

MOLECULAR EPIDEMIOLOGY OF *STAPHYLOCOCCUS AUREUS*, A CASE
STUDY OF KHYBER PAKHTUNKHWA, PAKISTAN



Thesis submitted to

CENTRE OF BIOTECHNOLOGY AND MICROBIOLOGY, UNIVERSITY OF
PESHAWAR

In partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in
Microbiology

By

Farman Ullah

CENTRE OF BIOTECHNOLOGY AND MICROBIOLOGY, UNIVERSITY OF
PESHAWAR, KHYBER PUKHTUNKHWA, PAKISTAN

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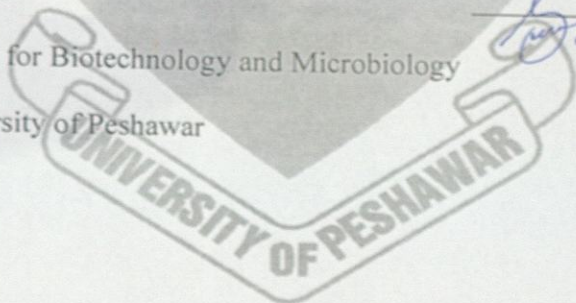
CERTIFICATE OF APPROVAL

This thesis titled

"Molecular epidemiology of *Staphylococcus aureus*, a case study of Khyber Pakhtunkhwa, Pakistan" submitted by Farman Ullah is hereby approved and recommended as partial fulfillment for the award of Degree of PhD in Microbiology

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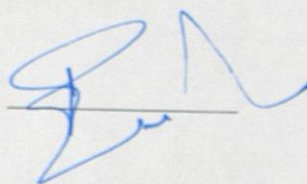
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AUTHOR'S DECLARATION

I solemnly declare that the research work presented in this thesis was carried out in accordance with the requirements of the University of Peshawar's regulations for Research Degree Programs. The author has not submitted this work for any other academic award. The work is original and of author's own data, while work done in collaboration with or with the assistance of, others, is indicated as such. The views expressed in this thesis, belongs to the author.

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Dedication

Dedicate to our Holy Prophet Muhammad (S.A.W.W) and humanity

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FARMAN ULLAH

LIST OF ABBREVIATIONS

Full name	Abbreviation	Full name	Abbreviation
Amoxicillin+Clavulannic Acid	AMC	Amoxicillin	AML
Ampicillin	AMP	Active Pharmaceutical Ingredient	API
American Type Culture Collection	ATCC	Aztreonam	ATM
Ceftazidime	CAZ	Chromosome recombinase A	<i>ccrA</i>
Chromosome recombinase B	<i>ccrB</i>	Cephadrine	CE
Cefaclor	CEC	Colony Forming Unit	CFU
Ciprofloxacin	CIP	Cystine Lactose Electrolyte Deficient agar	CLED agar
Clindamycin	CLI	Clinical Laboratory and Standards Institute	CLSI
Gentamicin	CN	Cefpodoxime	CPD
Congo Red Agar	CRA	Ceftriaxone	CRO
Chronic rhinosinusitis	CRS	Cefotaxime	CTX
Deoxyribonucleic acid	DNA	Deoxyribonuclease enzymes	DNase
Deoxy Nucleotide	dNTPs	Doxycycline	DO

Triphosphates			
<i>Escherichia coli</i>	<i>E. coli</i>	Erythromycin	E
Ethylene Diamine Tetra Amine	EDTA	Enterohemorrhagic <i>Escherichia coli</i>	EHEC
Enteroinvasive <i>Escherichia coli</i>	EIEC	European Methicillin Resistant <i>Staphylococcus aureus</i>	EMRSA
Enoxacin	ENX	Enteropathogenic <i>Escherichia coli</i>	EPEC
erythromycin ribosomal <i>methylase</i> genes	<i>Erm genes</i>	Erythromycin	ERY
Exfoliative toxin A	ETA	Exfoliative toxin B	ETB
Enterotoxigenic <i>Escherichia coli</i>	ETEC	Hydrochloric acid	HCl
Hemolytic Uremic Syndrome	HUS	Intercellular adhesion operon	<i>ica</i> operon
Imipenem	IPM	<i>Klebsiella pneumoniae</i>	<i>K. pneumoniae</i>
Potassium chloride	KCl	Potassium dihydrogen phosphate	KH ₂ PO ₄
Khyber Teaching Hospital	KTH	Meropenem	MEM
Magnesium	Mg	Major Histocompatibility complex	MHC
Minimum Inhibitory	MIC	Macrolides, lincosamides	MLS _B

concentration		and type B streptogramin	
Messenger ribonucleic acid	mRNA	Methicillin Resistant <i>Staphylococcus aureus</i>	MRSA
Microbial surface components recognising adhesive matrix molecules <i>msrA</i> gene	MSCRAMMs	Methicillin Sensitive <i>Staphylococcus aureus</i>	MSSA
Disodium hydrogen phosphate	Na ₂ HPO ₄	National Culture Type Collection	NCTC
optical density	OD	<i>Pseudomonas aeruginosa</i>	<i>P. aeruginosa</i>
Penicillin-binding proteins	PBPs	Phosphate buffered saline	PBS
Polymerase chain reaction	PCR	Polysaccharide intercellular adhesions	PIA
Polymorphonuclear neutrophils	PMNs	Polymer β-1, 6 linked N- acetylglucosamine	PNAG
Panton valentine leukocidin	PVL	Ribonucleic acid	RNA
Ribosomal ribonucleic acid	rRNA	<i>Staphylococcus aureus</i>	<i>S. aureus</i>
<i>Staphylococcus epidermidis</i>	<i>S. epidermidis</i>	<i>Staphylococcal</i> cassette chromosome <i>mec</i>	SCC <i>mec</i>
Staphylococcal enterotoxin a	<i>Sea</i>	Staphylococcal enterotoxin b	<i>Seb</i>
Staphylococcal enterotoxin c	<i>Sec</i>	Staphylococcal enterotoxin d	<i>Sed</i>

<i>Staphylococcal</i> enterotoxins	SEs	<i>Staphylococcal</i> food poisoning	SFP
Skin and soft-tissue infections	SSTIs	Trimethoprim+Sulphame thoxazole	SXT
Tris-Acetic acid-EDTA	TAE	Tris Ethylene Diamine Tetra Amine	TE
Tetracycline resistance gene K	Tet K	Tetracycline resistance gene L	Tet L
Tetracycline resistance gene M	Tet M	Tetracycline resistance gene O	Tet O
Tetracycline resistance gene A	<i>TetA</i> gene	Transfer ribonucleic acid	tRNA
Tryptic Soya Broth	TSB	Toxic shock syndrome	TSS
Toxic shock syndrome toxin-1	TSST-1	Toxin shock toxin	<i>Tst</i>
Urinary Tract Infections	UTIs	Vancomycin	VA

Molecular Epidemiology of *Staphylococcus aureus*, a case study of Khyber Pakhtunkhwa, Pakistan

Abstract

The present study was conducted from June 2010 to June 2014 in Khyber Teaching Hospital (KTH) and Hayatabad Medical Complex (HMC), Peshawar, Pakistan to know about the prevalence of resistance to antibiotics and molecular epidemiology of *Staphylococcus aureus*. The specimens include pus, blood and urine received for culture and sensitivity. *S. aureus* were identified at molecular level using polymerase chain reaction (PCR). The identified strains were subjected to antimicrobial testing by disc diffusion method and minimum inhibitory concentration (MIC) method according to the guide lines of clinical laboratory and standards institute (CLSI). Cefoxitin, 30 µg discs were also applied in the culture sensitivity to find methicillin resistant *S. aureus* (MRSA) strains. Majority of *S. aureus* were isolated from skin infections. A total of 273 (61.34%) isolates were found to be MRSA out of 445. The samples collected from KTH's clinical laboratory were named as KLS (n=167), KTH's environmental samples as KE (n=64), KTH's blood isolates as KLBS (n=15), KTH's currency notes as CRN (n=18), KTH's burn ward samples as KBRN (n=89) and the isolates collected from Hayatabad medical complex were named as HMC (n=92). As a whole, among the antibiotics tested against *S. aureus*, vancomycin was most potent among all antibiotics to which 89.89% isolates were susceptible. Next to vancomycin were fusidic acid, rifampicin, linezolid and chloramphenicol inhibiting 84.94, 83.37, 83.15 and 81.12 % of the isolates respectively. Among the 445 isolates, 73.03% were susceptible to doxycycline, 62.47 % to amikacin and 60% to meropenem. Gentamicin, clarithromycin and ciprofloxacin had average activity, i.e. 53.93, 44.04 and 41.57 % respectively. Rest of the antibiotics had below 40 % activities. MRSA strains were found more resistant as compared to MSSA. The reason for erythromycin resistance was genetically investigated. In the KLS

isolates *ermA*, *ermB* and *ermC* were found in 40 (23.95 %), 35 (20.95 %) and 39 (23.35 %) respectively. Most of these *erm* positive isolates were MRSA. In the KE isolates, the prevalence of *ermA* (17.19 %), *ermB* (17.19 %) and *ermC* (28.12 %) was low. The *ermA* 6 (66.67 %) and *ermC* 2 (22.22 %) were found in CRN isolates too. In the HMC isolates the *ermA* 30 (32.61 %), *ermB* 16 (17.39 %) and *ermC* 27 (29.35 %) were quite significant. The prevalence of *erm* genes were low in KBRN isolates. The Panton Valentine Leukocidin (*pvl*) gene was found more in the methicillin susceptible *S. aureus* MSSA compared to MRSA. In the KLS and CRN sample the prevalence of *pvl* in MSSA were 54 (80.60 %) and 8 (88.89 %) while in MRSA it was 48 (48%), 4 (44.44%). The presence of *Staphylococcal* enterotoxins (*sea*, *seb*, *sec*, *sed*) and toxic shock syndrome toxin-1 (*tst*) was determined by PCR. A highly diverse pattern was observed on the prevalence of the toxin genes. The most frequently detected gene was *sea* followed by *seb*, *sec*. The *sed* gene was not detected in all the 445 isolates. The prevalence of the *tst* gene was low and was only detected in 11 (0.02 %) strains. The MRSA (n=54) of KBRN isolates were studied by Pulse field gel electrophoresis (PFGE). 14 clusters were identified by PFGE. These clusters included 29 different pulso-types that were prevailing in the Burns Unit. Out of the total 29 types, 11 strains occurred two or more times as the same pulso-types. MRSA strains were found highly resistant to penicillin and cephalosporin (85–100 %). Among anti-staphylococcal agents that were tested, 17 % of the strains were resistant to both fusidic acid and linezolid. Susceptibility to vancomycin was 100%. Similar pulso-types were detected that were prevailing in the Burn Units. Along with similarities in the strains, diversity also exists as 29 pulso-types were detected in the total 54 MRSA strains isolated from burns patients.

1. Introduction

Staphylococcus aureus (*S. aureus*) is Gram-positive cocci which is a part of normal microbiota of human and frequently found in the respiratory tract and on the skin. Normally *S. aureus* remains non-pathogenic but still it has the ability to cause various diseases including skin infections, like scalded skin syndrome, pimples, boils (furuncles), folliculitis, cellulitis, abscesses, carbuncles, to life-threatening diseases such as bacteremia, sepsis pneumonia, osteomyelitis, meningitis, endocarditis, and toxic shock syndrome (TSS). *S. aureus* is one of the causative agents leading to nosocomial infections (hospital acquired) as well as postsurgical wound infections [1].

Scientific classification of *Staphylococcus aureus* is as under [2].

Domain:	Bacteria
Kingdom:	Eubacteria
Phylum:	Firmicutes
Class:	Coccus
Order:	Bacillales
Family:	Staphylococcaceae
Genus:	<i>Staphylococcus</i>
Specie:	<i>aureus</i>

S. aureus was first isolated from the pus in surgical abscess of a knee joint in 1980 and identified by Sir Alexander Ogston [3] and was named as *Staphylococcus aureus* by Rosenbach in accordance to biological nomenclature.

Staphylococcus aurous is combination of Greek *staphyle* which means a “cluster of grapes”, and *kokkos* which means “berry”. The word *aurous* is a Latin word which means “golden”.

This is because the *S. aureus* look like clusters of rounded bodies as bunches of grapes under the microscope and gives golden color colonies on the surface of blood agar which is helpful in its identification [4]. *S. aureus* when divided by binary fission do not separate completely and remain adhered to each other which results in the formation of grapes like clusters [5].

1.1 Culture and biochemical characteristics of *S. aureus*

S. aureus produces dome shaped golden color colonies on blood agar (Enriched media) because it divides in three planes Fig 1.1. It gives yellow color colonies on mannitol salt agar (MSA) which is a selective media for Staphylococci. MSA contain 7.5 % NaCl that allow growing *S. aureus* only, forming yellow-colored colonies because of mannitol fermentation and resulting in the drop of medium's pH [1]. *S. aureus* are catalase (formation of bubbles in H_2O_2), coagulase (fibrin clot formation) and DNase (zone of clearance on DNase agar) positive which help to identify it up to specie level [6].

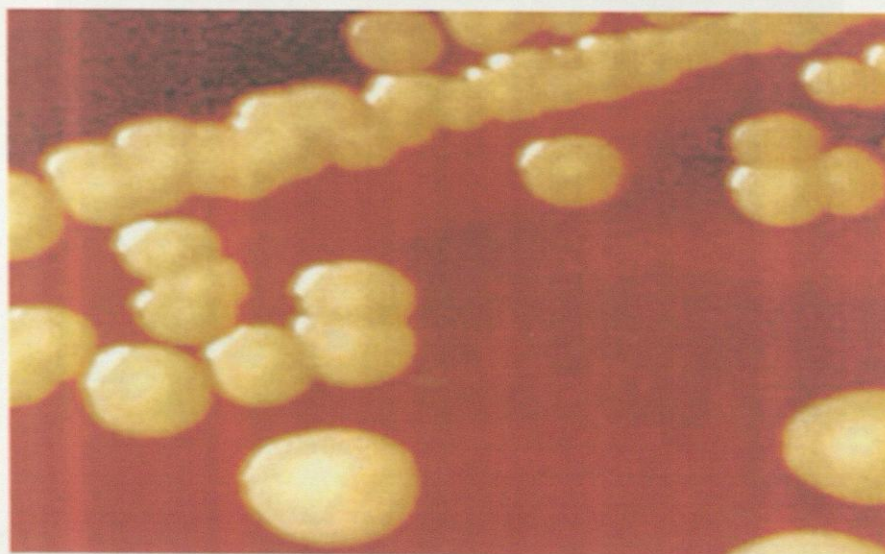


Fig 1.1: *S. aureus* produces golden color colonies on blood agar



Fig 1.2: *S. aureus* growth (yellow color colonies) on MSA



Fig 1.3: *S. aureus* growth on brilliance MRSA agar 2

1.2 Methicillin-Resistant *S. aureus* (MRSA)

Methicillin Resistance *S. aureus* (MRSA) is a resistant strain of *S. aureus* that has developed resistance to β -lactam antibiotics, including the penicillins (amoxicillin, oxacillin, methicillin, etc.) and the cephalosporin. The acquisition of such resistance does not lead to

more intrinsic virulence of the organism than strains of *S. aureus* that have no antibiotic resistance, but due to this resistance the MRSA infections are more difficult to eradicate with standard types of antibiotics and thus more dangerous [7].

