococcus aureus. *Journal of medical microbiology, 7*(2), 285-297.

Larsen, H., Aarestrup, F. M., & Jensen, N. (2002). Geographical variation in the presence of genes encoding superantigenic exotoxins and β-hemolysin among Staphylococcus aureus isolated from bovine mastitis in Europe and USA. *Veterinary microbiology, 85*(1), 61-67.

Laurent, F., Chardon, H., Haenni, M., Bes, M., Reverdy, M.-E., Madec, J.-Y., Tristan, A. (2012). MRSA harboring mecA variant gene mecC, France. *Emerging infectious diseases, 18*(9), 1465.

Leclercq, R., Derlot, E., Duval, J., & Courvalin, P. (1988). Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium. *New England Journal of Medicine, 319*(3), 157-161.

Lee, S.Y., Park, Y.J., Yu, J. K., Jung, S., Kim, Y., Jeong, S. H., & Arakawa, Y. (2012). Prevalence of acquired fosfomycin resistance among extended-spectrum β-lactamase-producing Escherichia coli and Klebsiella pneumoniae clinical isolates in Korea and IS 26-composite transposon surrounding fosA3. *Journal of antimicrobial chemotherapy, 67*(12), 2843-2847.

Lee, Y.C., Chen, P. Y., Wang, J. T., & Chang, S. C. (2020). Prevalence of fosfomycin resistance and gene mutations in clinical isolates of methicillin-resistant Staphylococcus aureus. *Antimicrobial Resistance & Infection Control, 9*(1), 1-7.

Letter, A. (2011). Ministry of National Health Services, Regulations & Coordination Government of Pakistan National Institute of Health, Islamabad, Pakistan. *Journal of global infecfious diseases, 3*(3), 285.

Levine, D. P., Crane, L. R., & Zervos, M. J. (1986). Bacteremia in narcotic addicts at the Detroit Medical Center. II. Infectious endocarditis: a prospective comparative study. *Reviews of infectious diseases, 8*(3), 374-396.

Li, M., Diep, B. A., Villaruz, A. E., Braughton, K. R., Jiang, X., DeLeo, F. R., Otto, M. (2009). Evolution of virulence in epidemic community-associated methicillin-resistant Staphylococcus aureus. *Proceedings of the National Academy of Sciences, 106*(14), 5883-5888.

Li, V., Chui, L., Louie, L., Simor, A., Golding, G. R., & Louie, M. (2013). Cost-effectiveness and efficacy of spa, SCCmec, and PVL genotyping of methicillin-resistant Staphylococcus aureus as compared to pulsed-field gel Electrophoresis. *PloS one, 8*(11), e79149.

Li, X., Fang, F., Zhao, J., Lou, N., Li, C., Huang, T., & Li, Y. (2018). Molecular characteristics and virulence gene profiles of Staphylococcus aureus causing bloodstream infection. *Brazilian Journal of Infectious Diseases, 22*(6), 487-494.

Li, X., Huang, T., Xu, K., Li, C., & Li, Y. (2019). Molecular characteristics and virulence gene profiles of Staphylococcus aureus isolates in Hainan, China. *BMC infectious diseases, 19*(1), 1-12.

Liang, Y., Tu, C., Tan, C., & El-Sayed, M. A. E.-G. (2019). Antimicrobial resistance, virulence genes profiling and molecular relatedness of methicillin-resistant Staphylococcus aureus strains isolated from hospitalized patients in Guangdong Province, China. *Infection and drug resistance, 12*, 447.

Lim, K., Hanifah, Y., Yusof, M., & Thong, K. L. (2012). ermA, ermC, tetM and tetK are essential for erythromycin and tetracycline resistance among methicillin-resistant Staphylococcus aureus strains isolated from a tertiary hospital in Malaysia. *Indian journal of medical microbiology, 30*(2), 203.

Lim, S. M., & Webb, S. A. R. (2005). Nosocomial bacterial infections in Intensive Care Units. I: Organisms and mechanisms of antibiotic resistance. *Anaesthesia, 60*(9), 887-902.

Liu, C., Chen, Z.-j., Sun, Z., Feng, X., Zou, M., Cao, W., Sun, M. (2015). Molecular characteristics and virulence factors in methicillin-susceptible, resistant, and heterogeneous vancomycin-intermediate Staphylococcus aureus from central-southern China. *Journal of Microbiology, Immunology and Infection, 48*(5), 490-496.

Liu, C., Chen, Z.-j., Sun, Z., Feng, X., Zou, M., Cao, W., Infection. (2015). Molecular characteristics and virulence factors in methicillin-susceptible, resistant, and heterogeneous vancomycin-intermediate Staphylococcus aureus from central-southern China. *Journal of Microbiology, Immunology and Infection, 48*(5), 490-496.

Liu, Y., Kong, F., Xiao, M., Wang, Q., O'Sullivan, M., Sintchenko, V., Gilbert, G. L. (2010). Unexpected diversity of staphylococcal cassette chromosome mec Type IV in methicillin-resistant Staphylococcus aureus strains. *Journal of clinical microbiology, 48*(10), 3628-3634.

Llarrull, L. I., Fisher, J. F., & Mobashery, S. (2009). Molecular basis and phenotype of methicillin resistance in Staphylococcus aureus and insights into new β-lactams that meet the challenge. *Antimicrobial agents and chemotherapy, 53*(10), 4051-4063.

Loeffler, A., Boag, A. K., Sung, J., Lindsay, J. A., Guardabassi, L., Dalsgaard, A., Lloyd, D. H. (2005). Prevalence of methicillin-resistant Staphylococcus aureus among staff and pets in a small animal referral hospital in the UK. *Journal of antimicrobial chemotherapy, 56*(4), 692-697.

Löffler, B., Hussain, M., Grundmeier, M., Brück, M., Holzinger, D., Varga, G., Peters, G. (2010). Staphylococcus aureus panton-valentine leukocidin is a very potent cytotoxic factor for human neutrophils. *PLoS pathogens, 6*(1).

Loncaric, I., Kubber-Heiss, A., Posautz, A., Stalder, G. L., Hoffmann, D., Rosengarten, R., & Walzer, C. (2013). Characterization of methicillin-resistant Staphylococcus spp. carrying the mecC gene, isolated from wildlife. *Journal of antimicrobial chemotherapy, 68*(10), 2222-2225.

Long, K. S., & Vester, B. (2012). Resistance to linezolid caused by modifications at its binding site on the ribosome. *Antimicrobial agents and chemotherapy, 56*(2), 603-612.

Loomba, P. S., Taneja, J., & Mishra, B. (2010). Methicillin and vancomycin resistant S. aureus in hospitalized patients. *Journal of global infectious diseases, 2*(3), 275.

Loubet, P., Burdet, C., Vindrios, W., Grall, N., Wolff, M., Yazdanpanah, Y., Lescure, F.-X. (2018). Cefazolin versus anti-staphylococcal penicillins for treatment of methicillin-susceptible Staphylococcus aureus bacteraemia: a narrative review. *Clinical microbiology and infection, 24*(2), 125-132.

Lowder, B. V., Guinane, C. M., Zakour, N. L. B., Weinert, L. A., Conway-Morris, A., Cartwright, R. A., Fitzgerald, J. R. (2009). Recent human-to-poultry host jump, adaptation, and pandemic spread of Staphylococcus aureus. *Proceedings of the National Academy of Sciences, 106*(46), 19545-19550.

Lowy, F. D. (1998). Staphylococcus aureus infections. *New England journal of medicine, 339*(8), 520-532.

Lowy, F. D. (2003). Antimicrobial resistance: the example of Staphylococcus aureus. *The Journal of clinical investigation, 111*(9), 1265-1273.

Lu, C. L., Liu, C. Y., Huang, Y. T., Liao, C. H., Teng, L. J., Turnidge, J. D., & Hsueh, P.-R. (2011). Antimicrobial Susceptibility of Commonly Encountered Bacterial Isolates to Fosfomycin as Determined by the Agar Dilution and Disk Diffusion Methods. *Antimicrobial agents and chemotherapy*, AAC. 00349-00311.

Lu, J.-J., Perng, C.-L., Lee, S.-Y., & Wan, C.-C. (2000). Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. *Journal of clinical microbiology, 38*(6), 2076-2080.

Lunde, T. M., Hjerde, E., & Al-Haroni, M. (2021). Prevalence, diversity and transferability of the Tn 916-Tn 1545 family ICE in oral streptococci. *Journal of oral microbiology, 13*(1), 1896874.

Lyon, B. R., Gillespie, M. T., & Skurray, R. A. (1987). Detection and characterization of IS256, an insertion sequence in Staphylococcus aureus. *Microbiology, 133*(11), 3031-3038.

Lyon, G. J., Mayville, P., Muir, T. W., & Novick, R. P. (2000). Rational design of a global inhibitor of the virulence response in Staphylococcus aureus, based in part on localization of the site of inhibition to the receptor-histidine kinase, AgrC. *Proceedings of the National Academy of Sciences, 97*(24), 13330-13335.

Ma, X. X., Ito, T., Tiensasitorn, C., Jamklang, M., Chongtrakool, P., Boyle-Vavra, S., Hiramatsu, K. (2002). Novel type of staphylococcal cassette chromosome mec identified in community-acquired methicillin-resistant Staphylococcus aureus strains. *Antimicrobial agents and chemotherapy, 46*(4), 1147-1152.

Ma, Y., Xu, Y., Yestrepsky, B. D., Sorenson, R. J., Chen, M., Larsen, S. D., & Sun, H. (2012a). Novel inhibitors of Staphylococcus aureus virulence gene expression and biofilm formation. *PloS one, 7*(10).

Madigan, M. T., J. M. Martinko, and T. D. Brock. (2006). *Brock Biology of Microorganisms* (11 ed. ed.). Pearson, Prentice Hall, London: Upper Saddle River, NJ.

Magiorakos, A.-P., Srinivasan, A., Carey, R., Carmeli, Y., Falagas, M., Giske, C., Olsson-Liljequist, B. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical microbiology and infection, 18*(3), 268-281.

Majerczyk, C. D., Sadykov, M. R., Luong, T. T., Lee, C., Somerville, G. A., & Sonenshein, A. L. (2008). Staphylococcus aureus CodY negatively regulates virulence gene expression. *Journal of bacteriology, 190*(7), 2257-2265.

Marais, E., Aithma, N., Perovic, O., Oosthuysen, W., Musenge, E., & Dusé, A. (2009). Antimicrobial susceptibility of methicillin-resistant Staphylococcus aureus isolates from South Africa. *SAMJ: South African Medical Journal, 99*(3), 170-173.

Martineau, F., Picard, F. J., Lansac, N., Ménard, C., Roy, P. H., Ouellette, M., & Bergeron, M. G. (2000). Correlation between the Resistance Genotype Determined by Multiplex PCR Assays and the Antibiotic Susceptibility Patterns of Staphylococcus aureus andStaphylococcus epidermidis. *Antimicrobial agents and chemotherapy, 44*(2), 231-238.