MRSA began as a hospital-acquired infection, but has developed limited endemic status and is now sometimes community-acquired. The terms HA-MRSA (healthcare-associated MRSA) and CA-MRSA (community-associated MRSA) reflect this distinction.

1.3 Virulence and pathogenesis of *S. aureus*

The virulence of *S. aureus* depends upon the presence of some attachment proteins on its surface such as fibronectin, laminin, and fibrinogen binding factor which are involved in adherence to cell surfaces and biomaterials, thereby causing colonisation [8]. The secretion of invasins including staphylokinase and coagulase [9] by *S. aureus* also play a role in host cells penetration.

There are strains of *S. aureus* that produces surface proteins, for example protein A, that prevents opsonisation by binding to immunoglobulin G in wrong orientation and thus resist phagocytosis by host's defence cells [10]. The same action is performed by capsular polysaccharide (microcapsule) attached to cell surface [11]. Golden colour carotenoid pigment which is produced by *S. aureus* confers them to resist phagocytosis by their antioxidant properties [12; 13]. Various enzymes, for example, catalase and coagulase produced by *S. aureus* are responsible for their survival inside the phagocytic cells. Thus the immune system of a host is bypassed by all these factors for survival [14].

Exfoliating toxins are involved in the disease staphylococcal scalded skin syndrome (SSSS). These toxins have esterase and protease activity causes peeling of the skin. These toxins occur in two 2 antigenically different forms ETA and ETB [15; 16]. Some strains of *S.*

aureus produce Toxic Shock Syndrome Toxin (TSST-1) and staphylococcal enterotoxin, which have six antigenic types, from A to G. These toxins are said to have superantigenic properties. Due to these properties a large number of T-cells are non-specifically stimulated without normal recognition of the antigen. This results in massive release of cytokines and symptoms of TSS [17].

S. aureus may also produce α -haemolysin, β -toxin, δ -toxin, Υ -toxin and leukocidin which are membrane damaging toxins. They attach itself to the host cell α -toxin receptor which results in small pores in the cell ultimately lysing the cell by osmotic pressure. In this regard, human platelets and monocytes are particularly susceptible to such toxins [18].

The production of these virulent factors are signalled by environmental conditions around them, for example in meningitis and wound infections caused by *S. aureus* [19].

1.4 Biofilm Formation in *S. aureus*

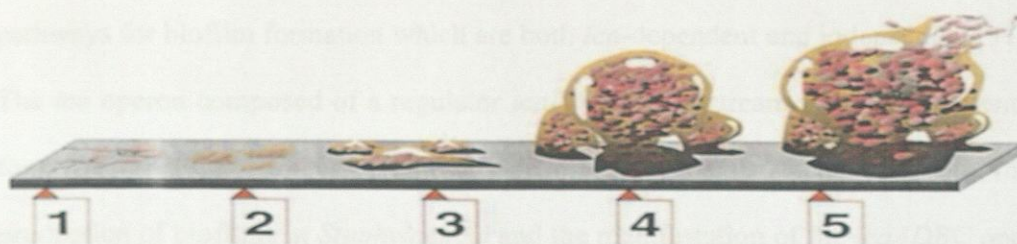
Biofilms are organized microbial systems basically consists of microbial cells layers associated with surfaces. The bacterial cells are embedded in self-produced matrix of extracellular polymeric substance (EPS) referred as slime and is generally composed of polysaccharides, extracellular DNA, and proteins. Biofilms might form on living or non-living surfaces and could be prevalent in natural, hospital and industrial settings [20; 21; 22].

Biofilms produced by staphylococci on the devices that are implanted in patients are responsible for chronic infections in the hospitals [23]. Investigators have demonstrated that *S. aureus* also forms biofilms in addition to the *S. epidermidis* [24; 25; 26; 27].

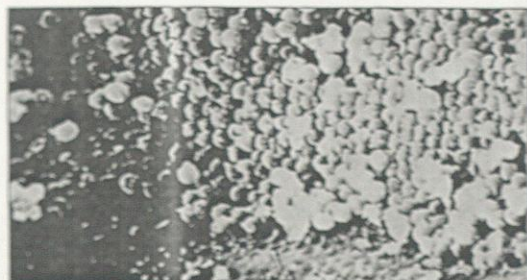
In the biofilm formation the microorganism first attach to a surface (Fig1.4, stage 1) followed by sticking to each other (Fig1.4, stage 2) to form multi-layered cell clusters [20;

25; 28]. After these events, a complex maturation process (Fig1.4 stage 3 and 4) occurs that builds functioning biofilm having proper three dimensional designs with sophisticated channels for circulation of nutrients and signalling molecules. Finally, dispersion occurs (Fig1.4, stage5) that releases cells for new destinations and the cycle is completed.

The bacterial cells get attached to the surface weakly and reversibly. The temporarily attached cells attempt to permanently occupy the surface by secreting some surface proteins which are known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [29]. After this intercellular sticking starts by various mechanisms such as secretion of Polysaccharides Intercellular Adhesions (PIA), a glycocalyx [30]. PIA are polymer of β -1, 6 linked N-acetylglucosamine (PNAG), the production of which is controlled by the *ica* (intercellular adhesion) operon [31; 20; 24; 25; 28; 30]. It is known that PIA negative strains cause much less catheter related infections as compare to PIA positive mutants in animal models [32].



a) "Five stages involved in the process of biofilm development and maturation" [33] (Image Credit: D. Davis)



b) "Formation of a biofilm on the end of a catheter"

"Electron microscopy of a biofilm formed on the end of a catheter. The adhesive substrate can be seen where some of these colonies have become free shown by the arrow."

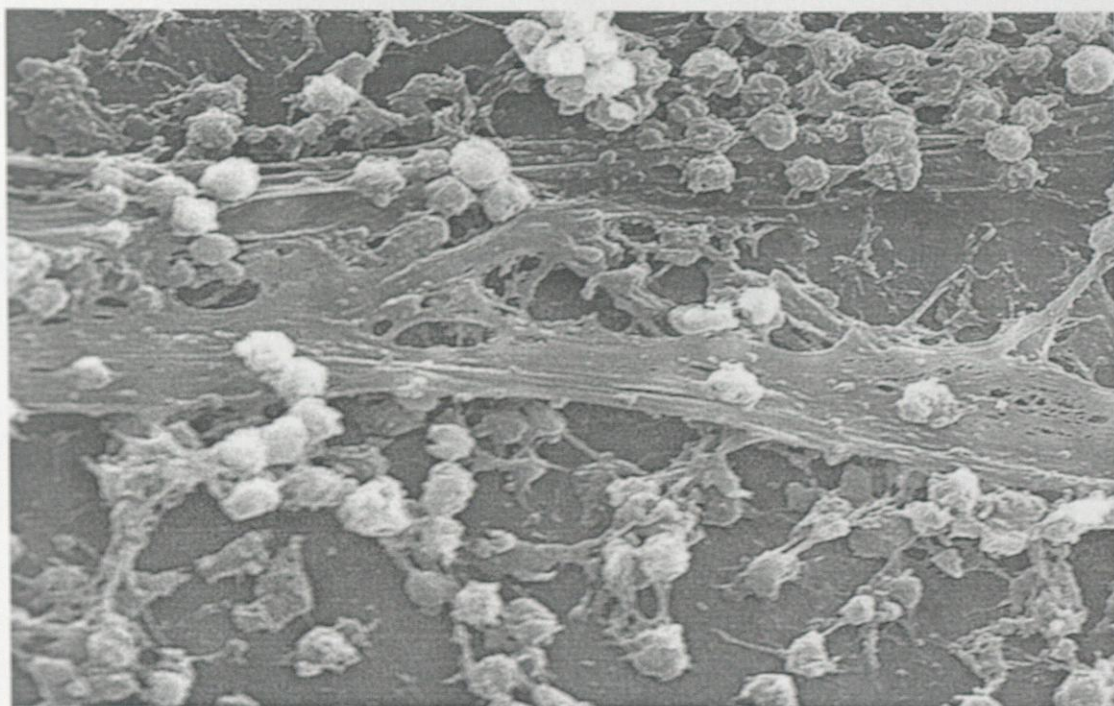


Fig 1.4: Formation of biofilm. Image credit: CDC/ Rodney M. Donlan, Ph.D.; Janice Carr (PHIL #7488), 2005.

Some investigators have proved that biofilm formation has been observed independent of PIA production [34], and even if the *ica* is not present, this suggests that there must be pathways for biofilm formation which are both *ica*-dependent and independent [31; 35].

The *ica* operon composed of a regulator *icaR* located upstream in opposite orientation with four open reading frames. Numerous regulatory proteins; penetrance are involved in production of biofilms in *Staphylococci* and the manifestation of the *icaADBC* operon is just one factor [36]. Studies in which the exploring the genes and the environmental factors that affects the biofilm creation are given in the Table 1.1. These examples are a few illustrations, but still there are many more.

There might be a possibility that the mechanisms responsible for the production of biofilm i.e. *ica* gene manifestation and polysaccharide creation happens in response to the environmental factors similar to other virulence factors.



Fig 1.5: "The *ica* Operon [30].

This theory support a low prevalence of biofilm formation from clinical isolates of *Staphylococci*, compared with *in vitro*" [26]. The occurrence of the *ica* operon in *Staphylococci* collected from infected areas have been studied in many cases, which are presented in Table 1.1. It is suggested that the occurrence of the *ica* operon in the clinical strains of *S. aureus* could be consider as a virulence marker in routine identification of certain virulent strains [31] which highlight the worth of this gene loci and a need for further research.

Table 1.1: Various studies on genes and the environmental factors that affect biofilms, and the presence of the *ica* loci in strains collected from different infected areas

Gene studied	What the genes code	Reference
<i>CAN</i>	Collagen adhesin gene: 29% of the strains were <i>cna</i> -positive and also able to bind collagen <i>in vitro</i> . 83% of the strains were slime forming.	[27]
<i>SarA</i>	<i>Staphylococcal</i> accessory regulator: activates the development of <i>S. aureus</i> biofilm by increasing transcription of the <i>ica</i> operon and suppression of transcription (or a repression of its synthesis) of a protein involved in the turnover of PIA/PNAG.	[37]
<i>SigmaB</i>	Transcription factor, has a role in the formation of biofilm however its effect on <i>ica</i> is unknown.	[38]
<i>Bap</i>	Findings suggest Bap protein compensates for a PIA-PNAG (<i>ica</i> product) deficiency.	[25]
<i>IcaR</i>	Found upstream of <i>icaADBC</i> acting as an important environmental regulator in <i>ica</i> expression.	[39]
Environmental factor	Effect of environmental factor	
Osmotic stress	Induced biofilm formation	[38]
Aerobic environment	Aerobic environment stimulates the production of PIA/PNAG.	[40]
Site of infection	Experimental findings and prevalence of <i>ica</i> operon	
Cochlear implants	Only <i>S. aureus</i> was detected growing on cochlear implants removed from patients with infections. Extracellular polymeric substances were observed demonstrating the presence of biofilm and need for research in this area.	[41]
Orthopaedic steel implants	Most <i>S. aureus</i> strains were capable of forming biofilm and the established biofilms were resistant to gentamicin.	[42]
Catheter associated	61% of <i>S. aureus</i> strains were <i>icaA</i> and <i>icaD</i> positive.	[31]

infections		
Prosthetic joint infections	100% of <i>S. aureus</i> (N=55) isolated were <i>icaA</i> positive.	[43]
Bovine mastitis	35 out of 35 tested positive for <i>icaA</i> and <i>icaD</i> , whereas only 24 tested positive on Congo red agar.	[44]
Clinical isolates	35.2% of <i>S. aureus</i> <i>icaA</i> and <i>icaD</i> positive	
Hospital staff carriers	22.2% of <i>S. aureus</i> <i>icaA</i> and <i>icaD</i> positive	[45]
Clinical isolates	68.9% of the MRSA were positive for <i>ica</i> and <i>mecA</i>	[46]
Clinical isolates and carriers	91% of MRSA were <i>icaD</i> positive and all <i>icaD</i> negative strains were <i>icaA</i> positive. 47% of MRSA and 69% of MSSA produced biofilm <i>in vitro</i> .	[47]

1.5 Panton Valentine Leukocidin (PVL)

Leukocidin is a, "Pathogenic bacteria's toxin damaging the white blood cells". It provide defense to bacteria against damaging effects of various immune system as well as help to occupy and adhere in the hosts tissue to cause a disease. *S. aureus* produces various extracellular proteins [48] and PVL is one such agent [49; 50]. It was first isolated from strains of *S. aureus* with V8 proteases in a patient suffering from chronic furunculosis by Pantan and Valentine [51]. It belongs to the family of pore-forming toxins in recent times described as synergohymenotropic toxins [52]. It comprised of two components toxin (*lukS* PV and *lukF* PV) which are differentiated by elution in an ion-exchange column, as S stand for slow and F stand for fast elution [53; 54]. For leucotoxic activity the presence of both the components is necessary [55]. Another toxin produced by *S. aureus* is the gamma hemolysin which is also comprised of two-components. The PVL action is haemolysins-independent i.e., a strain may produce hemolysin and not leukocidin and vice versa [51; 56].

Both *LukS*-PV and *LukF*-PV belong to a single operon. The PVL toxin is very common virulence factor and this is linked with community-acquired-MRSA (CA-MRSA). It is one the cause of high mortality rate in case of necrotizing-pneumonia. It is also known that CA-MRSA can harbor toxic-shock-syndrome toxin 1 and rarely exfoliative toxins [57]. CA-MRSA can cause an outbreak and also may turn to be virulent. However production of Pantan-Valentine leukocidin (PVL) toxins by CA-MRSA is of particular concern which is considered to contribute to their increased virulence [58].

Some data shows that less than 5% of *S. aureus* strains produce PVL toxin in Western Europe. The cytotoxin is known for causing destruction of leukocytes and tissue necrosis, particularly polymorphonuclear neutrophils (PMNs) and macrophages [59]. The S-

component of the leukotoxins binds directly to a Ca^{2+} channel or to a receptor that is linked to a Ca^{2+} channel. There after the F-component causes Ca^{2+} channel opening leading to Ca^{2+} influx. Then the toxin get penetrated resulting a pore in the membrane and consequently leading to release of inflammatory-mediators such as histamine, leukotriene B4 and IL-8 [60; 61; 50; 62].

Prevost *et al.*, in 1995 cloned and sequenced the genes for the first time, which encode PVL. The *lukF*-PV and *lukS*-PV genes encode proteins of molecular weight 34,000 (composed of 325 amino acids) and 32,000 (composed of 312 amino acids) respectively. The later contains a 28-residue signal peptide while the former contains a 24-residue peptide whose cleavage results in mature proteins. Each of them arises from two different loci within the *S. aureus* genome. The isolates which produce both the gamma-hemolysin and PVL contain two F components (*lukF*-PV and HigB) and three S components (*lukS*-PV, HigA and HigC) [59].

Destruction of neutrophils predisposes host to bacterial infections because these are the main cellular defense against pathogens. The production of PVL toxin by *S. aureus* is associated with cutaneous abscesses, severe necrotic skin infections and furuncles. Although it is still difficult to find the exact roles of various *Staphylococcal* factors in invasive infections [50].

A specific association between the presence of the *pvl*-gene and furunculosis has been described in that 86%-93% of *S. aureus* strains produce the PVL toxin isolated from primary necrotic infections [50]. Association of PVL with epidemic-furunculosis and not involved all types of furuncles has also been proposed. Later on a series of studies have been conducted in the whole world for determination of of PVL-genes distribution among *S. aureus*. In most studies the PVL was correlated with community onset *staph*-infections. In France, Issartel *et al.*, found PVL-genes in 89% *S. aureus* strains causing soft tissue

abscesses (furuncles), with no difference in primary and secondary skin infections [63]. Out of 415 *S. aureus* from 15 countries screened by Strauss *et al.*, found 47% PVL positive in skin infections [64], however 92.4% PVL were found Tunisian isolates from the community. Until 2003 it was known that some *pvl*-positive CA-MRSA clones which were considered as continent-specific have now reached to other continents and have been detected in several other countries. In some countries the *pvl*-positive CA-MRSA-strains, have become resistant to anti-staphylococcal-antimicrobial agents to which they were previously-susceptible [57]. It was observed that the *pvl*-positive-*S. aureus* isolates respond-differently to different-antibiotics. As some antibiotics may down and others may up regulate the PVL. Fusidic acid, clindamycin and linezolid decrease PVL production in *S. aureus* in a concentration-dependent-fashion at sub-inhibitory-level, however vancomycin has no effect. The PVL production is increased at subinhibitory-oxacillin-concentrations [65]. Thus, PVL positive *S. aureus* caused infections should be treated with antibiotics having inhibitory-effect on PVL-production [66].

1.6 Staphylococcal Enterotoxins

Variety of heat stable exoproteins produced by *S. aureus* found to act as virulence factors causing food poisoning. The *Staphylococcal*- enterotoxins (SEs) and toxic-shock-syndrome-toxin-1 (TSST-1) are extensive studied and explored among these exoproteins [67]. The SEs are categorized into five toxin groups designated as *sea* - *see* and are responsible for food poisoning. Recently other *Staphylococcal*-enterotoxins (*seg-ser* and *seu*) have also been reported [68; 69; 70]. Until now more than 18 serologically-distinct SEs and enterotoxin-like (*sei*) toxins have been reported. These toxins have been identified with the characteristics of superantigenic, have low molecular weights, are stable at boiling temperature and are

resistant to proteolysis [71]. However some of them (like *seg*, *she*, *sei*, *sej* and *seu*) are still under investigation to find their role in instigating human disease and pathogenicity [72]. *Staphylococcal*-enterotoxin-genes are harbored on mobile genetic elements. Gene coding for *seb* and *sec* are localized on the chromosome, genes for *sea* is harbored by a temperate-bacteriophage. The genes for *sed*, *sej*, *ser* and all other SEs excluding SEH are carried on plasmids and pathogenicity-islands (SaPIs). Several SEs genes are located in most SaPIs and play a role in their horizontal transfer amongst *Staphylococcal* strains [73].

Staphylococcal disease and pathogenicity caused by emetic and superantigenic toxins is a result of multiple expression of toxins by *S. aureus* [74]. The massive growth of *S. aureus* in food lead to production of SEA, which is commonly implicated in *Staphylococcal* food-poisoning (SFP) [75]. Individuals suffering from SFP are characterized by nausea, diarrhoea, violent vomiting and abdominal-cramps in between 1–8 hours after consumption of food. The IgE-reactivity to food-antigens consequently after administration of food antigens and *seb* [76]. However, There are studies in Japan that *S. aureus*-strains isolated from individual of food-poisoning cases were negative for all *Staphylococcal*-enterotoxin-genes reported so far [77]. The PCR can be used for detection of the presence of enterotoxin-genes in *S. aureus* isolates but the ability of such strains to produce enterotoxins and their tendency to lead disease is demonstrated by bioassay or immunologic-methods [78]. The distribution of enterotoxin-genes amongst *S. aureus*-strains is not uniform [79; 80]. Furthermore, their distributions varies from country to country in the *S. aureus* population [81].

The acute or chronic rhinosinusitis may be contributed by *S. aureus*. The nasal secretions of patients with chronic rhinosinutitis (CRS) has been reported to have high levels of *sea* and

seb [82] which are respectively coded by the *sea* and *seb*-genes. In the CRS patients the *Seb* could move to the nasal-cavity along with the secretions, where it contaminate the airway-mucosa or if compelled backwards, it might be swallowed-down the gastrointestinal-tract, which can disturb local tissues immune-homeostasis [83]. The permeability of the airway epithelial barrier is increased by *Seb* which facilitate epithelium crossing [84]. Furthermore, the SEB also contributes to CRS by activating T cells. CRS can be a potential source of *seb* in the body as *S. aureus* can easily colonise the rhinosinuses [76]. The nasal colonisation of *S. aureus* is also associated with the inter-patients transmission and subsequent invasive infection of patients and plays a potential role in the epidemiology and pathogenesis of *Staphylococcal* infections. In the adult population the nasal carriage rate of *S. aureus* infections is 30-50% [85]. Food-allergy in rhinosinusitis derived from *seb* is associated-with maintenance and augmenting of polarised T-helper (Th2)-responses. Such a food-allergy symptoms are inhibited in patients with CRS food-allergy by the removal of *seb*-producing-pathogens from the rhinosinuses. In addition, in patients of food-allergy and chronic-rhinosinusitis, significant amelioration of food allergy has been demonstrated. Therefore, controlling colonisation of *S. aureus* and nasal infection could be beneficial in conditions related to these [83].

T lymphocytes CD8+ and CD4+ are strongly induced by *seb* even at extreme low concentrations [86]. Exposing the airway to *seb* aerosols can strongly activate the immune system. This can also result in a condition clinical similar to toxic shock [87]. Moreover, the immune system is greatly activated when the the conjunctiva is exposed to to SEB [88]. Binding of *seb* at a particular site to the major histocompatibility complex (MHC) assists its biological effects. The toxin is presented to a T-cell-antigen-receptor by MHC-2 which

elicits T-cell-proliferation and cytokine-production upon formation of ternary-complexes [89; 90].

1.7 Toxic Shock Syndrome Toxin-1 (TSST-1)

This is a superantigenic-toxin and is encoded by the *tst* gene. Toxic-shock-syndrome-toxin 1 (TSST-1) is a key virulence-factor of some *S. aureus* isolates. The prevalence of the *tsst* gene amongst *S. aureus* is very low [80]. TSST-1 is responsible for toxic-shock-syndrome (TSS), neonatal-toxic-shock-like exanthematous-diseases (NTED) and *staphylococcal*-scarlet-fever. TSST-1 positive MRSA causes many diseases including toxic-shock-syndrome and suppurative infections [57]. Hypotension, rash, myalgia, multiple organ failure, fever and late desquamation of feet and hands are reported with TSS. A *tsst*-positive MRSA genotype primarily found in Japan has been evidenced in France recently. This MRSA is associated with bloodstream infections and is spreading in rest of the Europe reportedly [89]. Menstrual toxic shock syndrome (mTSS) caused by *S. aureus* has been associated with menstruation and tampon use in menstruating women. Although prevalence of mTSS is low but may prove to be fatal due to extensive use of tampons by the menstruating women [91]. According to a study conducted by Czerwinski, about 80% of the women participants used tampons at some point in the study during menstruation. TSST-1 causes about 100% of mTSS cases and almost 50% of non-menstrual TSS. While the remaining 50% of non-menstruation TSS cases are caused by *staphylococcal* enterotoxin B or C [92]. SEB causes toxic-shock at low-serum-concentrations and can also prompt-multiple-organ failure and hypotension [17]. Majority of non-menstruation-associated-TSS is reported from both the community and hospitals secondary to local-infections by *S. aureus* [93].

1.8. Use of antibiotics for the treatment of infections cause by *S. aureus*

Bacterial infections are treated by the use of antibiotics. The word antibiotic traditionally implicated for the substances-produced by microorganisms to leading to growth-inhibition of the other microorganisms. The term antimicrobial is extensive in meaning, since it comprised the synthetic-agents in the laboratory (i.e., sulfa drugs) as well as the antibiotics of natural-origin (i.e., penicillins) produced by microorganisms. Antimicrobials agents might be either bactericidals (i.e., killed the organisms) or bacteriostatic (i.e., prevented growth of microorganism) [94]. The Greek stem derived word, antibiotic means "against life". A French researcher Paul Vuillemin, in the late 19th century, described a substance isolated some years earlier from the bacteria, *Pseudomonas aeruginosa* and demonstrated "antibiotic". Latter on another substance, called pyocyanin, inhibited the growth of other bacteria in test-tubes but rejected for disease therapy due to its high toxicity. The Vuillemin's discovery leaded to the current era, his word "antibiotic" are now considered to be chemical and natural products that are inhibitory to other organisms [95].

After the discovery of penicillin by Alexander Fleming in 1927, The Howard Florey, Ernst-Chain and Norman-Heatly purified and evaluated penicillin against-streptococci and *staphylococci* in 1940. For this discovery, they received Nobel-prize in 1945 [1].

The penicillin discovery motivated the serial search for other antibiotics. In 1944, streptomycin was discovered by Selman Walsman. In 1953, the microorganisms producing chloramphenicol, neomycin, terramycin and tetracyclin were isolated. Thereafter, the chemotherapeutic agents and newer drugs discovery have revolutionized modern medicine and greatly minimized the human suffering [1]. Since appearing in early 1940's antibiotics are the greatest-success-achievements in the clinical-medicine, reducing morbidity and

mortality dramatically from bacterial-infections and is largely responsible for average 10 years life-span extension of normal human-beings in the developed-countries [96].

1.8.1 Mechanism of action of antibiotics

The bacterial cell-wall is made up of polysaccharides and polypeptides known as peptidoglycan (murein, mucopeptide). The cell wall polysaccharide consists of alternating amino-sugars, *N*-acetylglucosamine and *N*-acetylmuramic acid. The latter sugar is attached with a five amino acid peptide which further terminates in D-ala-D-ala. The terminal alanine is removed to form a crosslink with another peptide (transpeptidase reaction) which is catalyzed by an enzyme known as penicillin-binding proteins (PBPs). β -lactam antibiotics bound to PBPs through strong covalent bound due to structural similarity with the natural D-ala-D-ala, leading to inhibition of transpeptidation reaction. β -lactam antibiotic inhibit peptidoglycan synthesis, weakening the bacterial cell wall and finally bacterial cell dies [97].

Vancomycin which is a glycopeptide antibiotic, binds to the free carboxyl end of pentapeptide resulting in steric interference with peptidoglycan backbone prolongation thus inhibiting cell wall synthesis in susceptible strains of bacteria. Another glycopeptide, Teicoplanin interacts with the D-alanyl-D-alanine terminal of the muramylpentapeptide which fits into a cleft inside the antibiotic molecule thereby inhibits polymerization of peptidoglycan [98; 94]. The quinolones antibiotics inhibit the bacterial DNA gyrase or topoisomerase II which produces negative twists in DNA, and assists DNA strands separation thus blocks DNA replication, repair, and transcription. Another enzyme responsible for DNA unwinding during replication, topoisomerase IV is inhibited by fluoroquinolones [99; 100].

The macrolides antibiotics, containing the macrocyclic lactone structure, were the first choice as an alternative-to-*penicillin* in individuals who are allergic to β -lactam-antibiotics. Generally the macrolides are considered to be bacteriostatic but they might be cidal in higher concentrations. They inhibit the bacterial protein synthesis by blocking the translocation step via irreversible binding 50S ribosomal subunit of bacteria [101].

Tetracyclines, containing four fused rings in their basic structure, act by binding to 30S ribosomes, thereby preventing-attachment of the aminoacyl-tRNA to the acceptor-site on the mRNA-ribosomal-complex. This binding ultimately prevents the addition of amino-acids to the pre-synthesized peptide-chain. The activity of individual tetracycline is altered by its ability to penetrate lipid membranes of the bacteria. They access in cytoplasm of gram-positive bacteria through an energy-dependent-process, however, in gram-negative-organisms, the diffusion through porins in the outer-membrane is reported. In contrast, the minocycline and doxycycline they can enter gram-negative-cells through simple-diffusion as well as through the porins due to its higher-lipophilicity. From the periplasmic-space, the tetracyclines are further transported across the inner-cytoplasmic-membrane by a protein-carrier-system [94].