Masaisa, F., Kayigi, E., Seni, J., Bwanga, F., & Muvunyi, C. M. (2018). Antibiotic resistance patterns and molecular characterization of methicillin-resistant Staphylococcus aureus in clinical settings in Rwanda. *The American journal of tropical medicine and hygiene, 99*(5), 1239-1245.

Mascaro, V., Squillace, L., Nobile, C. G., Papadopoli, R., Bosch, T., Schouls, L. M., Pavia, M. (2019). Prevalence of methicillin-resistant Staphylococcus aureus (MRSA) carriage and pattern of antibiotic resistance among sheep farmers from Southern Italy. *Infection and Drug Resistance, 12*, 2561.

Mawalla, B., Mshana, S. E., Chalya, P. L., Imirzalioglu, C., & Mahalu, W. (2011). Predictors of surgical site infections among patients undergoing major surgery at Bugando Medical Centre in Northwestern Tanzania. *BMC surgery, 11*(1), 21.

Mayville, P., Ji, G., Beavis, R., Yang, H., Goger, M., Novick, R. P., & Muir, T. W. (1999). Structure-activity analysis of synthetic autoinducing thiolactone peptides from Staphylococcus aureus responsible for virulence. *Proceedings of the National Academy of Sciences, 96*(4), 1218-1223.

Mazi, W., Sangal, V., Sandstrom, G., Saeed, A., & Yu, J. (2015). Evaluation of spa-typing of methicillin-resistant Staphylococcus aureus using high-resolution melting analysis. *International journal of infectious diseases, 38*, 125-128.

McClure-Warnier, J.-A., Conly, J. M., & Zhang, K. (2013). Multiplex PCR assay for typing of staphylococcal cassette chromosome mec types I to V in methicillin-resistant Staphylococcus aureus. *Journal of visualized experiments: JoVE*(79).

McClure, J.-A., Conly, J. M., Lau, V., Elsayed, S., Louie, T., Hutchins, W., & Zhang, K. (2006). Novel multiplex PCR assay for detection of the staphylococcal virulence marker Panton-Valentine leukocidin genes and simultaneous discrimination of methicillin-susceptible from-resistant staphylococci. *Journal of clinical microbiology, 44*(3), 1141-1144.

McDougal, L. K., Steward, C. D., Killgore, G. E., Chaitram, J. M., McAllister, S. K., & Tenover, F. C. (2003). Pulsed-field gel electrophoresis typing of oxacillin-resistant Staphylococcus aureus isolates from the United States: establishing a national database. *Journal of clinical microbiology, 41*(11), 5113-5120.

Medhus, A., Slettemeås, J. S., Marstein, L., Larssen, K. W., & Sunde, M. (2012). Methicillin-resistant Staphylococcus aureus with the novel mecC gene variant isolated from a cat suffering from chronic conjunctivitis. *Journal of antimicrobial chemotherapy, 68*(4), 968-969.

Mehmood, A., Butt, T., & Usman, M. (2007). A study on MRSA isolates to detect reduced susceptibility to vancomycin: A preliminary report.

Mehrotra, M., Wang, G., & Johnson, W. M. (2000). Multiplex PCR for detection of genes forStaphylococcus aureus enterotoxins, exfoliative toxins, toxic shock

syndrome toxin 1, and methicillin resistance. *Journal of clinical microbiology, 38*(3), 1032-1035.

Meier, S., Goerke, C., Wolz, C., Seidl, K., Homerova, D., Schulthess, B., . . . Bischoff, M. (2007). σB and the σB-dependent arlRS and yabJ-spoVG loci affect capsule formation in Staphylococcus aureus. *Infection and immunity, 75*(9), 4562-4571.

Merino, N., Toledo-Arana, A., Vergara-Irigaray, M., Valle, J., Solano, C., Calvo, E., Lasa, I. (2009). Protein A-mediated multicellular behavior in Staphylococcus aureus. *Journal of bacteriology, 191*(3), 832-843.

Micek, S. T., Kollef, K. E., Reichley, R. M., Roubinian, N., & Kollef, M. H. (2007). Health care-associated pneumonia and community-acquired pneumonia: a single-center experience. *Antimicrobial agents and chemotherapy, 51*(10), 3568-3573.

Michalopoulos, A. S., Livaditis, I. G., & Gougoutas, V. (2011). The revival of fosfomycin. *International journal of infectious diseases, 15*(11), e732-e739.

Mick, V., Domínguez, M. A., Tubau, F., Liñares, J., Pujol, M., & Martín, R. (2010). Molecular characterization of resistance to Rifampicin in an emerging hospital-associated Methicillin-resistant Staphylococcus aureus clone ST228, Spain. *BMC microbiology, 10*(1), 1.

Milheiriço, C., Oliveira, D. C., & de Lencastre, H. (2007a). Multiplex PCR strategy for subtyping the staphylococcal cassette chromosome mec type IV in methicillin-resistant Staphylococcus aureus:'SCCmec IV multiplex'. *Journal of antimicrobial chemotherapy, 60*(1), 42-48.

Milheiriço, C., Oliveira, D. C., & de Lencastre, H. (2007b). Update to the multiplex PCR strategy for assignment of mec element types in Staphylococcus aureus. *Antimicrobial agents and chemotherapy, 51*(9), 3374-3377.

Miller, L. G., Eells, S. J., Taylor, A. R., David, M. Z., Ortiz, N., Zychowski, D., Daum, R. S. (2012). Staphylococcus aureus colonization among household contacts of patients with skin infections: risk factors, strain discordance, and complex ecology. *Clinical Infectious Diseases, 54*(11), 1523-1535.

Mir, Z., Farahani, N. N., Abbasian, S., Alinejad, F., Sattarzadeh, M., Pouriran, R., Darban-Sarokhalil, D. (2019). The prevalence of exotoxins, adhesion, and biofilm-related genes in Staphylococcus aureus isolates from the main burn center of Tehran, Iran. *Iranian Journal of Basic Medical Sciences, 22*(11), 1267.

Mirani, Z. A., Aqeel, A., & Naz, S. (2017). Prevalence of staphylococci in commercially processed food products in Karachi-Pakistan. *Journal of Microbiology and Infectious Diseases, 7*(02), 83-87.

Mirkarimi, S. F., Hasani, A., Abdinia, B., Barhaghi, M. H. S., Nikbakht, M., & Rezaee, M. A. (2016). High diversity of methicillin-resistant Staphylococcus aureus (MRSA) isolates based on hypervariable region polymorphisms. *Archives de Pédiatrie, 4*(4), e36433.

Mirza, S. (2007). Minimising Antibiotic Resistance–Is There a Way Forward. *Infectious Disease Journal, 7*, 5-9.

Mitchell, G., Lafrance, M., Boulanger, S., Séguin, D. L., Guay, I., Gattuso, M., . . . Malouin, F. (2011). Tomatidine acts in synergy with aminoglycoside antibiotics against multiresistant Staphylococcus aureus and prevents

virulence gene expression. *Journal of antimicrobial chemotherapy, 67*(3), 559-568.

Moellering Jr, R. C. (2008). Current treatment options for community-acquired methicillin-resistant Staphylococcus aureus infection. *Clinical Infectious Diseases, 46*(7), 1032-1037.

Mohammadi, S., Sekawi, Z., Monjezi, A., Maleki, M.-H., Soroush, S., Sadeghifard, N., Asadollahi, K. (2014). Emergence of SCCmec type III with variable antimicrobial resistance profiles and spa types among methicillin-resistant Staphylococcus aureus isolated from healthcare-and community-acquired infections in the west of Iran. *International journal of infectious diseases, 25*, 152-158.

Mohsenzadeh, M., Ghazvini, K., & Azimian, A. (2015). *Frequency of specific agr groups and antibiotic resistance in Staphylococcus aureus isolated from bovine mastitis in the northeast of Iran.* Paper presented at the Veterinary Research Forum.

Monecke, S., Gavier-Widen, D., Mattsson, R., Rangstrup-Christensen, L., Lazaris, A., Coleman, D. C., Ehricht, R. (2013). Detection of mecC-positive Staphylococcus aureus (CC130-MRSA-XI) in diseased European hedgehogs (Erinaceus europaeus) in Sweden. *PloS one, 8*(6), e66166.

Monecke, S., Müller, E., Büchler, J., Stieber, B., & Ehricht, R. (2014). Staphylococcus aureus in vitro secretion of alpha toxin (hla) correlates with the affiliation to clonal complexes. *PloS one, 9*(6), e100427.

Monecke, S., Skakni, L., Hasan, R., Ruppelt, A., Ghazal, S. S., Hakawi, A., Ehricht, R. (2012). Characterisation of MRSA strains isolated from patients in a hospital in Riyadh, Kingdom of Saudi Arabia. *BMC microbiology, 12*(1), 146.

Moore, P., & Lindsay, J. (2001). Genetic variation among hospital isolates of methicillin-sensitive Staphylococcus aureus: evidence for horizontal transfer of virulence genes. *Journal of clinical microbiology, 39*(8), 2760-2767.

Moosavian, M., Dehkordi, P. B., & Hashemzadeh, M. (2020). Characterization of SCCmec, Spa Types and Multidrug Resistant of Methicillin-Resistant Staphylococcus aureus Isolates in Ahvaz, Iran. *Infection and Drug Resistance, 13*, 1033.

Mootz, J. M., Benson, M. A., Heim, C. E., Crosby, H. A., Kavanaugh, J. S., Dunman, P. M., Horswill, A. R. (2015). R ot is a key regulator of S taphylococcus aureus biofilm formation. *Molecular microbiology, 96*(2), 388-404.

Moremi, N., Mshana, S. E., Kamugisha, E., Kataraihya, J., Tappe, D., Vogel, U., Claus, H. (2012). Predominance of methicillin resistant Staphylococcus aureus-ST88 and new ST1797 causing wound infection and abscesses. *The Journal of Infection in Developing Countries, 6*(08), 620-625.

Morikawa, K., Inose, Y., Okamura, H., Maruyama, A., Hayashi, H., Takeyasu, K., & Ohta, T. (2003). A new staphylococcal sigma factor in the conserved gene cassette: functional significance and implication for the evolutionary processes. *Genes to Cells, 8*(8), 699-712.

Morinaga, N., Kaihou, Y., & Noda, M. (2003). Purification, cloning and characterization of variant LukE-LukD with strong leukocidal activity of

staphylococcal bi-component leukotoxin family. *Microbiology and immunology, 47*(1), 81-90.

Moroney, S. M., Heller, L. C., Arbuckle, J., Talavera, M., & Widen, R. H. (2007). Staphylococcal cassette chromosome mec and Panton-Valentine leukocidin characterization of methicillin-resistant Staphylococcus aureus clones. *Journal of clinical microbiology, 45*(3), 1019-1021.

Morosini, M.-I., Díez-Aguilar, M., & Cantón, R. (2019). Mechanisms of action and antimicrobial activity of ceftobiprole. *Revista Española de Quimioterapia, 32*(Suppl 3), 3.