The penetration of aminoglycosides in bacterial-cells follows several steps. In the first step, the cationic aminoglycosides interact with anionic cell surface. Then it penetrates through the porin channel or the water-filled-areas of the peptidoglycan-wall in Gram-negative-bacteria or Gram-positive-bacteria respectively. Another penetration pathway of aminoglycoside is to disrupt the outer-membrane and enhance their own uptake through nonporin-type-channels in the phosphate-rich gram-negative-bacteria. Thereafter the aminoglycosides cross the cytoplasmic membrane by binding to a transport molecule that is

either part of or directly linked to the electron transport chain. The electrical potential gradient across the membrane is responsible for the movement of the drug-transporter complex. Such a transport mechanism needs energy which is favorable in aerobic environment but does not occur in anaerobic environment. In the cytoplasm the aminoglycoside bind to ribosome which maintains a low concentration free aminoglycosides intracellular which supports the continued transfer of drug from the cytoplasmic membrane to the ribosomes, which results in a loss of membrane integrity and death of the bacteria. Furthermore the aminoglycosides inhibit the protein synthesis by binding to ribosomes. Streptomycin, the most thoroughly studied aminoglycoside, binds to the 30S subunit where it interferes with protein synthesis in two ways [102; 94] as given below:

(1) Restricting polysome formation

(2) Misreading mRNA

Chloramphenicol, one of the most potent antibiotics, binds to the bacterial 50S ribosomal subunit and inhibits protein synthesis at the peptidyl transferase reaction [103; 104]. Fusidic acid is a protein synthesis inhibitor by preventing the turnover of elongation factor G (EF-G) from the ribosome. It is primarily effective on Gram-positive bacteria such as staphylococci and streptococci [105]. Fusidic acid inhibits bacterial replication and does not kill the bacteria, and is therefore termed "bacteriostatic".

1.8.2. Resistance to antibiotics

The bacteria are considered to be resistant if their growth is not halted by the maximum level of antibiotic that is tolerated by the host [103]. The emergence of antibiotic resistance is a serious problem in all kinds of pathogenic bacteria [106].

Bacteria use different strategies to combat environmental pressures for survival and their

response to antibiotic pressure is a consequence of this behavior. Resultantly antimicrobial usage causes selection of resistant bacteria. Misuse of antibiotics results in multi-drug resistant pathogens may endanger the antibiotic era. Development of new antibiotics has been sluggish despite of high demand in the recent years. Therefore extensive effort will be required to maintain the efficacy of these drug groups due to the persistently growing resistance [36].

As many antibiotics are produced by other microorganism the antibiotic resistance genes were present in the pre-antibiotic era in bacteria to detoxify antibiotics. Nucleotide sequences of several antibiotic resistance genes show regions of homology with the genes for antibiotic production [107; 108]. Until penicillin discovery and even before sufficient penicillin G had been produced on small scale to treat patients. Then it was reported that an enzyme in *Bacillus coli* (now known as *Escherichia coli*) that inactivated penicillin G by Oxford group. It had been reported that *S. aureus* strains were capable of penicillin G inactivation. Resistance to streptomycin soon followed [94].

Soon after the production of penicillin in the 1940's by pharmaceuticals, microbes acquired resistance mechanisms [109].

Furthermore the former reports led to genetic studies of bacterial resistance and identification of plasmids that confer resistance and are widely distributed in nature. Resistance also is present in the form of genes on transposons and can be transferred to plasmids or become integrated into chromosomes. Chromosomal genes that code for resistance also can be transferred in the reverse direction and become attached to transposons and the resistance transferred by plasmids [94].

The use of antibiotics leads to inhibition of susceptible strains and allow the proliferation of

intrinsically resistant bacteria or that have acquired extra chromosomal resistance. From an epidemiological point of view, plasmid resistance has got importance in the sense that resistance in this form is transmissible and may be associated with other properties that enable a microorganism to colonize and invade a susceptible host [94].

The theory of antibiotic resistance has propagated globally beyond the professionals involved in the diagnosis and management of infection and is highly reported in the media [110]. In remote communities the expression of organism or a novel gene coding for drug resistance has implications for the developed world. Once a resistant organism is introduced into a population, it is rapidly disseminated [111].

Antibiotic resistance depends on the time interval and use of antibiotic. It should be administered in a way to avoid or minimize development of resistance such as used as therapeutic or for prophylaxis [110]. The pattern of microbial-resistance varies from large hospitals to small hospitals, country to country, community to community and state-to-state. It is worthy to get the information about susceptibility pattern of pathogens in prescribing area, the cost of the drug and the trend of the susceptibility. Like other developing world, in Pakistan, the graph of antibiotic resistance is generally increasing to all commonly used antibiotics [112; 113]. This increase in resistance associated with many factors. Inappropriate dosage and prolonged treatment with antibiotics because of self-medication or negligence of health professionals is one of contributing factor in Pakistan. The spread of antibiotic resistance is also led by the poor hygienic conditions in most of the country's hospital settings and health facilities providing an excellent environment for the origination and expanse of antibiotic resistance [114].

1.8.3. Mechanism of resistance to antibiotics in bacteria

Bacteria adopt resistance by various mechanisms. It is noteworthy to understand that certain mechanism of resistance is not confined to a single class of drugs. Against the same chemotherapeutic agent, two bacteria may adopt two different resistance mechanisms. Furthermore, resistant mutants are not created by direct exposure to a drug but they arise spontaneously and are then selected [1].

The basic underlying mechanisms of microbial resistance development are:

1.8.3.1. Resistance based on altered receptors for drug

By this mechanism, bacteria produce another alternative target (might be receptor or enzyme) along with original sensitive target thereby protecting them from antibiotic. Thus alternative enzyme “bypasses” the effect of the antibiotic which allows bacteria to survive. The well-explained example of such mechanism is methicillin-resistant *S. aureus* (MRSA), which produce alternative penicillin binding protein (PBP2a) along with “normal” penicillin binding proteins (PBPs). This altered protein has low affinity for the β -lactams, cephalosporins and penicillins and encoded by the *mecA* gene. Consequently the bacterial cells remain alive and continue to synthesize peptidoglycan [115; 116].

1.8.3.2. Inactivation of drug

The production of β -lactamases is the most high-lighted mechanism bacterial resistance to antibiotics. β -lactamase enzymes catalyze the hydrolysis of β -lactam antibiotics. The subsequent products are inactive. In over 200 known types of β -lactamase, many β -lactamases show activity against both penicillins and cephalosporins up to some degree; rest of them are more specific, namely, cephalosporinases (for example, AmpC enzyme found in *Enterobacter* spp.) or penicillinases (for example, *S. aureus* penicillinase) [117].

The chloramphenicol transacetylase enzyme producing gram-positive and gram-negative bacteria are resistant to chloramphenicol. However, the acetylated chloramphenicol binds poorly to the ribosome compared to non-acetylated. So nonacetylated drug inhibit protein synthesis more effectively, and is unaffected by chloramphenicol transacetylase [118].

1.8.3.3. Altered rates of entry or removal of drug

Some bacteria prevent the antibiotic from entrance to cell or propelling it out faster than it can move in (rather like a bilge pump in a boat) thereby protecting the target of antibiotic action [117].

Beta-Lactam antibiotics may enter in gram negative bacteria through water filled hollow membrane protein known as a porin. In the case of imipenem resistant *Pseudomonas aeruginosa*, imipenem cannot penetrate the cell due lack of the specific D2 porin confers resistance. Fluoroquinolones and aminoglycosides share the former mechanism with low level resistance to aminoglycosides and fluoroquinolones. Augmented efflux via an active transport pump is a well-recognized tool for resistance to tetracyclines and is encoded by an extensive range of related genes, such as *tet* (A), that have become dispersed in the enterobacteriaceae [119].

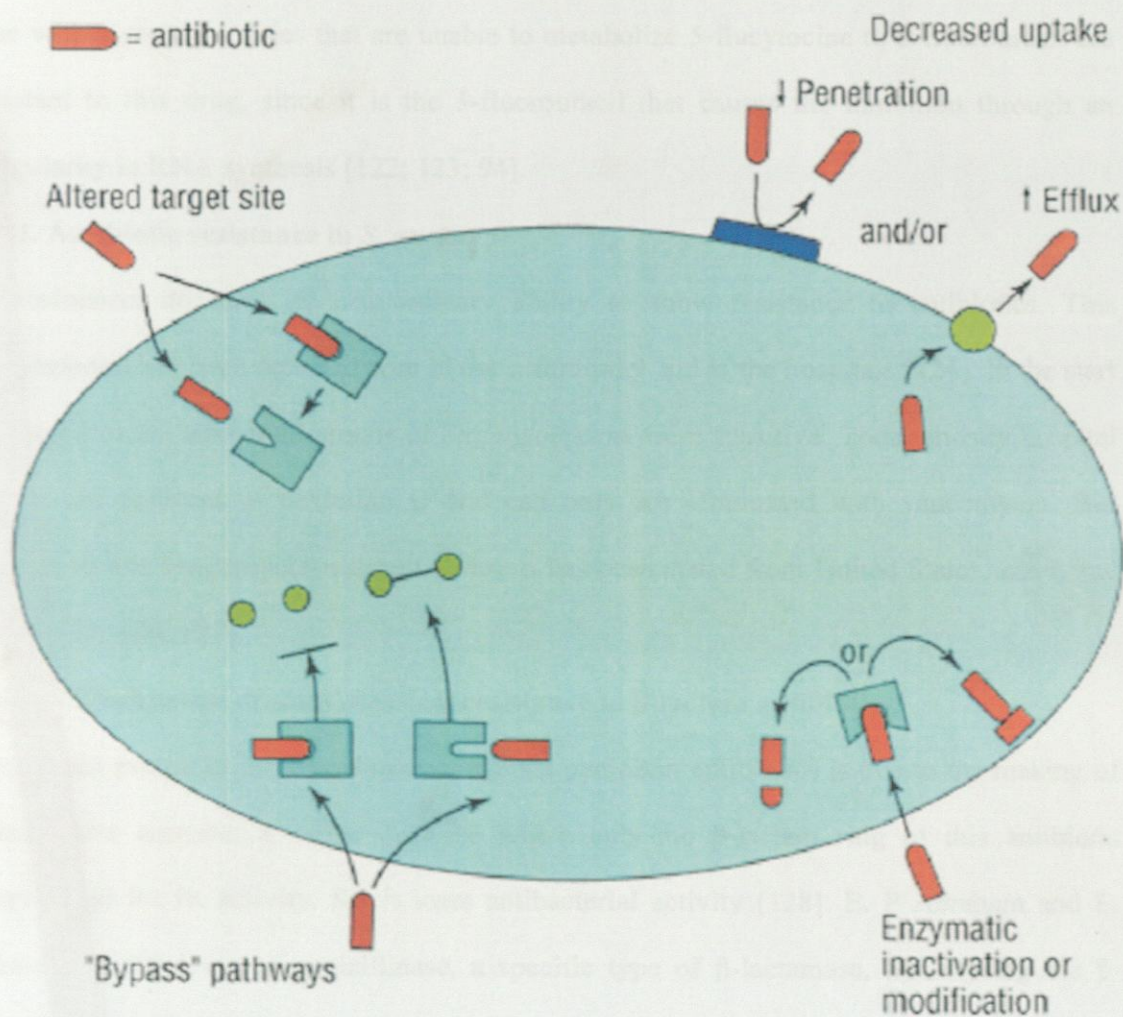


Fig 1.6: Major biochemical mechanisms of antibiotic resistance [120].

Similarly, the efflux pump coded by the *msrA* gene expels erythromycin out of the cells. This results in decreased concentration of the drug inside the cell and hence less or no effect on the cells [121].

1.8.3.4. Failure to metabolize the drug

Anaerobic bacteria such as the rare *Bacteriodes fragilis* are resistant to metronidazole and nitroimidazole because they do not metabolize these antibiotics to the active metabolites,

which eventually results in DNA damage, and are not killed by metronidazole. Same is the case with *Candida* species that are unable to metabolize 5-flucytocine to 5-fluorouracil are resistant to this drug, since it is the 5-fluorouracil that causes the inhibition through an irregularity in RNA synthesis [122; 123; 94].

1.8.4. Antibiotic resistance in *S. aureus*

Staphylococci do have an extraordinary ability to show resistance to antibiotics. This phenomenon has been detected both in the community and in the hospitals [124]. In the start of penicillin era almost all strains of *Staphylococcus* were sensitive. Today mostly hospital strains are resistant to penicillin G and can only be eliminated with vancomycin. But parallel to this vancomycin resistant *S. aureus* has been stated from United States, Japan and India [125; 126; 127].

1.8.4.1. Mechanism of *staphylococcal* resistance to β -lactam antibiotics

Resistance primarily in *staphylococcal* against penicillin antibiotics is due to the making of β -lactamase enzyme, a serine protease which cuts the β -lactam ring of this antibiotic responsible for its activity. So, it loses antibacterial activity [128]. E. P Abraham and E. China in 1940 revealed penicillinase, a specific type of β -lactamase, hydrolysing the β -lactam ring of penicillins specifically [81]. To fight such a resistance, a research group in UK presented a penicillinase resistant antibiotic, methicillin in 1961. However, resistance to methicillin developed the same year [129], and MRSA (Methicillin-Resistant *S. aureus*) was originated, a *S. aureus* isolate resistant to methicillin remains resistant to all β -lactams. The primary difference of MRSA versus Methicillin-sensitive *S. aureus* (MSSA) is the production of altered PBP i.e., PBP2a by the former [128]. This PBP2a is encoded by *mecA* gene and reduced attraction for β -lactam antibiotics [130; 131; 132; 133]. This changed

PBP2a can retain functions of the regular PBPs at the same antibiotic concentrations which causes to inactivate the regular PBPs [134].

Initially, it was considered that MRSA are only prevailing in hospitals but latter on the MRSA was reported even from the community [135]. Depending upon the route of acquisition, they were named as hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA). There are several explanations of HA-MRSA and CA-MRSA.

It was reported that *mecA* gene that codes for the transformed PBP2a is positioned on a mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*) [136; 137]. Primarily it was considered that the SCC*mec* might be dispersed via lysogenic bacteriophage [138]. However, later chromosome recombinase A and B (*ccrA* and *ccrB*) genes were revealed which encode recombinases of the resolvase/invertase family which integrate SCC*mec* into the bacterial chromosome [136]. There are four known types of SSC*mec*, i.e. I-IV. The SCC*mec* I-III are prevalent in the HA-MRSA and SSC*mec* is mostly found in the CA-MRSA [139].