Mossong, J., Gelhausen, E., Decruyenaere, F., Devaux, A., Perrin, M., Even, J., & Heisbourg, E. (2013). Prevalence, risk factors and molecular epidemiology of methicillin-resistant Staphylococcus aureus (MRSA) colonization in residents of long-term care facilities in Luxembourg, 2010. *Epidemiology & Infection, 141*(6), 1199-1206.

Motamedi, H., Asghari, B., Tahmasebi, H., & Arabestani, M. R. (2018). Identification of hemolysine genes and their association with antimicrobial resistance pattern among clinical isolates of Staphylococcus aureus in West of Iran. *Advanced biomedical research, 7*.

Mullarky, I., Su, C., Frieze, N., Park, Y., & Sordillo, L. (2001). Staphylococcus aureus agr genotypes with enterotoxin production capabilities can resist neutrophil bactericidal activity. *Infection and immunity, 69*(1), 45-51.

Mylonakis, E., Moreno, R., El Khoury, J. B., Idnurm, A., Heitman, J., Calderwood, S. B., (2005). Galleria mellonella as a model system to study Cryptococcus neoformans pathogenesis. *Infection and immunity, 73*(7), 3842-3850.

Nadig, S., Velusamy, N., Lalitha, P., Kar, S., Sharma, S., & Arakere, G. (2012). Staphylococcus aureus eye infections in two Indian hospitals: emergence of ST772 as a major clone. *Clinical Ophthalmology (Auckland, NZ), 6*, 165.

Naeem, M., Adil, M., Naz, S. M., Abbas, S. H., Khan, I., Khan, A., & Khan, M. U. (2013). Resistance and sensitivity pattern of Staphylococcus aureus; A study in Lady Reading Hospital Peshawar. *JPMI: Journal of Postgraduate Medical Institute, 27*(1).

Nagaev, I., Björkman, J., Andersson, D. I., & Hughes, D. (2001). Biological cost and compensatory evolution in fusidic acid-resistant Staphylococcus aureus. *Molecular microbiology, 40*(2), 433-439.

Nahar, N., Rashid, R. B., Kabir, A. H., & Rahman, M. S. (2017). In Silico Virulence and Resistance Profile Analysis of Staphylococcus species. *Bangladesh Pharmaceutical Journal, 20*(1), 71-84.

Nahvi, M. D., Fitzgibbon, J. E., John, J. F., & Dubin, D. T. (2001). Sequence analysis of dru regions from methicillin-resistant Staphylococcus aureus and coagulase-negative staphylococcal isolates. *Microbial Drug Resistance, 7*(1), 1-12.

Naimi, H. M., Rasekh, H., Noori, A. Z., & Bahaduri, M. A. (2017). Determination of antimicrobial susceptibility patterns in Staphylococcus aureus strains recovered from patients at two main health facilities in Kabul, Afghanistan. *BMC Infectious diseases, 17*(1), 737.

Naqvi, Z. A., Hashmi, K., & Kharal, S. A. (2007). Methicillin resistant Staphylococcus aureus (MRSA) in burn patients. *Pakistan Journal of Pharmacology, 24*(2), 7-11.

Neela, V., Ehsanollah, G. R., Zamberi, S., Van Belkum, A., & Mariana, N. S. (2009). Prevalence of Panton–Valentine leukocidin genes among carriage and invasive Staphylococcus aureus isolates in Malaysia. *International journal of infectious diseases, 13*(3), e131-e132.

Neu, H. C. (1992). The crisis in antibiotic resistance. *Science, 257*(5073), 1064-1073.

Nguyen, D. M., Mascola, L., & Brancoft, E. (2005). Recurring methicillin-resistant Staphylococcus aureus infections in a football team. *Emerging infectious diseases, 11*(4), 526-532. doi:10.3201/eid1104.041094

Nia, N. Z., Pourmand, M. R., & Afrough, P. (2013). Comparison of hypervariable region (hvr) of meca gene in staphylococcus aureus isolated from nasal carriers and clinical samples. *Jundishapur Journal of Microbiology, 6*(9), A1.

Nimmo, G. R., Bell, J. M., Mitchell, D., Gosbell, I. B., Pearman, J. W., & Turnidge, J. D. (2003). Antimicrobial resistance in Staphylococcus aureus in Australian teaching hospitals, 1989-1999. *Microbial Drug Resistance, 9*(2), 155-160.

Nishi, J., Miyanohara, H., Nakajima, T., Kitajima, I., Yoshinaga, M., Maruyama, I., & Miyata, K. (1995a). Molecular typing of the methicillin resistance determinant (mec) of clinical strains of Staphylococcus based on mec hypervariable region length polymorphisms. *The Journal of laboratory and clinical medicine, 126*(1), 29.

Nishi, J., Miyanohara, H., Nakajima, T., Kitajima, I., Yoshinaga, M., Maruyama, I., & Miyata, K. (1995b). Molecular typing of the methicillin resistance determinant (mec) of clinical strains of Staphylococcus based on mec hypervariable region length polymorphisms. *The Journal of laboratory clinical medicine, 126*(1), 29-35.

Nizet, V. (2007). Understanding how leading bacterial pathogens subvert innate immunity to reveal novel therapeutic targets. *Journal of Allergy and Clinical Immunology, 120*(1), 13-22.

Normark, B. H., & Normark, S. (2002). Evolution and spread of antibiotic resistance. *Journal of internal medicine, 252*(2), 91-106.

Novick, R., Projan, S., Kornblum, J., Ross, H., Ji, G., Kreiswirth, B., Moghazeh, S. (1995). Theagr P2 operon: An autocatalytic sensory transduction system inStaphylococcus aureus. *Molecular and General Genetics MGG, 248*(4), 446-458.

Novick, R. P., & Geisinger, E. (2008). Quorum sensing in staphylococci. *Annual review of genetics, 42*, 541-564.

Ntirenganya, C., Manzi, O., Muvunyi, C. M., & Ogbuagu, O. (2015). High prevalence of antimicrobial resistance among common bacterial isolates in a tertiary healthcare facility in Rwanda. *The American journal of tropical medicine and hygiene, 92*(4), 865-870.

Nulens, E., Stobberingh, E. E., van Dessel, H., Sebastian, S., van Tiel, F. H., Beisser, P. S., & Deurenberg, R. H. (2008). Molecular characterization of Staphylococcus aureus bloodstream isolates collected in a Dutch University

Hospital between 1999 and 2006. *Journal of clinical microbiology, 46*(7), 2438-2441.

Nurjadi, D., Olalekan, A. O., Layer, F., Shittu, A. O., Alabi, A., Ghebremedhin, B., Caumes, E. (2014). Emergence of trimethoprim resistance gene dfrG in Staphylococcus aureus causing human infection and colonization in sub-Saharan Africa and its import to Europe. *Journal of antimicrobial chemotherapy, 69*(9), 2361-2368.

Nüsing, R., Goerig, M., Habenicht, A., & Ullrich, V. (1993). Selective eicosanoid formation during HL-60 macrophage differentiation: Regulation of thromboxane synthase. *European journal of biochemistry, 212*(2), 371-376.

O'Gara, J. P. (2007). ica and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aureus. *FEMS microbiology letters, 270*(2), 179-188.

O'Hara, F. P., Suaya, J. A., Ray, G. T., Baxter, R., Brown, M. L., Mera, R. M., Amrine-Madsen, H. (2016). spa typing and multilocus sequence typing show comparable performance in a macroepidemiologic study of Staphylococcus aureus in the United States. *Microbial Drug Resistance, 22*(1), 88-96.

O'Keeffe, K. M., Wilk, M. M., Leech, J. M., Murphy, A. G., Laabei, M., Monk, I. R., Geoghegan, J. A. (2015). Manipulation of autophagy in phagocytes facilitates Staphylococcus aureus bloodstream infection. *Infection and immunity, 83*(9), 3445-3457.

O'Neill, A. J., & Chopra, I. (2006). Molecular basis of fusB-mediated resistance to fusidic acid in Staphylococcus aureus. *Molecular microbiology, 59*(2), 664-676.

Ogston, A. (1882). Micrococcus poisoning. *Journal of anatomy and physiology, 16*(Pt 4), 526.

Ojulong, J., Mwambu, T., Jolobo, M., Agwu, E., Bwanga, F., Najjuka, C., & Kaddu-Mulindwa, D. (2008). Prevalence of Methicillin resistant Staphylococcus aureus (MRSA) among isolates from surgical site infections in Mulago hospital-Kampala, Uganda. *The Internet Journal of Infectious Diseases, 7*(2), 34-45.

Okuma, K., Iwakawa, K., Turnidge, J. D., Grubb, W. B., Bell, J. M., O'Brien, F. G., Kapi, M. (2002). Dissemination of new methicillin-resistant Staphylococcus aureus clones in the community. *Journal of clinical microbiology, 40*(11), 4289-4294.

Oliveira, D. C., & de Lencastre, H. (2002). Multiplex PCR strategy for rapid identification of structural types and variants of the mec element in methicillin-resistant Staphylococcus aureus. *Antimicrobial agents and chemotherapy, 46*(7), 2155-2161.

Oliveira, D. C., Milheiriço, C., & de Lencastre, H. (2006). Redefining a structural variant of staphylococcal cassette chromosome mec, SCCmec type VI. *Antimicrobial agents and chemotherapy, 50*(10), 3457-3459.

Olsen, R. J., Watkins, M. E., Cantu, C. C., Beres, S. B., & Musser, J. M. (2011). Virulence of serotype M3 Group A Streptococcus strains in wax worms (Galleria mellonella larvae). *Virulence, 2*(2), 111-119.

Omuse, G., Zyl, K. N., Hoek, K., Abdulgader, S., Kariuki, S., Whitelaw, A., & Revathi, G. (2016). Molecular characterization of Staphylococcus aureus

isolates from various healthcare institutions in Nairobi, Kenya: a cross sectional study. *Annals of Clinical Microbiology and Antimicrobials, 15*(1), 51.

Ong, M. H. L., Ho, W. Y., Ng, W. W., & Chew, C.-H. (2017). High prevalence of tetM as compared to tetK amongst methicillin-resistant Staphylococcus aureus (MRSA) isolates from hospitals in Perak, Malaysia. *Jundishapur Journal of Microbiology, 10*(6).

Oscarsson, J., Tegmark-Wisell, K., & Arvidson, S. (2006). Coordinated and differential control of aureolysin (aur) and serine protease (sspA) transcription in Staphylococcus aureus by sarA, rot and agr (RNAIII). *International journal of medical microbiology, 296*(6), 365-380.

Osman, K. M., Amer, A. M., Badr, J. M., Helmy, N. M., Elhelw, R. A., Orabi, A., . . . Saad, A. S. (2016). Antimicrobial resistance, biofilm formation and mecA characterization of methicillin-susceptible S. aureus and non-S. aureus of beef meat origin in Egypt. *Frontiers in microbiology, 7*, 222.

Otto, M. (2008). Staphylococcal biofilms. *Bacterial biofilms*, 207-228.

Ouchenane, Z., Smati, F., Rolain, J.-M., & Raoult, D. (2011). Molecular characterization of methicillin-resistant Staphylococcus aureus isolates in Algeria. *Pathologie Biologie, 59*(6), e129-e132.

Paddock, F. B. (1918). *The beemoth or waxworm*: Texas Agricultural Experiment Stations.

Pal, N., Sharma, B., Sharma, R., & Vyas, L. (2010). Detection of inducible clindamycin resistance among Staphylococcal isolates from different clinical specimens in western India. *Journal of postgraduate medicine, 56*(3), 182.

Pandya, N., Chaudhary, A., Mehta, S., & Parmar, R. (2014). Characterization of methicillin resistant Staphylococcus aureus from various clinical samples at tertiary care hospital of rural Gujarat. *Journal of research in medical and dental sciences, 2*, 49-53.

Panton, P., Came, M., & Valentine, F. (1932). Staphylococcal toxin. *The Lancet*.

Paterson, G., Morgan, F., Harrison, E., Cartwright, E., Török, M., Zadoks, R., Holmes, M. (2014). Prevalence and characterization of human mecC methicillin-resistant Staphylococcus aureus isolates in England. *Journal of antimicrobial chemotherapy, 69*(4), 907-910.

Paterson, G. K., Larsen, A., Robb, A., Edwards, G., Pennycott, T., Foster, G., Peacock, S. (2012). The newly described mecA homologue, mecA LGA251, is present in methicillin-resistant Staphylococcus aureus isolates from a diverse range of host species. *Journal of antimicrobial chemotherapy, 67*(12), 2809-2813.

Patot, S., Imbert, P. R., Baude, J., Simoes, P. M., Campergue, J.-B., Louche, A., Laurent, F. (2017). The TIR homologue lies near resistance genes in Staphylococcus aureus, coupling modulation of virulence and antimicrobial susceptibility. *PLoS pathogens, 13*(1).

Paulin, J., & Samson, R. (1973). *feu bacterien en France. II. Caracteres des souches d'Erwinia amylovora (Burril) Winslow et al., 1920, isolees du foyer franco-belge.* Paper presented at the Annales de phytopathologie.

Peacock, S. J., de Silva, I., & Lowy, F. D. (2001). What determines nasal carriage of Staphylococcus aureus? *Trends in microbiology, 9*(12), 605-610.

Peerayeh, S. N., Azimian, A., Nejad, Q. B., & Kashi, M. (2009). Prevalence of agr specificity groups among Staphylococcus aureus isolates from university hospitals in Tehran. *Laboratory Medicine, 40*(1), 27-29.

Peleg, A. Y., Jara, S., Monga, D., Eliopoulos, G. M., Moellering, R. C., & Mylonakis, E. (2009). Galleria mellonella as a model system to study Acinetobacter baumannii pathogenesis and therapeutics. *Antimicrobial agents and chemotherapy, 53*(6), 2605-2609.

Peleg, A. Y., Monga, D., Pillai, S., Mylonakis, E., Moellering Jr, R. C., & Eliopoulos, G. M. (2009). Reduced susceptibility to vancomycin influences pathogenicity in Staphylococcus aureus infection. *The Journal of infectious diseases, 199*(4), 532-536.

Périchon, B., & Courvalin, P. (2009). VanA-type vancomycin-resistant Staphylococcus aureus. *Antimicrobial agents and chemotherapy, 53*(11), 4580-4587.

Perumal, N., Murugesan, S., & Krishnan, P. (2016). Distribution of genes encoding aminoglycoside-modifying enzymes among clinical isolates of methicillin-resistant staphylococci. *Indian journal of medical microbiology, 34*(3), 350.

Perveen, I., Majid, A., Knawal, S., Naz, I., Sehar, S., Ahmed, S., & Raza, M. A. (2013). Prevalence and Antimicrobial Susceptibility Pattern of Methicillin-Resistant Staphylococcus aureus and Coagulase-Negative Staphylococci in Rawalpindi, Pakistan. *British Journal of Medicine and Medical Research*.

Perwaiz, S., Barakzi, Q., Farooqi, B. J., Khursheed, N., & Sabir, N. (2007). Antimicrobial susceptibility pattern of clinical isolates of methicillin resistant Staphylococcus aureus. *Journal of Pakistan Medical Association, 57*(1), 2.

Petersen, A., Stegger, M., Heltberg, O., Christensen, J., Zeuthen, A., Knudsen, L.,Larsen, J. (2013a). Epidemiology of methicillin-resistant Staphylococcus aureus carrying the novel mecC gene in Denmark corroborates a zoonotic reservoir with transmission to humans. *Clinical microbiology and infection, 19*(1), E16-E22.

Petrelli, D., Repetto, A., D'ercole, S., Rombini, S., Ripa, S., Prenna, M., & Vitali, L. A. (2008). Analysis of meticillin-susceptible and meticillin-resistant biofilm-forming Staphylococcus aureus from catheter infections isolated in a large Italian hospital. *Journal of medical microbiology, 57*(3), 364-372.

Pichon, B., Hill, R., Laurent, F., Larsen, A. R., Skov, R. L., Holmes, M., Kearns, A. M. (2012). Development of a real-time quadruplex PCR assay for simultaneous detection of nuc, Panton–Valentine leucocidin (PVL), mecA and homologue mecA LGA251. *Journal of antimicrobial chemotherapy, 67*(10), 2338-2341.

Pillar, C. M., Draghi, D. C., Sheehan, D. J., & Sahm, D. F. (2008). Prevalence of multidrug-resistant, methicillin-resistant Staphylococcus aureus in the United States: findings of the stratified analysis of the 2004 to 2005 LEADER Surveillance Programs. *Diagnostic microbiology and infectious disease, 60*(2), 221-224.

Pinheiro, L., Brito, C. I., De Oliveira, A., Martins, P. Y. F., Pereira, V. C., & Da Cunha, M. D. L. R. (2015). Staphylococcus epidermidis and Staphylococcus haemolyticus: molecular detection of cytotoxin and enterotoxin genes. *Toxins, 7*(9), 3688-3699.

Pinho, M. G., Kjos, M., & Veening, J.-W. (2013). How to get (a) round: mechanisms controlling growth and division of coccoid bacteria. *Nature reviews microbiology, 11*(9), 601-614.

Pirko, E. Y., Tektook, N. K., sheet Saleh, M. M., & Jaffar, Z. A. (2019). Prevalence of methicillin resistance Staphylococcus aureus (MRSA) and methicillin sensitivity Staphylococcus aureus (MSSA) among hospitalized Iraqi patients. *Biomedical Research, 30*(6).

Pohl, K., Francois, P., Stenz, L., Schlink, F., Geiger, T., Herbert, S., Wolz, C. (2009). CodY in Staphylococcus aureus: a regulatory link between metabolism and virulence gene expression. *Journal of bacteriology, 191*(9), 2953-2963.

Pokhrel, R. H., Aung, M. S., Thapa, B., Chaudhary, R., Mishra, S., Kawaguchiya, M., . . . Kobayashi, N. (2016). Detection of ST772 Panton-Valentine leukocidin-positive methicillin-resistant Staphylococcus aureus (Bengal Bay clone) and ST22 S. aureus isolates with a genetic variant of elastin binding protein in Nepal. *New microbes and new infections, 11*, 20-27.

Pourcel, C., Hormigos, K., Onteniente, L., Sakwinska, O., Deurenberg, R. H., & Vergnaud, G. (2009). Improved multiple-locus variable-number tandem-repeat assay for Staphylococcus aureus genotyping, providing a highly informative technique together with strong phylogenetic value. *Journal of clinical microbiology, 47*(10), 3121-3128.

Prabhu, K., Rao, S., & Rao, V. (2011). Inducible clindamycin resistance in Staphylococcus aureus isolated from clinical samples. *Journal of laboratory physicians, 3*(1), 25.

Proctor, R. A., Balwit, J. M., & Vesga, O. (1994). Variant subpopulations of Staphylococcus aureus as cause of persistent and recurrent infections. *Infectious agents and disease, 3*(6), 302-312.

Qiu, J., Feng, H., Lu, J., Xiang, H., Wang, D., Dong, J., Deng, X. (2010). Eugenol reduces the expression of virulence-related exoproteins in Staphylococcus aureus. *Applied Enviromental Microbiology, 76*(17), 5846-5851.

Quiblier, C., Seidl, K., Roschitzki, B., Zinkernagel, A. S., Berger-Bächi, B., & Senn, M. M. (2013). Secretome analysis defines the major role of SecDF in Staphylococcus aureus virulence. *PloS one, 8*(5).

Rafiq, M. S., Rafiq, M. I., Khan, T., Rafiq, M., & Khan, M. M. (2015). Effectiveness of simple control measures on methicillin resistant Staphylococcus aureus infection status and characteristics with susceptibility patterns in a teaching hospital in Peshawar. *Journal of the Pakistan Medicine Association, 65*(9), 915-920.

Rahimi, F., Bouzari, M., Katouli, M., & Pourshafie, M. R. (2013). Antibiotic resistance pattern of methicillin resistant and methicillin sensitive Staphylococcus aureus isolates in Tehran, Iran.

Rahimi, F., & Shokoohizadeh, L. (2018). Characterization of virulence factors and prophage profiles of methicillin-resistant Staphylococcus aureus strains isolated from a referral hospital in Tehran, Iran. *Archives of Clinical Infectious Diseases, 13*(5).

Rajaduraipandi, K., Mani, K., Panneerselvam, K., Mani, M., Bhaskar, M., & Manikandan, P. (2006). Prevalence and antimicrobial susceptibility pattern

of methicillin resistant Staphylococcus aureus: A multicentre study. *Indian journal of medical microbiology, 24*(1), 34.

Rajan, V., Schoenfelder, S. M., Ziebuhr, W., & Gopal, S. (2015). Genotyping of community-associated methicillin resistant Staphylococcus aureus (CA-MRSA) in a tertiary care centre in Mysore, South India: ST2371-SCCmec IV emerges as the major clone. *Infection, Genetics and Evolution, 34*, 230-235.

Rammelkamp, C. H., & Maxon, T. (1942). Resistance of Staphylococcus aureus to the Action of Penicillin. *Proceedings of the Society for Experimental Biology and Medicine, 51*(3), 386-389.

Rana-Khara, R., Lakhani, S. J., Vasava, S., Shah, K., & Panjwani, D. (2016). Methicillin Resistant Staphylococcus aureus (MRSA) and Vancomycin Resistant Staphylococcus aureus (VRSA) from a rural based tertiary care and teaching hospital in Vadodara district, Gujarat. *International Archives of Integrated Medicine, 3*(7), 187-195.

Raymond, D. P., Kuehnert, M. J., & Sawyer, R. G. (2002). Preventing antimicrobial-resistant bacterial infections in surgical patients. *Surgical infections, 3*(4), 375-385.

Rayner, C., & Munckhof, W. (2005). Antibiotics currently used in the treatment of infections caused by Staphylococcus aureus. *Internal medicine journal, 35*, S3-16.

Reddy, P. S., & Suresh, R. (2017). Phenotypic detection of Inducible Clindamycin resistance among the clinical isolates of staphylococcus aureus by using the lower limit of inter disk space. *Journal of Microbiology and Biotechnology Research, 2*(2), 258-264.