Soon after the first discovery of MRSA in 1961 [129], its prevalence was increased exponentially. In 2006, Orrett and Land, reported 60.1% MRSA from surgical/burn wounds from Trinidad, West Indies, which were highly resistant to clindamycin (75.3%), tetracycline (78.7%) and ciprofloxacin (59.1%) [140]. Bell and Turnridge reported from 1998-99 SENTRY program 4.1 to 34.4% MRSA from Australia, 64.8 to 74.5% from Japan, 57 to 75% from Taiwan, 69.8% from Hong Kong, 62.3% from Singapore and 41.5% from South Africa [141]. Hsueh reported 66% prevalence of MRSA from nosocomial origin from Taiwan during 1986 to 2001 [142], 95% of these were MDR (resistant to 3 or more).

Randrianirina *et al.*, reported 6.5% prevalence rate for CA-MRSA and 4.4% for HA-MRSA from Madagascar in 2007 [143]. In 2006, Bratu *et al.*, from USA reported 44% MRSA prevalence in community [144]. Baddour *et al.*, from Saudi Arabia in 2006, reported 77.5% MRSA prevalence among inpatients and 22.5% among outpatients [145]. Dar *et al.*, found 35% prevalence of MRSA in hospital in 2006 [146]. Brown *et al.*, found 23% prevalence rate of MRSA in Jamaica in 2007 10% MDR [130]. Subedi *et al.*, reported 15.4% MRSA prevalence from Nepal in 2005 [147].

1.8.4.2. Macrolides resistance in *S. aureus*

Macrolides inhibit protein synthesis by binding to the peptidyl transferase region of the 50S ribosome subunit which results in dissociation of the peptidyl t-RNA [148]. The macrolides, such as erythromycin, azithromycin have been extensively used and studied antibiotics [149]. Macrolide-lincosamide-streptograminB (MLS_B) antibiotics are valuable antibiotics for penicillin-allergic patients having *staphylococcal* infections [150]. Macrolides-resistant *staphylococci* generally remain co-resistant to lincosamides and Type B Streptogramins. This phenomenon is known as MLS_B. Strains which exhibit an MLS_B resistance possess erythromycin ribosomal methylase (*erm*) genes [151; 152]. The *erm* genes encode rRNA methylases [152]. The products of these genes act by target site modification through methylation of an adenine residue 2058 (*E. coli* numbering system) in the 23S rRNA component of peptidyl transferase region. This results in blocking the binding of 14, 15 and 16 membered ring macrolides, lincosamides and type B streptogramins to the ribosomes [153]. Expression of methylase gene is controlled through translation attenuation, leading to inducible expression. The inducible strains appear to be resistant to erythromycin but sensitive to other MLS_B antibiotics, without prior exposure to inducing antibiotics e.g,

erythromycin. Deletions or mutations within the control region can also result in constitutive gene expression [154].

The *ermA*, *ermB* and *ermC* are typically found in *staphylococci* [89] among over 30 distinctive *erm* genes [155; 150]. *ermC* is common than *ermA* in clinical isolates of *S. aureus* and coagulase-negative *staphylococci* [156].

msrA gene is present in strains that are inducibly resistant to only 14 and 15 membered ring macrolides and type B streptogramins and are sensitive to lincosamides (MSB) [151]. This gene was discovered by Ross [153] in a *S. epidermidis* isolate, located on a large (>30Kb) *staphylococcal* plasmid. The *msrA* gene mediates resistance to erythromycin via an efflux pump and is a member of ABC transporter family [157; 153]. Lina *et al.*, (1999) while analysing the distribution of genes encoding MLS resistance amongst *staphylococci*, found that *msrA* gene conferring macrolide resistance was comparatively higher in coagulase-negative *staphylococci* (14.6%) than in *S. aureus* (2.1%). Other data shows that some strains can harbour a combination of both *erm* and *msrA* genes [151; 158]. The strains harbouring both sets of genes were phenotypically MLS resistant [151], indicating that phenotype is masked by *erm* genes. The reason for multiple resistance genes in the same cell is unclear at present.

Macrolide resistance is also conferred by a number of inactivating enzymes such as esterases (encoded by *ere* gene) and macrolide phosphotransferases (encoded by *mph* genes) that disrupt the structure of erythromycin. These are largely found in gram negative bacteria and are rarely present in gram positive ones [150].

The *erm* genes (*ermA*, *ermB* and *ermC*) are either expressed constitutively (CMLS_B) or inducibly (iMLS_B) [159], therefore, there is a reluctance to use clindamycin against

erythromycin-resistant isolates [160]. Broth dilution susceptibility cannot detect inducible resistance but these phenotypes can be identified in-vitro by a disc diffusion test using ERY and CLI discs (D-test) [161]. This test is performed by placing a 15ug ERY disc in proximity (15-20mm) to a 2ug CLI disc on an agar plate that has been inoculated overnight [161; 162]. A distorted (D-shaped) zone of inhibition around the CLI disc is indicative of positive inducible phenotype. *ErmA* and *ermC* are the predominant genetic determinants for ERY while *msrA* is rare [11], therefore, ERY-R *S.aureus* may be considered CLI-R in most geographical regions and further if an organism exhibits the iMLS_B phenotype, CLI should be used cautiously because resistance may develop during therapy.

Regular surveillance studies are carried throughout the world to know about the resistance genes to macrolides circulating in the area and to determine whether these are expressed constitutively or inducibly. In 2007, Otsuka *et al.*, from Japan reported 61.3% MLS_B constitutive and 38.7% MLS_B inducible resistance in MRSA with *ermA* (95%) gene being predominant, while in MSSA these were 1.3% and 94% respectively with *ermC* gene predominant (42%) [163]. A study from Turkey in 2007, found 58.3% MLS_B constitutive, 20.8% MLS_B inducible resistance and 20.8% MS phenotype in *S. aureus*, while gene distribution was as, *ermC* 62.5%, *ermA* 50%, *ermB* 8.3%, *ermA+ermC* 37.5% and *msrA* 12.5%. They also found one MRSA carrying four resistance determinants, i.e. *ermA+ermB+ermC+msrA* [164]. In Greece, *ermC* is the predominant gene (96.5%) mediating resistance to MLS_B antibiotics and is mostly expressed constitutively (94.7%) [165]. A large scale study conducted by the SENTRY Antimicrobial Surveillance Programme on isolates collected from 24 European university hospitals found *ermA* as the predominant resistance determinant in MRSA being 88% mostly in MLS_B constitutive

resistance phenotype while *ermC* was mainly found in MSSA in strains with inducible expression [166]. In Korea, *ermA* alone was present in majority (75.8%) of MRSA as compared to 6.7% in MSSA [167].

1.8.4.3. Tetracycline resistance in *S. aureus*

Tetracyclines are broad spectrum antibiotics which were discovered in the 1940's. These antibiotics are used against a wide variety of bacterial infections, caused by both gram positive and gram negative bacteria. Tetracyclines antibiotics are used in humans as well as in animals [168]. These antibiotics inhibit protein synthesis by preventing bacterial ribosome association with aminoacyl-tRNA [169]. After discovery of tetracyclines, more resreach driven development of minocycline and doxycycline [168].

Tetracycline resistance determinants have been identified. Roberts has reported 38 known, acquired tetracycline resistance genes and these use one of three strategies for mediating resistance [170]. These include 1: efflux proteins, 2: ribosomal protection proteins and 3: enzymatic inactivation of tetracycline.

The majority of these genes (60%) code for energy dependent efflux proteins. Tetracycline resistance genes can be exchanged amongst the bacterial population because different bacterial species have been found to have same resistance genes [171]. Actually, bacteria become tetracycline-resistant by acquiring new genes present on plasmids and transposons [168]. In contrast to common infections in which tetracyclines antibiotics are used for a few days, acne patients often use tetracycline for several months [172]. In this way, the targeted bacteria and also the other normal flora of the skin is exposed for a long time to selective pressure of antibiotics.

In gram positive bacteria, tetracycline resistance is mostly caused by *tetK*, *tetM*, *tetO* and

tetL, tetracycline resistance genes. The *tetK* and *tetL* genes encode efflux proteins, which are proteins associated with membrane, are energy dependent and allow entry of tetracycline inside the cell [171]. The *tetM* and *tetO* genes encode ribosomal protection proteins, which reduce the affinity of tetracycline for the ribosome. Other gram positive bacteria i.e., coagulase negative *staphylococci* also harbours these tetracycline resistance genes [173].

Surveillance for detection of various *tet* determinants is done worldwide. In 2006, Jones *et al.*, found 74% *tetK* and 13% *tetM* in MRSA and 73.8% *tetK* and 21.4% *tetM* in MSSA in a worldwide collection of *S. aureus* [174]. A study conducted by the SENTRY Antimicrobial Surveillance Programme between 1997 and 1999, found 57% and 10% resistance to tetracycline in MRSA and MSSA respectively. They recorded high prevalence of *tetM* in MRSA (76%) and high prevalence of *tetK* in MSSA (96%) [148]. Warsa *et al.*, tested 97 tetracycline resistant *S. aureus* isolates collected from five Asian countries (Japan, China, Thailand, Indonesia and Korea) for *tetK* and *tetM* and found that 80% tetracycline resistance was caused by *tetM*. Similarly Sekiguchi *et al.*, from Japan have reported 100% prevalence of *tetM* in the tetracycline resistant *S. aureus* [175].

1.9. Molecular epidemiology using Pulse field gel electrophoresis (PFGE)

It is obvious that the environment may play a substantial role in the spread of microorganisms, comprising multi drug resistant (MDR) strains. Alike other microorganisms MRSA are also known to live in dry conditions and can persist in clinical areas that are incompetently cleaned [176]. Inquiries of many epidemics have proved this phenomenon that MRSA can be easily spread by interaction, chiefly in hospital [177; 11]. MRSA carriage and upper respiratory tract infections could favor the spread of MRSA through air droplets [178; 179; 180; 181]. Bed making generates dust particle which also can

harbor MRSA shed from a preceding MRSA positive patient [182]. Thus, floating spread from patients with MRSA in respiratory secretions permits further study, not only in terms of infection control for patients, but also for the warning and use of personal protective strategies by healthcare workers.

Patients with burn injuries, after surgeries and trauma are prone to microbial colonization and infections because areas of the skin which provides a physical barrier to opportunistic pathogens are destroyed. Consequently a favorable, nutrient-rich environment is available to microbes that may come from the normal-flora of healthcare personnel, patients and the hospital environment [183; 184].

Burn patients with a weakened immune system can get methicillin-resistant *S. aureus* (MRSA) which is one of the most attentive pathogens globally and is propagated and transmitted in hospital setting [185; 186]. MRSA colonization and infection in burn patients is widely recognized and it has been detected in many cases that the same types of HA-MRSA are accountable for the outbreaks that prevail in burn units [187; 188]. As a result the infection caused by these multi drug resistant MRSA strains make them difficult and costly to treat [189; 190]. As it is evident that the routes of transmission and causes of MRSA infections in burn units include patients susceptibility [191;], surgical techniques [192; 193], health-care worker who are transporter for MRSA [194], the capability of *S. aureus* to live on surfaces [195; 196] and air-borne propagation [197] therefore the chance of occurrence of MRSA infection are more likely for these immune-compromised patients. Due to the ability of *S. aureus* to reside on dry surfaces and linings the prior stay of a MRSA positive patients in a room and substances used for such patients are also means of transmission if the room is not disinfected properly [198; 199].

Because the spread of MRSA strains in the hospital setting can result in severe threats to immunocompromised patients. Therefore it is of utmost importance to conduct an epidemiological investigation of the outbreaks in order to overcome their propagation and transmission [200]. Different techniques have been used to study the molecular epidemiology of *S. aureus* isolates, including pulse field gel electrophoresis (PFGE), multi locus sequence typing (MLST), Staphylococcal cassette chromosome mec (SCC mec) typing and polymerase chain reaction (PCR)-based typing. MLST, which examines the slowly evolving genomic core, is useful for defining ancestry and, when coupled with SCCmec typing, is used to discriminate between different clones of MRSA [201]. PFGE is considered the gold standard in comparison to these other techniques in the study of the epidemiology of bacterial strains responsible for an outbreak in the hospital settings [202; 203; 204; 205].

As the infrastructure and expertise for PFGE typing was available, In the Department of Infectious Diseases, College of Veterinary Medicine, Seoul National University, South Korea, so it was chosen over other aforementioned typing methods to study the molecular epidemiology of MRSA prevailing in the burn units of Khyber Teaching Hospital Peshawar (KTH, a tertiary care hospital). KTH provides health care facilities to a large number of patients in the Khyber Pukhtunkhwa province of Pakistan.

The purpose of this study is to determine the clonal similarities of MRSA strains prevailing in burn units, which are transmitted from patients to patients by different means. The results of this might be recommended for the development of planning and strategies and adaptation of guidelines [206] that may help to overcome HA-MRSA infections in burn patients.

1.10. Antibiotic Resistance in *S. aureus* reported from Pakistan and neighbor countries

Antibiotic resistance in bacteria is high in this region [207; 208; 209]. Mostly the isolated pathogens in this region are multi-drug resistant. Studies conducted in India have reported MRSA prevalence up to 51% [210], in Iran up to 41% [211], in Nepal up to 69.1% [127]. In Pakistan, MRSA prevalence of up to 51% has been reported [212]. The incidence of healthcare-associated infections produced by MRSA in the Khyber Pukunkhwa province, Pakistan has not been well assessed and the data presented is limited. However studies showed recently in the whole country recommended that 42–51% of healthcare-associated *S. aureus* infections may be produced by MRSA [213; 212]. The results of an earlier research from the Khyber Teaching Hospital Peshawar also presented a 46 % MRSA prevalence, among which many were resistant to different tetracycline antibiotics [214]. Multidrug resistant MRSA has also been stated in other hospitals of Peshawar [215] with high level of resistance to beta lactam antibiotics.

Aims of the study

The aims of the current study was:

- Evaluation of epidemiological data concerning the prevalence of *S. aureus*.
- Isolation, characterization and preservation of *S. aureus* strains.
- Determination of resistance pattern against commonly used antibiotics by disc diffusion method and MICs determination.
- Molecular characterization of methicillin-resistance *S. aureus* (MRSA) isolates
- Comparison of resistance genes, identified in the bacterial isolates with the identified resistant genes of other geographic regions.
- Molecular epidemiology methicillin-resistance *S. aureus* MRSA.