Reed, A. V. (2015). Connecting supply, short-sellers and stock returns: Research challenges. *Journal of Accounting and Economics, 60*(2-3), 97-103.

Reyes, J., Rincón, S., Díaz, L., Panesso, D., Contreras, G. A., Zurita, J., Adachi, J. (2009). Dissemination of methicillin-resistant Staphylococcus aureus USA300 sequence type 8 lineage in Latin America. *Clinical Infectious Diseases, 49*(12), 1861-1877.

Reynolds, P. E. (1989). Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *European Journal of Clinical Microbiology and Infectious Diseases, 8*(11), 943-950.

Rohrer, S., & Berger-Bachi, B. (2003). FemABX peptidyl transferases: a link between branched-chain cell wall peptide formation and β-lactam resistance in gram-positive cocci. *Antimicrobial agents and chemotherapy, 47*(3), 837-846.

Romeeza, T., Naeem, R., & Nadeem, A. (2010). Multiplex Polymerase Chain Reaction Based Typing of Staphylococcal Chromosomal Cassette mec of Methicillin Resistant Staphylococcus aureus from Selected Hospitals in Lahore. *The International Medical Journal of Malaysia, 9*(1).

Rosec, J., & Gigaud, O. (2002). Staphylococcal enterotoxin genes of classical and new types detected by PCR in France. *International journal of food microbiology, 77*(1-2), 61-70.

Rossato, A. M., Reiter, K. C., & d'Azevedo, P. A. (2018). Coexistence of virulence genes in methicillin-resistant Staphylococcus aureus clinical isolates. *Revista da Sociedade Brasileira de Medicina Tropical, 51*(3), 361-363.

Rubinstein, E., Kollef, M. H., & Nathwani, D. (2008). Pneumonia caused by methicillin-resistant Staphylococcus aureus. *Clinical Infectious Diseases, 46*(Supplement_5), S378-S385.

Rusenova, N., Gebreyes, W., Koleva, M., Mitev, J., Penev, T., Vasilev, N., & Miteva, T. (2013). Comparison of three methods for routine detection of Staphylococcus aureus isolated from bovine mastitis. *Kafkas Universitesi Veteriner Fakultesi Dergisi, 19*(4), 709-712.

Ryffel, C., Bucher, R., Kayser, F., & Berger-Bächi, B. (1991). The Staphylococcus aureus mec determinant comprises an unusual cluster of direct repeats and codes for a gene product similar to the Escherichia coli sn-glycerophosphoryl diester phosphodiesterase. *Journal of bacteriology, 173*(23), 7416-7422.

Saba, C. K. S., Amenyona, J. K., & Kpordze, S. W. (2017). Prevalence and pattern of antibiotic resistance of Staphylococcus aureus isolated from door handles and other points of contact in public hospitals in Ghana. *Antimicrobial Resistance & Infection Control, 6*(1), 1-6.

Sabat, A., Malachowa, N., Miedzobrodzki, J., & Hryniewicz, W. (2006). Comparison of PCR-based methods for typing Staphylococcus aureus isolates. *Journal of clinical microbiology, 44*(10), 3804-3807.

Sabat, A., Melles, D. C., Martirosian, G., Grundmann, H., van Belkum, A., & Hryniewicz, W. (2006). Distribution of the serine-aspartate repeat protein-encoding sdr genes among nasal-carriage and invasive Staphylococcus aureus strains. *Journal of clinical microbiology, 44*(3), 1135-1138.

Sabat, A. J., Koksal, M., Akkerboom, V., Monecke, S., Kriegeskorte, A., Hendrix, R., Friedrich, A. W. (2012). Detection of new methicillin-resistant Staphylococcus aureus strains that carry a novel genetic homologue and important virulence determinants. *Journal of clinical microbiology, 50*(10), 3374-3377.

Sabbagh, P., Riahi, S. M., Gamble, H. R., & Rostami, A. (2019). The global and regional prevalence, burden, and risk factors for methicillin-resistant Staphylococcus aureus colonization in HIV-infected people: a systematic review and meta-analysis. *American journal of infection control, 47*(3), 323-333.

Sabouni, F., Mahmoudi, S., Bahador, A., Pourakbari, B., Sadeghi, R. H., Ashtiani, M. T. H., Mamishi, S. (2014). Virulence factors of Staphylococcus aureus isolates in an Iranian referral children's hospital. *Osong public health and research perspectives, 5*(2), 96-100.

Saeed, A., Ahsan, F., Nawaz, M., Iqbal, K., Rehman, K. U., & Ijaz, T. (2020). Incidence of vancomycin resistant phenotype of the methicillin resistant Staphylococcus aureus isolated from a tertiary care hospital in Lahore. *Antibiotics, 9*(1), 3.

Saeed, M., Rasheed, F., Hussain, S., Ahmad, M., & Arif, M. (2016). Hospitalized patients; Methicillin-resistant Staphylococcus aureus Prevalence and Susceptibility patron. *Professional Medical Journal, 23*(7).

Sahebnasagh, R., Saderi, H., & Owlia, P. (2014). The Prevalence of Resistance to Methicillin in Staphylococcus aureus Strains Isolated from Patients by PCR Method for Detec-tion of mecA and nuc Genes. *Iranian journal of public health, 43*(1), 84.

Saiki, A. Y., Shen, L. L., Chen, C.-M., Baranowski, J., & Lerner, C. G. (1999). DNA cleavage activities of Staphylococcus aureus gyrase and topoisomerase IV stimulated by quinolones and 2-pyridones. *Antimicrobial agents and chemotherapy, 43*(7), 1574-1577.

Sakoulas, G., Eliopoulos, G. M., Moellering Jr, R. C., Wennersten, C., Venkataraman, L., Novick, R. P., & Gold, H. S. (2002). Accessory gene regulator (agr) locus in geographically diverse Staphylococcus aureus isolates with reduced susceptibility to vancomycin. *Antimicrobial agents and chemotherapy, 46*(5), 1492-1502.

Sakoulas, G., Eliopoulos, G. M., Moellering, R. C., Wennersten, C., Venkataraman, L., Novick, R. P., & Gold, H. S. (2002). Accessory gene regulator (agr) locus in geographically diverse Staphylococcus aureus isolates with reduced susceptibility to vancomycin. *Antimicrobial agents and chemotherapy, 46*(5), 1492-1502.

Saleem, A. J. (2009). High Frequency of Hemolysin Associated Genes among Staphylococcus Aureus Clinical Isolates in Iraq. *Journal of Global Pharma Technology*.

Saleem, F., Fasih, N., & Zafar, A. (2017). Susceptibility pattern of methicillin resistant Staphylococcus aureus to vancomycin and other alternate agents: report from a private sector hospital laboratory. *Journal of Pakistan Medical Association*.

Salyers, A. A., & Whitt, D. D. (2002). A molecular approach. *Bacterial pathogenesis*, 53-100.

Sangvik, M., Olsen, R. S., Olsen, K., Simonsen, G. S., Furberg, A.-S., & Sollid, J. U. E. (2011). Age-and gender-associated Staphylococcus aureus spa types found among nasal carriers in a general population. The Tromsø Staph and Skin Study. *Journal of clinical microbiology*, JCM. 05290-05211.

Saravolatz, L. D., Markowitz, N., Arking, L., Pohlod, D., & Fisher, E. (1982). Methicillin-resistant Staphylococcus aureus: epidemiologic observations during a community-acquired outbreak. *Annals of internal medicine, 96*(1), 11-16.

Sarma, J., & Ahmed, G. (2010). Characterisation of methicillin resistant S. aureus strains and risk factors for acquisition in a teaching hospital in northeast India. *Indian journal of medical microbiology, 28*(2), 127.

Satta, G., Ling, C. L., Cunningham, E. S., McHugh, T. D., & Hopkins, S. (2013). Utility and limitations of Spa-typing in understanding the epidemiology of staphylococcus aureus bacteraemia isolates in a single University Hospital. *BMC research notes, 6*(1), 398.

Sattar, M., Mubashir, F., Ibrahim, U. M., Buksh, A. R., Siddiqui, F., & Hafiz, S. (2019). Fosfomycin an Alternative for the Treatment of Methicillin Resistant Staphylococcus aureus (MRSA). *South Asian Journal of Research in Microbiology*, 1-7.

Schaumburg, F., Köck, R., Mellmann, A., Richter, L., Hasenberg, F., Kriegeskorte, A., von Eiff, C. (2012b). Population dynamics among methicillin resistant Staphylococcus aureus in Germany during a 6-year period. *Journal of clinical microbiology*, JCM. 01174-01112.

Schleifer, K. H., & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriological reviews, 36*(4), 407.

Schmitt, D. M., O'Dee, D. M., Cowan, B. N., Birch, J. W.-M., Mazzella, L. K., Nau, G. J., & Horzempa, J. (2013). The use of resazurin as a novel antimicrobial agent against Francisella tularensis. *Frontiers in cellular and infection microbiology, 3*, 93.

Schmitz, F.-J., Fluit, A. C., Gondolf, M., Beyrau, R., Lindenlauf, E., Verhoef, J., Jones, M. E. (1999). The prevalence of aminoglycoside resistance and corresponding resistance genes in clinical isolates of staphylococci from 19 European hospitals. *Journal of antimicrobial chemotherapy, 43*(2), 253-259.

Schmitz, F.-J., Sadurski, R., Kray, A., Boos, M., Geisel, R., Köhrer, K., Fluit, A. C. (2000). Prevalence of macrolide-resistance genes in Staphylococcus aureus and Enterococcus faecium isolates from 24 European university hospitals. *Journal of antimicrobial chemotherapy, 45*(6), 891-894.

Schulthess, B., Bloes, D. A., François, P., Girard, M., Schrenzel, J., Bischoff, M., & Berger-Bächi, B. (2011). The σB-dependent yabJ-spoVG operon is involved in the regulation of extracellular nuclease, lipase, and protease expression in Staphylococcus aureus. *Journal of bacteriology, 193*(18), 4954-4962.

Seas, C., Hernandez, K., Ramos, R., Bazan, E., Rodriguez, I., Torres, A., Gotuzzo, E. (2006). Oxacillin-resistant and multidrug-resistant Staphylococcus aureus in Lima, Peru. *Infection Control & Hospital Epidemiology, 27*(2), 198-200.

Senna, J., Pinto, C., Carvalho, L., & Santos, D. (2002). Comparison of pulsed-field gel electrophoresis and PCR analysis of polymorphisms on the mec hypervariable region for typing methicillin-resistant Staphylococcus aureus. *Journal of clinical microbiology, 40*(6), 2254-2256.

Senok, A. C., Somily, A. M., Slickers, P., Raji, M. A., Garaween, G., Shibl, A., Ehricht, R. (2017). Investigating a rare methicillin-resistant Staphylococcus aureus strain: first description of genome sequencing and molecular characterization of CC15-MRSA. *Infection and Drug Resistance, 10*, 307.

Seral, C., Van Bambeke, F., & Tulkens, P. (2003). Quantitative analysis of the activity of antibiotics (gentamicin, azithromycin, telithromycin, ciprofloxacin, moxifloxacin, oritavancin [LY333328]) against intracellular Staphylococcus aureus in mouse J774 macrophages. *Antimicrobial agents and chemotherapy, 47*, 2283-2292.

Seral, C., Van Bambeke, F., & Tulkens, P. M. (2003). Quantitative analysis of gentamicin, azithromycin, telithromycin, ciprofloxacin, moxifloxacin, and oritavancin (LY333328) activities against intracellular Staphylococcus aureus in mouse J774 macrophages. *Antimicrobial agents and chemotherapy, 47*(7), 2283-2292.

Serruto, D., Rappuoli, R., Scarselli, M., Gros, P., & Van Strijp, J. A. (2010). Molecular mechanisms of complement evasion: learning from staphylococci and meningococci. *Nature Reviews Microbiology, 8*(6), 393-399.

Seto, S., Tsujimura, K., & Koide, Y. (2011). Rab GTPases regulating phagosome maturation are differentially recruited to mycobacterial phagosomes. *Traffic, 12*(4), 407-420.

Shabir, S., Hardy, K. J., Abbasi, W. S., McMurray, C. L., Malik, S. A., Wattal, C., & Hawkey, P. M. (2010). Epidemiological typing of meticillin-resistant

Staphylococcus aureus isolates from Pakistan and India. *Journal of medical microbiology, 59*(3), 330-337.

Shallcross, L. J., Williams, K., Hopkins, S., Aldridge, R. W., Johnson, A. M., & Hayward, A. C. (2010). Panton–Valentine leukocidin associated staphylococcal disease: a cross-sectional study at a London hospital, England. *Clinical microbiology and infection, 16*(11), 1644-1648. doi:10.1111/j.1469-0691.2010.03153.x

Shang, W., Hu, Q., Yuan, W., Cheng, H., Yang, J., Hu, Z., Yang, Y. (2016). Comparative fitness and determinants for the characteristic drug resistance of ST239-MRSA-III-t030 and ST239-MRSA-III-t037 strains isolated in China. *Microbial Drug Resistance, 22*(3), 185-192.

Shi, C., Xiao, Y., Zhang, Q., Li, Q., Wang, F., Wu, J., & Lin, N. (2018). Efficacy and safety of cefazolin versus antistaphylococcal penicillins for the treatment of methicillin-susceptible Staphylococcus aureus bacteremia: a systematic review and meta-analysis. *BMC Infectious diseases, 18*(1), 508.

Shopsin, B., Mathema, B., Alcabes, P., Said-Salim, B., Lina, G., Matsuka, A., Kreiswirth, B. (2003). Prevalence of agr specificity groups among Staphylococcus aureus strains colonizing children and their guardians. *Journal of clinical microbiology, 41*(1), 456-459.

Shore, A. C., Deasy, E. C., Slickers, P., Brennan, G., O'Connell, B., Monecke, S., . . . Coleman, D. C. (2011). Detection of staphylococcal cassette chromosome mec type XI carrying highly divergent mecA, mecI, mecR1, blaZ, and ccr genes in human clinical isolates of clonal complex 130 methicillin-resistant Staphylococcus aureus. *Antimicrobial agents and chemotherapy, 55*(8), 3765-3773.

Shore, L. M., Randel, A. E., Chung, B. G., Dean, M. A., Holcombe Ehrhart, K., & Singh, G. (2011). Inclusion and diversity in work groups: A review and model for future research. *Journal of management, 37*(4), 1262-1289.

Shukla, S. K., Karow, M. E., Brady, J. M., Stemper, M. E., Kislow, J., Moore, N., Reed, K. D. (2010). Virulence genes and genotypic associations in nasal carriage, community-associated methicillin-susceptible and methicillin-resistant USA400 Staphylococcus aureus isolates. *Journal of clinical microbiology, 48*(10), 3582-3592.

Siddiqui, G., Karamat, K., & Hanan, A. (1999). Prevalence of methicillin resistant Staphylococcus aureus: A study at PAF hospital Sargodha. *Pakistan journal of Pathology, 10*, 26-28.

Siddiqui, T., Muhammad, I. N., Khan, M. N., Fatima, S., Alam, N., Masood, R., Naqvi, T. J. P. J. P. S. (2018). Prevalence of mecA: Genotyping screening of community acquired-MRSA isolates in Karachi, Pakistan. *Pakistan journal of pharmaceutical sciences, 5*, 2091-2094.

Siddiqui, T., Muhammad, I. N., Khan, M. N., Naz, S., Bashir, L., Sarosh, N., Naqvi, T. (2017). MRSA: Prevalence and susceptibility pattern in health care setups of Karachi. *Pakistan journal of pharmaceutical sciences, 30*, 2417-2421.

Sieradzki, K., Roberts, R. B., Haber, S. W., & Tomasz, A. (1999). The development of vancomycin resistance in a patient with methicillin-resistant Staphylococcus aureus infection. *New England Journal of Medicine, 340*(7), 517-523.

Sierra, J., Marco, F., Ruiz, J., de Anta, M. J., & Vila, J. (2002). Correlation between the activity of different fluoroquinolones and the presence of mechanisms of quinolone resistance in epidemiologically related and unrelated strains of methicillin-susceptible and-resistant Staphylococcus aureus. *Clinical microbiology and infection, 8*(12), 781-790.

Silva, N. d., & Cardoso, H. (2000). The production of hemolysin by Staphylococcus aureus isolated from bovine mastitis. *Veterinária Notícias, 6*(2), 63-67.

Sipahi, O. R., Bardak-Ozcem, S., Turhan, T., Arda, B., Ruksen, M., Pullukcu, H., Sipahi, H. (2013). Vancomycin versus linezolid in the treatment of methicillin-resistant Staphylococcus aureus meningitis. *Surgical infections, 14*(4), 357-362.

Sirkhazi, M., Sarriff, A., Aziz, N. A., Almana, F., Arafat, O., & Shorman, M. (2014). Bacterial spectrum, isolation sites and susceptibility patterns of pathogens in adult febrile neutropenic cancer patients at a specialist hospital in Saudi Arabia. *World journal of oncology, 5*(5-6), 196.

Skinner, D., & Keefer, C. S. (1941). Significance of bacteremia caused by Staphylococcus aureus: a study of one hundred and twenty-two cases and a review of the literature concerned with experimental infection in animals. *Archives of Internal Medicine, 68*(5), 851-875.

Sohail, M., & Latif, Z. (2018). Molecular analysis, biofilm formation, and susceptibility of methicillin-resistant Staphylococcus aureus strains causing community-and health care-associated infections in central venous catheters. *Revista da Sociedade Brasileira de Medicina Tropical, 51*(5), 603-609.

Sohail, M., & Latif, Z. (2018a). Molecular typing of Methicillin Resistance Staphylococcus aureus (MRSA) isolated from device related infections by SCCmec and PCR-RFLP of coagulase gene. *J Advancements in Life Sciences, 6*(1), 34-40.

Sohail, M., & Latif, Z. (2018b). Molecular typing of Methicillin Resistance Staphylococcus aureus (MRSA) isolated from device related infections by SCCmec and PCR-RFLP of coagulase gene. *Advancements in Life Sciences, 6*(1), 34-40.

Soltani, E., Farrokhi, E., Zamanzad, B., Abadi, M. S. S., Deris, F., Soltani, A., & Gholipour, A. (2019). Prevalence and distribution of adhesins and the expression of fibronectin-binding protein (FnbA and FnbB) among Staphylococcus aureus isolates from Shahrekord Hospitals. *BMC research notes, 12*(1), 49.

Son, N. T., Huong, V. T. T., Lien, V. T. K., Nga, D. T. Q., Au, T. T. H., Nga, T. T., Binh, T. Q. (2019). First Report on Multidrug-Resistant Methicillin-Resistant Staphylococcus aureus Isolates in Children Admitted to Tertiary Hospitals in Vietnam. *Journal of microbiology and biotechnology, 29*(9), 1460-1469.

Song, J.-H., Hsueh, P.-R., Chung, D. R., Ko, K. S., Kang, C.-I., Peck, K. R., Kim, Y.-S. (2011). Spread of methicillin-resistant Staphylococcus aureus between the community and the hospitals in Asian countries: an ANSORP study. *Journal of antimicrobial chemotherapy, 66*(5), 1061-1069.

Stefani, S., Chung, D. R., Lindsay, J. A., Friedrich, A. W., Kearns, A. M., Westh, H., & MacKenzie, F. M. (2012). Meticillin-resistant Staphylococcus aureus

(MRSA): global epidemiology and harmonisation of typing methods. *International journal of antimicrobial agents, 39*(4), 273-282.

Stegger, á., Andersen, P., Kearns, A., Pichon, B., Holmes, M., Edwards, G., Larsen, A. (2012). Rapid detection, differentiation and typing of methicillin-resistant Staphylococcus aureus harbouring either mecA or the new mecA homologue mecALGA251. *Clinical microbiology and infection, 18*(4), 395-400.

Strandén, A., Frei, R., Adler, H., Flückiger, U., & Widmer, A. (2009). Emergence of SCCmec type IV as the most common type of methicillin-resistant Staphylococcus aureus in a university hospital. *Infection, 37*(1), 44-48.

Stratchounski, L., Dekhnich, A., Kretchikov, V., Edelstain, I., Narezkina, A., Afinogenov, G., Gudkova, L. (2005). Antimicrobial resistance of nosocomial strains of Staphylococcus aureus in Russia: results of a prospective study. *Journal of chemotherapy, 17*(1), 54-60.

Strobel, M., Pförtner, H., Tuchscherr, L., Völker, U., Schmidt, F., Kramko, N., Peters, G. (2016). Post-invasion events after infection with Staphylococcus aureus are strongly dependent on both the host cell type and the infecting S. aureus strain. *Clinical microbiology and infection, 22*(9), 799-809.

Strommenger, B., Kettlitz, C., Werner, G., & Witte, W. (2003). Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in Staphylococcus aureus. *Journal of clinical microbiology, 41*(9), 4089-4094.

Sugino, Y., Iinumab, Y., Ichiyama, S., Ito, Y., Ohkawa, S., Nakashima, N., Hasegawa, Y. (2000). In vivo development of decreased susceptibility to vancomycin in clinical isolates of methicillin-resistant Staphylococcus aureus. *Diagnostic microbiology and infectious disease, 38*(3), 159-167.

Suhaili, Z., Lean, S.-S., Mohamad, N. M., Rachman, A. R. A., Desa, M. N. M., & Yeo, C. C. (2016). Draft genome sequence of Staphylococcus aureus KT/312045, an ST1-MSSA PVL positive isolated from pus sample in East Coast Malaysia. *Genomics data, 9*, 111.

Syed, M. A., Shah, S. H. H., Sherafzal, Y., Shafi-ur-Rehman, S., Khan, M. A., Barrett, J. B., Jackson, C. R. (2018). Detection and molecular characterization of methicillin-resistant Staphylococcus aureus from table eggs in Haripur, Pakistan. *Foodborne Pathogens and disease, 15*(2), 86-93.