- The distribution of genes encoding the various *staphylococcal* enterotoxins, Pantone-valentine Leukocidin toxin and toxic shock syndrome toxin-1 among clinical *S. aureus* strains using reliable Polymerase Chain reaction (PCR)

2. Materials and Methods

The current study was conducted in the Microbiology Section of Clinical Laboratory of Khyber Teaching Hospital (KTH) Peshawar, Center of Biotechnology and Microbiology (COBAM), University of Peshawar and Departments of Infectious Disease, College of Veterinary Medicine, Seoul National University, Republic of Korea from June 2010 to June 2014.

The study comprised of the following steps:

- Collection of specimen.
- Isolation, Identification and Preservation of *S. aureus* from the specimens collected.
- Confirmation of MRSA.
- Genetical verification of *S. aureus* and MRSA by the detection of *nuc* and *mecA* genes.
- Antimicrobial susceptibility testing.
- Detection of toxin genes by PCR.
- Detection of *ermA*, *ermB* and *ermC* genes by PCR.
- Molecular epidemiology of *S. aureus* isolated from burn patients.

2.1. Collection of specimen

In this study, a total of 11,136 samples were screened, collected from Khyber Teaching Hospital (KTH) and Hayatabad Medical Complex (HMC). KTH and HMC are the two tertiary care Hospitals which provide services in the public sector for the resident of Peshawar and close cities. Patients from the far flung towns and villages of Khyber Pakhtunkhwa are also referred to these big hospitals.

The suspected specimens included of pus, blood and urine received both from in patients and outdoor patients (OPD). A performa was designed for patient's history which includes

age, address, gender and history of antibiotic usage (appendix i).

445 positive *S. aureus* out of the total samples screened were collected. We have named the positive samples after the name of hospital, object, and specific ward, from where we have collected these samples. Therefore, the sample collected from the clinical laboratory of Khyber Teaching Hospital sent for culture sensitivity and positive samples were labelled as Khyber Teaching Hospital Lab samples (KLS). Similarly the specimens collected from the orthopaedic ward of Hayatabad Medical Complex (HMC) were screened and the positive samples of *S. aureus* were named as HMC. Likewise the positive samples collected from the KTH Burn Units were named as KBRN. In addition 64 environmental samples were collected from the floors, table, apparatus and inanimate objects in OT and burn patient's beds which were named as Khyber Teaching Hospital environmental samples (KE).

Furthermore, some currency notes were also screened for the presence of *S. aureus* which were collected from the hospital's cafeteria. The positive samples isolated were named as currency notes samples (CRN). *S. aureus* positive samples collected from the hospital personnel and university students were named as CAS. Blood specimens were collected in the KTH clinical laboratory and the positive *S. aureus* samples were named as KLBS (KTH Lab Blood Samples). Details of the sampling is shown in Table 2.1.

Table. 2.1: Information regarding labelling and collection of different strains

Samples collected from patients	Strains collection	Symbols of samples	Specimen
	Khyber Teaching Hospital LAB Samples collected from the clinical lab	KLS	Pus
	Khyber Teaching Hospital LAB Blood Samples.	KLBS	Blood
	Samples collected from Khyber Teaching Hospital Burn ward	KBRN	Pus
	Samples collected from orthopedic ward of Hayat Abad Medical Complex (HMC)	HMC	Pus
Environmental samples	Khyber Teaching Hospital environmental samples	KE	Swab wet with sterile normal saline
	Strains taken from currency notes collected from the cafeteria of Khyber Teaching Hospital (KTH)	CRN	Currency notes

2.2. Isolation, Identification of *S. aureus* and its preservation from the specimens

As we have collected the specimens separately from the two tertiary care hospitals therefore their isolation is discussed separately in detail.

2.2.1. Isolation of *S. aureus* from the KTH Clinical Laboratory

In our study, a total of 10136 specimen samples were received to the Microbiology section for culture and sensitivity testing including blood and other specimens for example urine and pus which were named as KLBS (KTH Lab Blood Samples) and KLS (KTH Lab Samples) respectively. The collection methods for the specimens are given as follows:

Urine samples were collected by taking midstream urine in sterile screw cap containers and were labeled. Pus specimens were collected in sterile syringes. Specimens from the open lesions were collected with sterile cotton swabs.

For blood samples commercially available trypton soya broth (TSB) in screw capped sterile bottles containing 45ml of media were used. 5ml of blood was added to each sterile bottle with the final dilution of 1:10 making total volume of 50 ml. Fresh specimens reaching to lab within 15 minutes of its collection were included in this study. TSB containing blood specimens were placed in the incubator at 37 °C and were observed for any turbidity. Consequent to the rise in turbidity the specimen was then applied on different differential and selective media such as MacConkey agar and Mannitol Salt Agar (MSA) (Oxoid, England).

Other specimens were inoculated by sterile inoculating wire loop on various culture media. Urine samples were inoculated on Blood agar and MacConkey agar (Oxoid, England). Whereas the pus samples and blood samples were inoculated on Blood agar, MacConkey agar and Mannitol Salt Agar (MSA) (Oxoid, England) media. The culture media were

incubated at 37 °C for 24 hours in aerobic condition; then the plates were examined for any bacterial growth. The grown colonies were isolated and identified by colony morphology, gram staining, catalase, coagulase and DNase tests. These isolates were preserved in tryptone soya broth (TSB) with 15% glycerol at - 40 °C for further studies [6].

All the media were sterilized in autoclave for 20 minutes at 121 °C at a pressure of 15 lbs/inch² before use. The media after cooling down in water bath to 50-55 °C were aseptically poured in petri dishes or test tubes, labeled and stored at 4 °C.

2.2.2. Isolation of *S. aureus* from the specimen collected from the burn patients (KBRN)

A special permission was granted from the hospital administration for collection of samples from the two burn units in KTH. One, ten-bed burn unit which is known as burn ward (BW), is for adult male and female patients. The other burn unit is for children which is located in the Pediatrics Surgical Ward (PSW) as a separate unit of 8 beds. The beds which were allocated for male and children were not surrounded by curtains to isolate them; however, the beds of female patients were surrounded by curtains. There was no air filtration system installed in the burn units. Therefore we intended to find out the molecular epidemiology and cases of horizontal transmission of *S. aureus* in these burn units. We have collected 400 samples from the burnt patients out of which 89 produced *S. aureus* strains. The pus specimens were collected with a sterile cotton swab under aseptic conditions. The swab sticks were covered in the plastic case and were named properly. These samples were brought to the Lab in Center of Biotechnology and Microbiology, University of Peshawar and were inoculated on MSA and Blood agar. The isolates were identified by colony morphology, gram staining, catalase, coagulase and DNase tests. These isolates were

preserved in tryptone soya broth (TSB) with 15% glycerol at - 40°C for further studies. Samples were collected after hospitalization ≥ 48 hours.

2.2.3. Isolation of *S. aureus* from the specimen collected from Hayatabad Medical Complex (HMC)

After taking permission we have collected a total of 250 pus specimens from Hayatabad Medical Complex (HMC). The specimens collected were screened as described previously and the positive samples of *S. aureus* were named as HMC, starting from HMC 001 to HMC 109.

2.2.4. Isolation of *S. aureus* from the hospital environment

A total of 200 samples from the hospital environment of KTH were collected. This collection yielded 64 positive cases of *S. aureus*. The samples were taken by a wet sterile swab in distilled water. The positions of each sample were mentioned on the cover of swab stick.

Air samples were taken by settle plate method by exposing the media plates for 2-4 hours in the operation theatre or other area. The area and time of exposure were mentioned on the plates and so on the pro-forma designed for this collection Appendix 2. *S. aureus* was isolated on the same way as mentioned above for other collections.

2.2.5. Isolation of *S. aureus* from the currency notes collected from the hospital cafeteria

We have collected 150 currency notes in our study from the main cafeteria situated at the basement of KTH main building. As these currency notes circulates in wards, nurses counter and doctors rooms therefore their study is also important. Samples were taken by a wet cotton swab in sterile distilled water inside the Laminar flow hood (LFH). These swabs were

immediately inoculated on the Mannitol salt agar, blood agar and MacConkey agar. The *S. aureus* were identified by methods as described above and were preserved.

2.3. Confirmation of methicillin-resistant *Staphylococcus aureus* (MRSA)

The isolates were subjected to phenotypic and genotypic identification of MRSA by the following methods.

2.3.1. Cefoxitin disc:

These strains were initially characterized as MRSA based on 30µg Cefoxitin disc (Oxoid) susceptibility method recommended by the Clinical Laboratory Standard Institute (CLSI) [216]. *S. aureus* ATCC 43300 and ATCC 25923 strains were used as positive and negative control respectively as recommended by CLSI [216].

2.3.2. Brilliance MRSA-2 agar:

All of the *S. aureus* isolates (445) were refreshed in Tryptone Soya Broth (TSB), then inoculated on Brilliance MRSA-2 agar (Oxoid), incubated over night at 37 °C to confirm MRSA. The petri plates were purchased from Oxoid UK with prepared media and were kept at 4°C in a dark room. Before inoculation the plates were kept in the laminar flow hood to reach to room temperature. *S. aureus* ATCC 43300 and ATCC 25923 strains were used as positive and negative control respectively. The expected results are given in the table 2.2.

Table No. 2.2: MRSA identification by means of brilliance MRSA agar 2 [217]

Positive Control applied:	Expected results
<i>Staphylococcus aureus</i> ATCC®43300	Blue colonies
Negative Controls applied:	
<i>Staphylococcus aureus</i> ATCC®25923	Inhibited
<i>Pseudomonas aeruginosa</i> ATCC®27853	Inhibited
<i>Staphylococcus epidermidis</i> ATCC®12228	Inhibited
<i>Proteus mirabilis</i> ATCC®10975	Inhibited

2.3.3. Genetical confirmation of *S. aureus* and MRSA by the detection of *nuc* and *mecA* genes

A determinant of methicillin resistance *mecA* gene and *nuc* gene, which encodes the *S. aureus*-specific region of the thermonuclease gene were amplified. The sequence of both the genes are given in the Table 2.3.

Table 2.3: PCR primer sequences, amplicon sizes and PCR conditions

Gene	Primer sequence (5'-3')	Product Size in bp	PCR conditions		References
			Annealing temp.	Cycles	
<i>mecA</i>	F-CTCAGGTACTGCTATCCACC	449	55°C	30	[218]
	R-CACTTGGTATATCTTCACC				
<i>Nuc</i>	F- GCGATTGATGGTGATACGGTT	280	55°C	30	[219]
	R- AGCCAAGCCTTGACGAACTA AAGC				

A standard protocol for extraction of DNA was used to provide a source of staphylococcal control DNA in PCR experiments [220]. For cell lysis, bacteria were either harvested from

tryptone soya agar (TSA) agar plates (one loopful, by using a 1 µl loop) or broth cultures (0.1 ml of an overnight culture, 10^8 bacteria). Cells from TSB were harvested by centrifuging for 30 seconds in a micro centrifuge (Eppendorf Microfuge; 16,000 xg). Cells were then suspended in 50 µl of lysostaphin (100 µg/ml in water; Sigma Chemical Germany). These suspensions were kept at 37°C for incubation. 50 µl of proteinase K solution (100 µg/ml; Sigma) along with 150 µl of 0.1 M Tris (pH 7.5) buffer were added after 10 minutes. After this, suspensions were incubated for an additional 10 minutes. The suspensions were then heated at 97 °C for 5 minutes. This treatment was important to efficiently lysed the *S. aureus* cells and to prevent any DNase activity.

For each PCR reaction a total of 20 µl of reaction mixture was prepared. The reaction mixture include 2 µl 10X PCR reaction buffer (containing 100 mM Tris-HCL pH 8.3, 500 mM KCl, 20 mM MgCl), 0.2 µl *Taq* DNA polymerase (5 U/µL, Intron Biotechnology Inc., Korea), 11.8 µl Nuclease-free water, 2 µl mixture of dNTPs (containing 2.5 mM each dNTP), 1µl each of forward and reverse primers (15 pmoles) (Table 2.3) and 2 µl of template DNA. The products of PCR were run in 1.5 % agarose gel. The buffer used for this purpose was 1X TAE. The bands were stained with Ethidium bromide and examined under an ultraviolet transilluminator. The gel was photographed by a gel documentation system (Bio Rad Milan, Italy). For the detection of *mecA* gene, ATCC 43300 and ATCC 25923 *S. aureus* strains were used as positive and negative control. For the detection of *nuc* gene, ATCC 25923 *S. aureus* and *S. epidermidis* were used as positive and negative control respectively.

2.4. Susceptibility testing for antimicrobial agents

The identified bacterial isolates were then subjected to antimicrobial susceptibility testing by

the following method:

- Disc Diffusion Susceptibility test method and
- MIC (Minimum Inhibitory Concentration) method

2.4.1. Disc Diffusion Susceptibility test

Culture sensitivity data by disc diffusion method were recorded according to the recommendation of the CLSI [216]. The disc diffusion method was carried out for each bacterial isolate on Mueller-Hinton agar (CM337-Oxoid, England) as growth medium. Medium was prepared according to manufacturer's instructions (38g in 1L distilled water) and sterilized by autoclaving at 121°C for 15 minutes at 15 psi. 25 ml of media was poured in 90 mm sterile Petri dishes and incubated at 37°C overnight to check sterility.

2.4.1.1. Inoculum preparation

Tryptone Soya broth (TSB), (OXOID UK) was made for inoculum preparation as recommended by the manufacturer (30g in 1L distilled water), 5ml of broth medium was dispensed in screw capped tubes and sterilized by autoclaving at 121°C for 15 minutes at 15psi. The media was allowed to cool and kept in an incubator for 24 hours at 35°C to check sterility. Next day one of each identified clinical isolate was inoculated in sterilized screw capped tube containing media, and placed in incubator for 2-6 hours at 35°C. The turbidity of broth cultures were adjusted according to 0.5 MacFarland standards by adding sterile saline against a white back ground with contrasting black lines.

2.4.1.2. Inoculation of plates

A sterile cotton swab was soaked by plummetering into standardized bacterial suspension; extra culture was removed by turning swab against the inner side of tube. Inoculum was spread evenly over the entire surface of Mueller-Hinton agar by swabbing back and forth

across the agar in three directions to give a uniform inoculum to entire surface.

2.4.1.3. Applications of antibiotic discs

The plates were allowed to dry for 15 minutes, then paper discs (OXOID UK) of antibiotics of given potencies were applied on inoculated plates with the help of the syringe needle or disc dispenser. Then plates were placed in incubator at 37°C for 18 hour in an inverted position. After 18-24 hours of incubation, plates were examined and zones of inhibition were measured with a scale. The lists of antibiotics with their potencies are given in Table 4. ATCC 43300 and ATCC 25923 strains were used as positive and negative control respectively as recommended by CLSI [216].

Table 2.4: Antimicrobial agents and their detail

S. No	Antimicrobial Agent	Symbol	Antibiotic Group	Disc Potency (μg)
1	Cefoxitin	FOX	Cephalosporin	30
2	Amoxicillin + Clualinic acid	AMC	Penicillin	20+10
3	Ampicillin	AMP	Penicillin	10
4	Cephadrine	CE	Penicillin	30
5	Cefaclor	CEC	Cephalosporin	30
6	Ceftazdime	CAZ	Cephalosporin	30
7	Cefixime	CFM	Cephalosporin	5
8	Cefepime	FEP	Cephalosporin	30
9	Cefpirome	CPO	Cephalosporin	30
10	Ciprofloxacin	CIP	Fluoroquinolone	5
11	Clarithromycin	CLR	Macrolide	15
12	Meropenem	MEM	Carbapenem	10
13	Gentamicin	CN	Carbapenem	10
14	Amikacin	AK	Aminoglycoside	10
15	Doxycycline	DO	Tetracycline	30
16	Trimethoprim+Sulphamethoxazole	SXT	Sulpha drug	1.25/23.75
17	Vancomycin	VA	Glycopeptide	30
18	Linezolid	LZD	oxazolidinones	30
19	Fusidic acid	FD	Fusidanes	10
20	Rifampicin	RD	antimycobacterials	5
21	Chloramphenicol	C	Chloramphenicol	30

2.4.2. Determination of minimum inhibitory concentrations (MIC) by broth micro dilution-method

The objective of broth dilution methods is to find out the lowest concentration of the antimicrobial agent (minimum inhibitory concentration, MIC) tested. MIC is the lowest concentration which under certain test conditions inhibits the growth of the bacterium being investigated. MIC helps to evaluate the susceptibilities of bacteria to drugs, and to judge the activity of new antimicrobial agents. We have used the broth micro dilution method [309] for detecting MICs. For broth microdilution 96-well microtiter plate was used. Bacteria are inoculated into a Tryptone soya broth (TSB) containing different concentrations of an antimicrobial agent. Growth was observed after incubation for a certain period of time (16-20 h) and the MIC value is noted.

TSB containing antimicrobial agent with concentrations in a twofold dilution series, was inoculated with a defined number of bacterial cells.

2.4.2.1. Stock solution of the antimicrobial agent

Antimicrobial powders were purchased from Sigma chemicals, Germany and stored at 4°C in a refrigerator. The antibiotics were supplied with the information about the potency (μg per mg powder), usually written on the container that were important to be taken into consideration when weighing the agent.

Stock solutions were prepared for broth micro-dilution tests with a concentration at least ten times higher than the highest concentration to be tested. This concentration is known as the stock solution and is prepared and stored in sterile 17.5 × 100 mm plastic tube (Corning USA). The solvent and diluent used for each antibiotic is given in Table 5. The following formula describe the right amount of antibiotic to be weighed:

$$W = \frac{(C \times V)}{P}$$

Where, C = final concentration of stock solution ($\mu\text{g/ml}$); P = potency given by the manufacturer ($\mu\text{g} / \text{mg}$); W = weight of antimicrobial agent in milligram to be dissolved and V = desired volume (ml).

Table 2.5: List of antibiotics used in the MIC test for *S. aureus*

S.NO	Antibiotic	Solvent	Diluent	MIC range ($\mu\text{g/ml}$)	S	I	R
1	Cefoxitin	Water	Water	0.125-256	≤ 4		≥ 8
2	Cephadrine	Phosphate buffer, pH 6.0, 0.1 mol/L	Water	0.125-256	≤ 8		≥ 32
3	Ciprofloxacin	Water	Water	0.125-256	≤ 1	2	≥ 4
4	Vancomycin	Water	Water	0.125-256	≤ 2	4-8	≥ 16
5	Lanezolid	Water	Water	0.125-256	≤ 4		≥ 8
6	Fusidic acid	Ethanol	Water	0.125-256	≤ 1		≥ 4
7	Erythromycin	Ethanol	Water	0.125-256	≤ 0.5	1-4	≥ 8

The stock solutions were further processed by diluting in sterile TSB as shown in Table 6.

The dilutions obtained were twice of the final concentration after mixing with an equal amount of bacteria suspension in TSB.

Series of dilutions were prepared for each of the seven antibiotics given in Table 5. Sterile broth was dispensed in thirteen sterile 17.5×100 mm plastic tube (Corning USA). Thus for seven antibiotics 91 plastic screw cap tubes were taken. Each of the tubes was labeled mentioning the respective concentration. Separate pipet tips were used for adding antibiotic stock solution to dilute it according to Table 2.6. Mixing was done with the help of a vortex

mixer.

Table 2.6: Scheme for the preparation of antibiotic dilutions in twofold series.

Stage	Antimicrobial Concentration (mg l^{-1}) in Stock solution	Source	Volume of antibiotic stock solution (ml)	Volume of sterile broth (ml)	Antimicrobial Concentration Obtained	Final concentration in test
1	5120	Stock	1	9	512	256
2	512	Stage 1	1	1	256	128
3	512	Stage 2	1	3	128	64
4	512	Stage 3	1	7	64	32
5	64	Stage 4	1	1	32	16
6	64	Stage 5	1	3	16	8
7	64	Stage 6	1	7	8	4
8	8	Stage 7	1	1	4	2
9	8	Stage 8	1	3	2	1
10	8	Stage 9	1	7	1	0.5
11	1	Stage 10	1	1	0.5	0.25
12	1	Stage 11	1	3	0.25	0.125
13	1	Stage 11	1	7	0.125	0.0625

Modified from Ericsson and Sherris [221].

2.4.2.2. Inoculum preparation:

For each isolate 3-5 colonies were selected of the same morphology from the fresh Muller-Hinton agar plate, touched them using a sterile loop and transferred into a sterile tube (17.5 X 100 mm; Corning USA) containing 3-4 ml of TSB. The bacterial cells were mixed with a vortex mixer.

The broth was then Incubated at 37 °C in a shaker incubator until the turbidity become equal to or greater than the turbidity of 0.5 McFarland Standard. The turbidity of the *S. aureus* isolates is checked in the tubes with the help of Densilameter II Erba Lachema (Czech Republic). The suspension is adjusted to 0.5 McFarland Standard by adding sterile broth if it is high, or by further incubation if the bacterial culture is low dense.

2.4.2.3. Broth micro dilution

Prepared antibiotic dilutions in sterile TSB as a result of dilution in stage 1 to 13 according to Table 4 were placed in a cleaned wrack inside a LFH (Laminar Flow Hood). These dilutions were twice the concentration of the final amount in test as the antibiotic solution is later inoculated with the same amount of bacterial cell suspension in broth.

Using a 96-well microtiter plate, that allows testing only ten different concentrations if the following outlines are followed as given in table 2.7. As we have to test 13 concentrations of each antibiotic therefore the remaining concentrations were tested in the other plates. In this procedure, 50 µl of each dilution is required for each bacterial isolate to be tested.

Table 2.7: Scheme for the application of different concentrations of antibiotics (in µg/ml) using a 96 wells plate for testing 8 strains.

Strain No	Concentrations of antibiotics (µg/ml)											
	1	2	3	4	5	6	7	8	9	10	11	12
1	256	128	64	32	16	8	4	2	1	0.5	GC	SC
2	256	128	64	32	16	8	4	2	1	0.5	GC	SC
3	256	128	64	32	16	8	4	2	1	0.5	GC	SC
4	256	128	64	32	16	8	4	2	1	0.5	GC	SC
5	256	128	64	32	16	8	4	2	1	0.5	GC	SC
6	256	128	64	32	16	8	4	2	1	0.5	GC	SC
7	256	128	64	32	16	8	4	2	1	0.5	GC	SC
8	256	128	64	32	16	8	4	2	1	0.5	GC	SC

GS: growth control, SC: sterility control.

A multichannel pipette was used to dispense the antibiotic solutions in the wells as we have tested one antibiotic at a time. 10 ml or more volume of each antibiotic concentration was poured into a sterile plastic tray for the collection of solution in an 8 nasal multichannel pipette. Similarly bacterial suspensions were poured into the sterile plastic tray for up taking in a 12 nasal multichannel pipette conveniently.

A 96-well microtiter plate was labeled inside the Laminar flow hood (LFH) with the respective antibiotic concentration according to Table 2.7. One row of wells is used for each strain with up to 10 different dilutions. 100 µl of TSB were transferred in the wells of column 12 and 50 µl in the wells of column 11 for sterility control and growth control respectively. For each test strain, 50 µl of each antibiotic dilution is added into the respective well as shown in Table 2.7. 50 µl of bacterial suspension adjusted to 1×10^8 cfu/ml is added to each well containing the antibiotic solution and to the growth control

well. This results in the final desired inoculum of 5×10^5 cfu/ml in each well. The plates are handled aseptically therefore we have piled the plates in a set of 5 and were covered in clean aluminum foil inside the LFH. These plated were incubated for 16-20 hours at 37 °C. The growth was observed as sediment (button size in ≥ 2 mm), need to occur [216; 222]. The MIC of KBRN and KE samples were performed in triplicate.

The MIC of Fusidic Acid was determined according to new criterion [223]. ATCC 43300 and ATCC 29213 *S. aureus* strains were used as positive and negative controls in the MIC tests respectively.

2.5. Detection of toxin genes by PCR.

The isolates were screened for Panton Valentine Leucocidin (PVL), *icaA*, *icaD*, *tsst*, *sea*, *seb*, *sec* and *sed* genes by PCR in Khyber Medical University, Peshawar and Seoul National University, South Korea. The DNA extracted for the detection of *nuc* and *mecA* genes with the help of lysostaphin (100 µg/ml in water; Sigma Chemical Germany) enzyme were used as a template DNA. The methods described as given in references in the Table 2.9 were followed. In case of no bands the methods were modified by trying various annealing temperatures (54 - 62 °C).

20 µl of PCR reaction mixture was prepared which contains 2 µl 10× PCR reaction buffer (containing 100 mM Tris-HCL pH 8.3, 500 mM KCl, 20 mM MgCl), 0.2 µl *Taq* DNA polymerase (5 U/µL, Intron Biotechnology Inc., Korea), 2 µl mixture of dNTPs (containing 2.5 mM each dNTP), 1µl each of forward and reverse primers (Oligonucleotides, Macrogen Korea) with 10-15 pmoles (Table 2.9), 11.8 µl Nuclease-free water, and 2 µl of template DNA. A known PVL positive strain, *S. aureus* ATCC 49775 was used as positive control while only PCR mixture without template DNA was used as negative control. In case no

positive standard were available then the amplicon obtained were sequenced (by MacroGen, Korea) and matched with the respective sequence of genes in the Gene bank database.

Table 2.8: Oligonucleotide primers used for amplification of various genes

Target Gene	Primer	PCR primer séquence 5'-3'	PCR conditions	Amplicon Size (bp)	Reference
<i>Pvl</i>	PVL-1	ATCATTAGGTAAAATGTCTGG	1	433	[50]
	PVL-2	ACATGATCC GCATCAACTGTATTGGATAGC AAAAGC			
<i>Tst</i>	TSST-1	ATGGCAGCATCAGCTTGATA	2	350	[80]
	TSST-2	TTTCCAATAACCACCCGTTT			
<i>Sea</i>	SEA-1	AAAGTCCCGATCAATTTATGG	2	216	[80]
	SEA-2	CTA GTAATTAACCGAAGGTTCTGT AGA			
<i>Seb</i>	SEB-1	TCGCATCAAACGACAAACG	2	278	[80]
	SEB-2	GCAGGTACTCTATAAGTGCC			
<i>Sec</i>	SEC-1	GACATAAAAGCTAGGAATTT	2	257	[80]
	SEC-2	AAATCGGCTTAACATTATCC			
<i>Sed</i>	SED-1	CTAGTTTGGTAATATCTCCT	2	317	[80]
	SED-2	TAATGCTATATCTTATAGGG			

1: 94 °C 5min then 35 x (94 °C 30 sec, 64 °C 30 sec, 72 °C 60 sec), 2: 30 x (94 °C 120 sec, 55 °C 120 sec, 72 °C 60 sec)

2.6. Detection of *ermA*, *ermB* and *ermC* genes by PCR.

The *ermA*, *ermB* and *ermC* genes which confer resistance to erythromycin were detected in *S. aureus* by PCR conducted in the Seoul National University, Korea.

For each PCR reaction 20ul mixture was prepared which contained:

- 2 μ l 10X PCR reaction buffer (containing 100 mM Tris-HCL pH 8.3, 500 mM KCl, 20 mM MgCl)
- 0.2 μ l *Taq* DNA polymerase (5 U/ μ L, Intron Biotechnology Inc., Korea)
- 2 μ l mixture of dNTPs (containing 2.5 mM each dNTP)
- 2ul template DNA
- 1 μ l each of forward and reverse primers (Oligonucleotides, Korea) with 10 - 15 pmoles
- 11.8 ul PCR grade water

The PCR reaction was performed in PCR Sprint Thermo Cycler (Thermo Electron Corporation).

Table 2.9: Primer used along with the PCR conditions to detect erythromycin resistance genes in *S. aureus*

Resistance gene	PCR primer sequence 5'-3'	PCR reaction conditions	Amplicon Size (bp)	Gene Bank accession no.	Reference
<i>ermA</i>	F-GTTCAAGAACAAT CAATACAGAG R-GGATCAGGAAAA GGACATTTTAC	30 cycles (30s at 94°C; 30s at 52°C; 1 min. at 72°C)	421	K02987	[224]
<i>ermB</i>	F-CCGTTTACGAAAT TGGAACAGGTAAA GGGC R-GAATCGAGACTT GAGTGTGC	As <i>erm(A)</i>	359	U35228	[317]
<i>ermC</i>	F-GCTAATATTGTTT AAATCGTCAATTCC R-GGATCAGGAAAA GGACATTTTAC	As <i>erm(A)</i>	572	X54338	[224]

1. 94 °C 5min then 50 x (94 °C 30 sec, 55.5 °C 30 sec, 72 °C 30 sec), 2.35 x (94 °C 30 sec, 64 °C 30 sec, 72 °C 60 sec), 3.30 x (94 °C 120 sec, 55 °C 120 sec, 72 °C 60 sec)

2.7. Agarose gel electrophoresis

After each PCR run, the samples were run on the agarose gel (Invitrogen, UK). Generally 1.5% agarose gel prepared in 1xTAE was used. The gel tank was filled with 1xTAE or 1xTBE 2-3mm above the gel slab. Before running on gel, the PCR products were mixed with 2µl loading buffer and 12µl from every tube was put in a single well in the gel already prepared. The gel was run in an electrophoresis gel tank for 45 minutes at 100V. A 1Kb DNA ladder (Fermentas, Lithuania) was run alongside the PCR products as a size marker. After running the gel, it was stained with 1µg/ml ethidium bromide for 15 minutes and then destained in water in plastic jar for 10 minutes. The bands stained with Ethidium bromide were examined under an ultraviolet transilluminator and photographed by a gel documentation system (Bio Rad Milan, Italy).