Ta, R., Rb, N., & Aa, N. (2010). Multiplex Polymerase Chain Reaction Based Typing of Staphylococcal Chromosomal Cassette mec of Methicillin Resistant Staphylococcus aureus from Selected Hospitals in Lahore. *International Medical Journal Malaysia*.

Taherirad, A., Jahanbakhsh, R., Shakeri, F., Anvary, S., & Ghaemi, E. A. (2016). Staphylococcal cassette chromosome mec types among methicillin-resistant Staphylococcus aureus in northern Iran. *Jundishapur Journal of Microbiology, 9*(8).

Taj, G., Agarwal, P., Grant, M., & Kumar, A. (2010). MAPK machinery in plants: recognition and response to different stresses through multiple signal transduction pathways. *Plant signaling & behavior, 5*(11), 1370-1378.

Taj, R., Muhammadzai, I., Ahmad, J., Khan, A., Syed, F., & Khan, Z. (2015). Frequency and antibiotic susceptibility pattern of methicillin resistant

Staphylococcus aureus in Abbottabad city of Pakistan. *Khyber Medical University Journal, 7*(4), 157-161.

Taj, R., Muhammadzai, I., Ahmad, J., Khan, A., Syed, F., & Khan, Z. (2016). Frequency and antibiotic susceptiblity pattern of methicillin resistant Staphylococcus aureus in Abottabad city of Pakistan. *Khyber Medical University Journal, 8*(4).

Taj, Y., Abdullah, F., & Kazmi, S. (2010). Current pattern of antibiotic resistance in Staphylococcus aureus clinical isolates and the emergence of vancomycin resistance. *Journal of the College of Physicians and Surgeons Pakistan, 20*(11), 728-732.

Taj, Y., Fatima, I., Ali, S., & Kazmi, S. (2014). Detection of genes for superantigen toxins in methicillin-resistant Staphylococcus aureus clinical isolates in Karachi. *Journal of the College of Physicians and Surgeons Pakistan, 24*(2), 101-105.

Takahata, S., Ida, T., Hiraishi, T., Sakakibara, S., Maebashi, K., Terada, S., Tomono, K. (2010). Molecular mechanisms of fosfomycin resistance in clinical isolates of Escherichia coli. *International journal of antimicrobial agents, 35*(4), 333-337.

Takano, T., Higuchi, W., Otsuka, T., Baranovich, T., Enany, S., Saito, K., Takano, M. (2008a). Novel characteristics of community-acquired methicillin-resistant Staphylococcus aureus strains belonging to multilocus sequence type 59 in Taiwan. *Antimicrobial agents chemotherapy, 52*(3), 837-845.

Takano, T., Higuchi, W., Otsuka, T., Baranovich, T., Enany, S., Saito, K., Takano, M. (2008b). Novel characteristics of community-acquired methicillin-resistant Staphylococcus aureus strains belonging to multilocus sequence type 59 in Taiwan. *Antimicrobial agents and chemotherapy, 52*(3), 837-845.

Takeuchi, S., Maeda, T., Hashimoto, N., Imaizumi, K., Kaidoh, T., & Hayakawa, Y. (2001). Variation of the agr locus in Staphylococcus aureus isolates from cows with mastitis. *Veterinary microbiology, 79*(3), 267-274.

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H., & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of clinical microbiology, 33*(9), 2233.

Tertiary, C. (2013). Prevalence and Antimicrobial Susceptibility of methicillin resistant staphylococcus aureus and coagulase-negative staphylococci in a tertiary care hospital. *Asian Journal of Pharmaceutical and Clinical Research, 6*(3), 231-234.

Thammavongsa, V., Kim, H. K., Missiakas, D., & Schneewind, O. (2015). Staphylococcal manipulation of host immune responses. *Nature Reviews Microbiology, 13*(9), 529-543.

Tharmalingam, N., Jayamani, E., Rajamuthiah, R., Castillo, D., Fuchs, B. B., Kelso, M. J., & Mylonakis, E. (2017). Activity of a novel protonophore against methicillin-resistant Staphylococcus aureus. *Future medicinal chemistry, 9*(12), 1401-1411.

Tiwari, H. K., Das, A. K., Sapkota, D., Sivrajan, K., & Pahwa, V. K. (2009). Methicillin resistant Staphylococcus aureus: prevalence and antibiogram in a

tertiary care hospital in western Nepal. *The Journal of Infection in Developing Countries, 3*(09), 681-684.

Tiwari, H. K., & Sen, M. R. (2006). Emergence of vancomycin resistant Staphylococcus aureus (VRSA) from a tertiary care hospital from northern part of India. *BMC Infectious diseases, 6*(1), 156.

Todar, K. (2005). Todar's Online Textbook of Bacteriology, Staphylococcus. In: Department of Bacteriology, University of Wincosin-Madison.

Tojo, S., Naganuma, F., Arakawa, K., & Yokoo, S. (2000). Involvement of both granular cells and plasmatocytes in phagocytic reactions in the greater wax moth, Galleria mellonella. *Journal of Insect Physiology, 46*(7), 1129-1135.

Tomasz, A. (1994). Multiple-Antibiotic-Resistant Pathogenic Bacteria--A Report on the Rockefeller University Workshop. *New England journal of medicine, 330*(17), 1247-1251.

Tong, S. Y., Davis, J. S., Eichenberger, E., Holland, T. L., & Fowler, V. G. (2015). Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clinical Microbiology Reviews, 28*(3), 603-661.

Tristan, A., Bes, M., Meugnier, H., Lina, G., Bozdogan, B., Courvalin, P., Etienne, J. (2007a). Global distribution of Panton-Valentine leukocidin--positive methicillin-resistant Staphylococcus aureus, 2006. *Emerging infectious diseases, 13*(4), 594-600.

Tristan, A., Bes, M., Meugnier, H., Lina, G., Bozdogan, B., Courvalin, P., Etienne, J. (2007b). Global distribution of Panton-Valentine leukocidin–positive methicillin-resistant Staphylococcus aureus, 2006. *Emerging infectious diseases, 13*(4), 594.

Trivedi, M. B., Vegad, M., & Soni, S. (2015). Prevalence of methicillin-resistant Staphylococcus aureus in various clinical samples in a tertiary-care hospital. *International Journal of Medical Science and Public Health, 4*(12), 1735-1738.

Truong-Bolduc, Q., Dunman, P., Eidem, T., & Hooper, D. (2011). Transcriptional profiling analysis of the global regulator NorG, a GntR-like protein of Staphylococcus aureus. *Journal of bacteriology, 193*(22), 6207-6214.

Trzcinski, K., Cooper, B. S., Hryniewicz, W., & Dowson, C. G. (2000). Expression of resistance to tetracyclines in strains of methicillin-resistant Staphylococcus aureus. *Journal of antimicrobial chemotherapy, 45*(6), 763-770.

Tsai, C. J.-Y., Loh, J. M. S., & Proft, T. (2016). Galleria mellonella infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence, 7*(3), 214-229.

Tu Quoc, P. H., Genevaux, P., Pajunen, M., Savilahti, H., Georgopoulos, C., Schrenzel, J., & Kelley, W. L. (2007). Isolation and characterization of biofilm formation-defective mutants of Staphylococcus aureus. *Infection and immunity, 75*(3), 1079-1088.

Tuchscherr, L., Bischoff, M., Lattar, S. M., Llana, M. N., Pförtner, H., Niemann, S., Cheung, A. L. (2015). Sigma factor SigB is crucial to mediate Staphylococcus aureus adaptation during chronic infections. *PLoS pathogens, 11*(4), e1004870.

Tuchscherr, L., Medina, E., Hussain, M., Völker, W., Heitmann, V., Niemann, S., Becker, K. (2011). Staphylococcus aureus phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. *EMBO molecular medicine, 3*(3), 129-141.

Udo, E., Al-Sweih, N., & Noronha, B. (2006). Characterisation of non-multiresistant methicillin-resistant Staphylococcus aureus (including EMRSA-15) in Kuwait Hospitals. *Clinical microbiology and infection, 12*(3), 262-269.

Udo, E., Pearman, J., & Grubb, W. (1993). Genetic analysis of community isolates of methicillin-resistant Staphylococcus aureus in Western Australia. *Journal of Hospital Infection, 25*(2), 97-108.

Udo, E. E., Al-Lawati, B.-H., Al-Muharmi, Z., & Thukral, S. (2014). Genotyping of methicillin-resistant Staphylococcus aureus in the Sultan Qaboos University Hospital, Oman reveals the dominance of Panton–Valentine leucocidin-negative ST6-IV/t304 clone. *New microbes and new infections, 2*(4), 100-105.

Udo, E. E., & Boswihi, S. S. (2017). Antibiotic Resistance Trends in Methicillin-Resistant Staphylococcus aureus Isolated in Kuwait Hospitals: 2011-2015. *Medical Principles and Practice, 26*(5), 485-490.

Ullah, A., Qasim, M., Rahman, H., Khan, J., Haroon, M., Muhammad, N., Muhammad, N. (2016b). High frequency of methicillin-resistant Staphylococcus aureus in Peshawar Region of Pakistan. *Springerplus, 5*(1), 600.

Ullah, F., Malik, S. A., Ahmed, J., Shah, S., Ayaz, M., Hussain, S., & Khatoon, L. (2012). Investigation of the genetic basis of tetracycline resistance in Staphylococcus aureus from Pakistan. *Tropical Journal of Pharmaceutical Research, 11*(6), 925-931.

Uzunović-Kamberović, S., Rijnders, M. I., Stobberingh, E. E., Ibrahimagić, A., Kamberović, F., & Ille, T. (2013). Molecular characterisation of methicillin-susceptible and methicillin-resistant Staphylococcus aureus in inpatients and outpatients in Bosnia and Herzegovina. *Wiener Medizinische Wochenschrift, 163*(1-2), 13-20.

Van Bambeke, F., Van Laethem, Y., Courvalin, P., & Tulkens, P. M. (2004). Glycopeptide antibiotics. *Drugs, 64*(9), 913-936.

Van Duijkeren, E., Wolfhagen, M. J., Box, A. T., Heck, M. E., Wannet, W. J., & Fluit, A. C. (2004). Human-to-dog transmission of methicillin-resistant Staphylococcus aureus. *Emerging infectious diseases, 10*(12), 2235.

van Leeuwen, W., van Nieuwenhuizen, W., Gijzen, C., Verbrugh, H., & van Belkum, A. (2000). Population studies of methicillin-resistant and-sensitive Staphylococcus aureus strains reveal a lack of variability in the agrD gene, encoding a staphylococcal autoinducer peptide. *Journal of bacteriology, 182*(20), 5721-5729.

Vandendriessche, S., Vanderhaeghen, W., Soares, F. V., Hallin, M., Catry, B., Hermans, K., Denis, O. (2013). Prevalence, risk factors and genetic diversity of methicillin-resistant Staphylococcus aureus carried by humans and animals across livestock production sectors. *Journal of antimicrobial chemotherapy, 68*(7), 1510-1516.

Vandenesch, F., Naimi, T., Enright, M. C., Lina, G., Nimmo, G. R., Heffernan, H., Reverdy, M.-E. (2003). Community-acquired methicillin-resistant Staphylococcus aureus carrying Panton-Valentine leukocidin genes: worldwide emergence. *Emerging infectious diseases, 9*(8), 978-984.

Vasconcelos, N. G., & Cunha, M. d. L. R. d. (2010). Staphylococcal enterotoxins: molecular aspects and detection methods. *Journal of Public Health and Epidemiology*, 29-42.

Vijayakumaran, V. (2013). *Characterization of Staphylococcus aureus Lipase.* The University of Western Ontario,

Vijayamohan, N., & Nair, S. P. (2014). A study of the prevalence of methicillin-resistant Staphylococcus aureus in dermatology inpatients. *Indian dermatology online journal, 5*(4), 441.

Walsh, C. (2003). *Antibiotics: actions, origins, resistance*: American Society for Microbiology (ASM).

Walsh, T. R., Efthimiou, J., & Dréno, B. (2016). Systematic review of antibiotic resistance in acne: an increasing topical and oral threat. *The Lancet infectious diseases, 16*(3), e23-e33.

Walther, B., Wieler, L. H., Vincze, S., Antão, E.-M., Brandenburg, A., Stamm, I., Lübke-Becker, A. (2012). MRSA variant in companion animals. *Emerging infectious diseases, 18*(12), 2017.

Wang, L., Liu, Y., Yang, Y., Huang, G., Wang, C., Deng, L., Shang, Y. (2012). Multidrug-resistant clones of community-associated meticillin-resistant Staphylococcus aureus isolated from Chinese children and the resistance genes to clindamycin and mupirocin. *Journal of medical microbiology, 61*(9), 1240-1247.

Wang, R., Braughton, K. R., Kretschmer, D., Bach, T.-H. L., Queck, S. Y., Li, M., Peschel, A. (2007). Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nature medicine, 13*(12), 1510.

Wang, X., Wang, X., Teng, D., Mao, R., Hao, Y., Yang, N., Wang, J. (2018). Increased intracellular activity of MP1102 and NZ2114 against Staphylococcus aureus in vitro and in vivo. *Scientific reports, 8*(1), 4204.

Wardenburg, J. B., & Schneewind, O. (2008). Vaccine protection against Staphylococcus aureus pneumonia. *Journal of Experimental Medicine, 205*(2), 287-294.

Wertheim, H. F., Vos, M. C., Ott, A., van Belkum, A., Voss, A., Kluytmans, J. A., Verbrugh, H. A. (2004). Risk and outcome of nosocomial Staphylococcus aureus bacteraemia in nasal carriers versus non-carriers. *The Lancet, 364*(9435), 703-705.

White, M. J., Boyd, J. M., Horswill, A. R., & Nauseef, W. M. (2014). Phosphatidylinositol-specific phospholipase C contributes to survival of Staphylococcus aureus USA300 in human blood and neutrophils. *Infection and immunity, 82*(4), 1559-1571.

Williamson, D. A., Carter, G. P., & Howden, B. P. (2017). Current and emerging topical antibacterials and antiseptics: agents, action, and resistance patterns. *Clinical Microbiology Reviews, 30*(3), 827-860.

Wilson, B. A., Garud, N. R., Feder, A. F., Assaf, Z. J., & Pennings, P. S. (2016). The population genetics of drug resistance evolution in natural populations of viral, bacterial and eukaryotic pathogens. *Molecular ecology, 25*(1), 42-66.

Wilson, D. N. (2014). Ribosome-targeting antibiotics and mechanisms of bacterial resistance. *Nature Reviews Microbiology, 12*(1), 35.

Winslow, C.-E., Rothberg, W., & Parsons, E. I. (1920). Notes on the classification of the white and orange staphylococci. *Journal of bacteriology, 5*(2), 145.

Wisplinghoff, H., Bischoff, T., Tallent, S. M., Seifert, H., Wenzel, R. P., & Edmond, M. B. (2004). Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clinical Infectious Diseases, 39*(3), 309-317.

Woodford, N., & Livermore, D. M. (2009). Infections caused by Gram-positive bacteria: a review of the global challenge. *Journal of Infection, 59*, S4-S16.

Wozniak, D. J., Tiwari, K. B., Soufan, R., & Jayaswal, R. K. (2012). The mcsB gene of the clpC operon is required for stress tolerance and virulence in Staphylococcus aureus. *Microbiology, 158*(Pt 10), 2568.

Wright, J. S., Traber, K. E., Corrigan, R., Benson, S. A., Musser, J. M., & Novick, R. P. (2005). The agr radiation: an early event in the evolution of staphylococci. *Journal of bacteriology, 187*(16), 5585-5594.

Wu, D., Wang, Q., Yang, Y., Geng, W., Wang, Q., Yu, S., Shen, X. (2010). Epidemiology and molecular characteristics of community-associated methicillin-resistant and methicillin-susceptible Staphylococcus aureus from skin/soft tissue infections in a children's hospital in Beijing, China. *Diagnostic microbiology and infectious disease, 67*(1), 1-8.

Wu, Q., Sabokroo, N., Wang, Y., Hashemian, M., Karamollahi, S., & Kouhsari, E. (2021). Systematic review and meta-analysis of the epidemiology of vancomycin-resistance Staphylococcus aureus isolates. *Antimicrobial Resistance & Infection Control, 10*(1), 1-13.

Wunderink, R. G., Niederman, M. S., Kollef, M. H., Shorr, A. F., Kunkel, M. J., Baruch, A., Chastre, J. (2012). Linezolid in methicillin-resistant Staphylococcus aureus nosocomial pneumonia: a randomized, controlled study. *Clinical Infectious Diseases, 54*(5), 621-629.

Xue, T., You, Y., Shang, F., & Sun, B. (2012). Rot and Agr system modulate fibrinogen-binding ability mainly by regulating clfB expression in Staphylococcus aureus NCTC8325. *Medical microbiology and immunology, 201*(1), 81-92.

Yilmaz, G., Aydin, K., Iskender, S., Caylan, R., & Koksal, I. (2007). Detection and prevalence of inducible clindamycin resistance in staphylococci. *Journal of medical microbiology, 56*(3), 342-345.

Yu, F., Li, T., Huang, X., Xie, J., Xu, Y., Tu, J., Hu, L. (2012). Virulence gene profiling and molecular characterization of hospital-acquired Staphylococcus aureus isolates associated with bloodstream infection. *Diagnostic microbiology and infectious disease, 74*(4), 363-368.

Yu, S., Fearnhead, P., Holland, B. R., Biggs, P., Maiden, M., & French, N. (2012). Estimating the relative roles of recombination and point mutation in the

generation of single locus variants in Campylobacter jejuni and Campylobacter coli. *Journal of molecular evolution, 74*(5), 273-280.

Zafar, A., Stone, M., Ibrahim, S., Parveen, Z., Hasan, Z., Khan, E., Bamford, K. (2011). Prevalent genotypes of meticillin-resistant Staphylococcus aureus: report from Pakistan. *Journal of medical microbiology, 60*(1), 56-62.

Zeinali, E., Moniri, R., Safari, M., & Mousavi, S. G. A. (2011). Molecular characterization and SCCmec typing in meticillin-resistant Staphylococcus aureus isolated from clinical samples. *KAUMS Journal (FEYZ), 14*(4), 439-446.

Zhang, K., McClure, J.-A., & Conly, J. M. (2012). Enhanced multiplex PCR assay for typing of staphylococcal cassette chromosome mec types I to V in methicillin-resistant Staphylococcus aureus. *Molecular and cellular probes, 26*(5), 218-221.

Zhang, K., McClure, J.-A., Elsayed, S., Louie, T., & Conly, J. M. (2005). Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome mec types I to V in methicillin-resistant Staphylococcus aureus. *Journal of clinical microbiology, 43*(10), 5026-5033.

Zhou, W., Shan, W., Ma, X., Chang, W., Zhou, X., Lu, H., & Dai, Y. (2012). Molecular characterization of rifampicin-resistant Staphylococcus aureus isolates in a Chinese teaching hospital from Anhui, China. *BMC microbiology, 12*(1), 240.

Zhu, F., Zhou, Y., Jiang, C., & Zhang, X. (2015). Role of JAK-STAT signaling in maturation of phagosomes containing Staphylococcus aureus. *Scientific reports, 5*, 14854.

Ziebandt, A. K., Becher, D., Ohlsen, K., Hacker, J., Hecker, M., & Engelmann, S. (2004). The influence of agr and σB in growth phase dependent regulation of virulence factors in Staphylococcus aureus. *Proteomics, 4*(10), 3034-3047.

Zimmerli, W., Widmer, A. F., Blatter, M., Frei, R., & Ochsner, P. E. (1998). Role of rifampin for treatment of orthopedic implant–related staphylococcal infections: a randomized controlled trial. *Jama, 279*(19), 1537-1541.

Turnitin Originality Report file:///C:/Users/ft/Downloads/Turnitin%20Originality%20Report.html

30/11/21

Turnitin Originality Report

Molecular Typing, Characterization and Pathogenicity of Clinical Multi-Drug Resistant Staphylococcus aureus isolates by Amir Afzal Khan .

From CL QAU (DRSML)

- Processed on 29-Nov-2021 09:15 PKT
- ID: 1714733524
- Word Count: 44877

Similarity Index
15%
Similarity by Source

Internet Sources:
9%
Publications:
10%
Student Papers:
3%

Focal Person (Turnitin)
Quaid-i-Azam University
Islamabad

sources:

1 < 1% match ()
Parisa Asadollahi, Narges Nodeh Farahani, Mehdi Mirzaii, Seyed Sajjad Khoramrooz et al. "Distribution of the Most Prevalent Types among Clinical Isolates of Methicillin-Resistant and -Susceptible around the World: A Review", Frontiers in Microbiology

2 < 1% match ()
Sadia Khan, Faisal Rasheed, Rabaab Zahra. "Genetic Polymorphism of Locus and Antibiotic Resistance of at two hospitals in Pakistan", Pakistan Journal of Medical Sciences

3 < 1% match ()
Front Microbiol. 2019 Jul 17; 10:1557

4 < 1% match (Internet from 24-Nov-2011)
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC66325/?tool=pmcentrez&re=

5 < 1% match ()
Ziver Sahin, Muge Bicakcigil, Kenan Aksu, Sevil Kamali et al. "Takayasu's arteritis is associated with , but not with , in Turkey", Arthritis Research & Therapy

6 < 1% match ()
Romen Singh Naorem, Jochen Blom, Csaba Fekete. "Genome-wide comparison of four MRSA clinical isolates from Germany and Hungary", PeerJ

7 < 1% match ()
Toms John Peedikayil Neetu, Sevanan Murugan. "Genotyping of Methicillin Resistant from Tertiary Care Hospitals in Coimbatore, South India", Journal of Global Infectious Diseases

8 < 1% match (Internet from 17-Apr-2016)
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4432697/

< 1% match (Internet from 07-Aug-2021)