2.8. Molecular epidemiology of *S. aureus* isolated from burn patients (KBRN samples) by PFGE method

Molecular epidemiology of KBRN samples is determined by Pulse Field Gel Electrophoresis method [225]. The method is optimized and includes the following steps.

2.8.1. Growing Fresh Cultures

The strains were refreshed on blood agar and one colony of each culture were inoculated in TSB in sterile 17.5 X 100 mm plastic tube (Corning USA). The bacterial cells were mixed by a vortex and incubated in a shaking incubator at 37 °C for 18-24 hours.

2.8.2. Preparation of Plugs

SeaKem Gold agarose (BMA #50152) was prepared with a concentration 1.8% W/V in Tris-EDTA (TE) buffer which gave the final desired 0.9% W/V as final concentration of plug after cell suspension is added.

Approximate amount of stock TE buffer (working TE buffer solution) was taken in a clean beaker that is required for preparing and washing plugs.

SeaKem Gold agarose, 1.8 gram was added to a 250 ml screw cap bottle (Scotch Duran, Germany). Working TE, 100 ml buffer was added to the bottle and gently tied to disperse agarose. The liquid was marked on the bottle to notice the meniscus. The cap of the bottle was removed and it was covered with plastic clear film loosely. The mixture was microwaved for 60 seconds, swirled gently. Heating was repeated for 10 second intervals until the whole agarose was completely dissolved.

Any loss in TE buffer and was replaced by the addition of pre-warmed Type I (reagent grade) water. The bottle was recapped and placed in 55°C in an incubator to equilibrate for 30 minutes.

At the same time a set of sterile microcentrifuge tubes and sterile 17.5 × 100 mm plastic tube (Corning USA) were marked according to the number of isolates.

Cell suspension of the isolates was checked by Densilameter II Erba Lachema and adjusted to 1.1-1.3 McFarland. 200 µl from the adjusted cell suspension in TSB was transferred to the labeled microcentrifuge tube and centrifuged at 13,000 rpm for 3-4 minutes. The liquid is aspirated.

The pellet is dispersed in 300 µl of TE (working) buffer, and the tubes were placed for equilibration in 37°C water bath for 10 minutes.

In case the numbers of tested isolates were 23, I used to take and labeled wells of plug mold with 30wells. The wells were sealed at the base side with the help of masking tape. The microcentrifuge tube were removed from the water bath and 4 µl conventional [Sigma #L7386] lysostaphin stock solution (1 mg/ml in 20 mM sodium acetate, pH 4.5) per tube

was added to the cell suspension directly, and mixed carefully.

300 μ l agarose (already equilibrated to 55°C) is poured into the cell suspension quickly but gently carefully to avoid shearing of the DNA and creating bubbles in the tube. Gently the mixture was mixed, and dispensed into the marked well(s) of the plug mold. All agarose cell mixtures were dispensed and the plugs were permitted to solidify at room temperature for 10-15 minutes.

2.8.3. Lysis of cells

With the help of a spatula plug were transferred into to the labeled sterile 17.5 X 100 mm plastic tube (Corning USA) tube in which at least 3 ml of EC lysis buffer was already added. These tubes were placed in incubator at 37°C for at least 4 hours.

2.8.4. Rinse Plugs

The EC lysis buffer was discarded while plugs were held with the help of a spatula in the tubes. At least 4 ml TE buffer was added to the sterile 17.5 X 100 mm plastic tube (Corning USA) and agitated in orbital rocker, at room temperature for 30 minutes. The plugs were remained immersed in TE buffer. Buffer was discarded and this process was repeated for at least three more times. 4 ml of working TE buffer was added after all washes in the tube containing the plugs and stored at 4 °C until the next step (preparation of enzyme digests).

2.8.5. *Sma*I Restriction Enzyme Digestion

For this purpose sterile 1.5 ml microcentrifuge tubes were labeled according to the isolate number given to it.

- Sufficient amount of 1 X water buffer mixture i.e. 200 μ l per plug was prepared (20 μ l of 10X T Buffer (Takara Japan) and 180 μ l of sterile reagent grade water). Thus for 23 plugs a total of 4600 μ l of water buffer was prepared in a sterile tube (17.5 X 100 mm

plastic tube Corning USA). From this mixture we added 200 μ l to the labeled microcentrifuge tubes.

- The plugs were removed from storage tube and placed in sterile petri dish. The plugs were trimmed 2 x 5 mm size.
- Plug slices were placed in labeled microcentrifuge containing water buffer mixture for 30-45 minutes at room temperature to equilibrate.
- At the meantime, sufficient enzyme water buffer was prepared in the tube that is also used for water buffer mixture. Mixture contains 20 μ l 10X T Buffer stock (Takara Japan) diluted with 177 μ l sterile, Type I water and 3 μ l *Sma*I enzyme (10 units/ml, Takara Japan). Thus for 23 plugs a total of 4600 μ l mixture was prepared.
- Water buffer were removed from the plug slices after 30 minutes equilibration with the help of a pipet fitted with a 250 μ l tip.
- After this 200 μ l water buffer enzyme mixture was added (already prepared in step number 4) to each tube. The tubes were closed and mixed by gentle tapping and incubated at 25°C for 3 hours.

2.8.6. Gel Running Process

- To prepare running gel, 1% w/v SeaKem Gold agarose (Lonza, Switzerland) was added to 150 ml of 0.5X TBE buffer for the large gel (21 x 14 cm). This molten agarose was placed in the water bath at 55 °C to equilibrate and to keep it remain molten for pouring into the gel tray.
- At the same time 2000 ml running buffer (0.5X TBE) was poured into the gel tank. The pump was switched on and set to 70 for a flow rate of 1 liter per minute. The cooling module was set at 14°C.

- Gel casting tray was assembled and placed at a level place to produce a uniform gel. The plugs were picked up with spatula and were placed directly on the very end of comb tooth. Lambda PFGE marker, 50-1000kb (*cl857 ind 1 Sam7*, New England BioLabs) was used as a DNA ladder and were placed on the different comb's tooth number 2, 9, 16, 23, 29. The comb was settled at its proper place into the comb holder and it was made sure that the plugs will remain in the gel after pouring agarose gel. The agarose was poured carefully into the gel try, as not to dislodge the gel slices. Gel was allowed for 45 minutes to solidify. After this the comb was removed by lifting straight up.
- The gel was removed from the casting platform (gel frame) but it was left as such on the removable black plate. The excess agarose was wiped away from the bottom and sides of the black try with tissue paper. The gel was carefully placed inside gel frame in electrophoresis chamber. At the end the cover of chamber was closed. We have checked that gel was covered by running buffer and the level of buffer was approximately 2mm above the gel surface.
- The run parameters for the CHEF-Mapper (made by Bio-Rad) were as follows: temp = 14°C, volts = 200 (6v/cm), initial switch = 5 seconds, final switch = 40 seconds and total time required = 21 hours for SeaKem Gold agarose.

2.8.7. Staining and Documentation

After completion of the run the CHEF- Mapper (Bio-Rad) was turned along with chiller. The gel was removed from the chamber and stained with ethidium bromide solution (AMRESCO X328, 10 mg/ml stock; 50 µl of stock was used in 500 ml distilled water) in a plastic container covered with aluminum foil for 20-30 minutes. The gel was removed and put into another container filled with fresh distilled water and let it for 45 - 60 minutes.

Images of the gel were taken by GEL DOC XR (Bio Rad Milan, Italy) and saved as tiff file for analysis.

2.8.8. Data processing and Analysis:

Gels images were taken by GEL DOC XR (Bio Rad Milan, Italy) and analyzed with BioNumerics software Gel Compar II (Applied Maths, Kortrijk, Belgium). Similarities in percentages were identified on a dendrogram derived from the unweighted pair group method using arithmetic averages based on Dice coefficients. Band position tolerance and optimization were set at 1.25 and 0.5%, respectively. A similarity coefficient of 80% was selected to define the pulso-types clusters.

2.9. BUFFERS

2.9.1. TAE buffer (10 X)

Tris-Acetic-acid-EDTA (TAE) buffer was prepared with the following composition:

	For 1000ml	Final conc.
Tris base (Sigma, Germany)	48.44 g	40 mM
EDTA (Sigma, Germany)	7.44 g	2 mM

The pH of the solution was brought to 7.9 with glacial acetic acid and the distilled water was added to 1000ml. For agarose gels, 1xTAE buffer was used. This buffer was also used as the running buffer for agarose gel electrophoresis.

2.9.2. TE (10 mM Tris HCl, 1 mM EDTA, pH 8) and NET buffers

From the stock solutions: For 2000 ml a total of 20 ml of 1M Tris HCl, pH 8 and 4ml of 0.5M EDTA, pH 8 was added in 2000ml cylinder. The volume was raised to 2000 ml in a graduated cylinder by the addition of Type I (reagent grade) water. This buffer was autoclaved on liquid cycle for 20 minutes. This solution was kept at room temperature for

further use. For 100 ml NET buffer preparation TE buffer was added to 99 ml PCR water and then 200ul of 5M NaCl is added.

2.9.3. EC lysis buffer

The required final concentration comprises of 6 mM Tris HCL, 100 mM EDTA, 1 M NaCl, 0.5% Polyoxyethylene 20 Cetyl Ether (Sigma #P5884), 0.2% Sodium deoxycholate and 0.5% Sodium lauroylsarcosine (Sigma #D6750)[53]. We have prepared 1800 ml EC lysis buffer by adding 10.8 ml of 1M Tris HCl (pH 8), 360 ml of 5M NaCl, 360 ml of 0.5 M EDTA (pH 8), (from the already prepared stock solution in our Lab, 9gm of Polyoxyethylene 20 Cetyl Ether, 3.6 gm Sodium deoxycholate, 9 gm of Sodium lauroyl sarcosine (Sigma #L5125) and 700ml of Type I water.

Foaming was avoided by adding each reagent carefully and the solution was heated for some time to allow facilitate solubalization. At the end quantity sufficient to 1800 ml of Type I water was added. This solution was autoclaved and then kept in shelf.

2.9.4. Tris Borate EDTA Buffer (TBE)

Tris Borate EDTA buffer (10 X) (TBE) was purchased from AccuGENE™ (Lonza) and diluted as given in the table 2.11

Table 2.10: Preparing 0.5X TBE from 10X TBE (AccuGENE™)

Reagents	Volume (ml)					
10 X TBE	100	105	110	115	120	125
Reagent grade water	1900	1995	2090	2185	2280	2375
Total Volume of 0.5X TBE	2000	2100	2200	2300	2400	2500

Table 2.11: Composition of Culture Media

Media	Company/Code	Ingredients	Amount (g/l)
Nutrient Agar	Oxoid, England	Yeast Extract	2.0
pH 7.4± 0.2 at 25°C	CM0003	Lab-Lemco` Powder	1.0
		Peptone	5.0
		Sodium chloride	5.0
		Agar	15.0
DNASE Agar	Oxoid, England	Tryptose	20.0
pH 7.3± 0.2 at 25°C	CM0321	Deoxyribonucleic acid	2.0
		Sodium chloride	5.0
		Agar	12.0
Blood Agar Base	Oxoid, England	`Lab-Lemco` Powder	10.0
pH 7.3± 0.2 at 25°C	CM	Peptone	10.0
		Sodium chloride	5.0
		Agar	15.0
MacConkey Agar	Oxoid, England	Peptone	20.0
pH 7.4± 0.2 at 25°C	CM0007	Lactose	10.0
		Bile Salts	5.0
		Sodium chloride	5.0
		Neutral red	0.075
		Agar	12.0
Nutrient Broth	Oxoid, England	`Lab-Lemco` Powder	1.0
pH 7.4± 0.2 at 25°C	CM0001	Yeast extract	2.0
		Peptone	5.0
		Sodium Chloride	5.0
Mannitol Salt Agar	Oxoid, England	`Lab-Lemco` Powder	1.0
pH 7.5 ± 0.2 at 25°C	CM0085	Peptone	10.0
		Mannitol	10.0
		Sodium chloride	75.0
		Phenol red	0.025
		Agar	15.0
Mueller-Hinton Agar	Oxoid, England	Beef extract, dehydrated infusion from	300.0
pH 7.3 ± 0.2 at 25°C	CM0337	Casein Hydrolysate	17.5
		Starch	1.5
		Agar	17.0
Tryptone soya broth	Oxoid, England	Pancreatic digest of casein	17.0
pH 7.3 ± 0.2 at 25°C	CM0129	Papaic digest of soybean meal	3.0
		Sodium chloride	5.0
		Dibasic potassium phosphate	2.5
		Glucose	2.5

Brilliance MRSA 2 AGAR pH 7.3 ± 0.2 at 25 °C	Oxoid, England PO1210 (UK)	Peptone mix	20.0
		Carbohydrates	4.0
		Kaolin	15.0
		Salts	5.0
		Agar	13.0
		Chromogenic mix	0.2
		Antibiotic cocktail	0.2

3.0 Results

Staphylococcus aureus isolated are listed in **table 3.1**. The data of these isolates is presented according to the area from where they are collected as described in the materials and methods section.

3.1 Khyber Teaching Hospital Lab samples (KLS)

The specimens that were received in the microbiology section of Khyber Teaching Hospital Laboratory (KLS) were screened for *Staphylococcus aureus*. A total of 167 isolates of *S. aureus* were collected and subjected to disc diffusion and MIC test for culture sensitivity. These samples were also screened for *nuc* and *mecA* genes for identification and selection of MRSA strains. Distributions of different toxins genes harbored by these isolates were studied. These isolates were further processed for the detection of different erythromycin resistant genes which are discussed below.

Table 3.1: Number of specimens and isolates collected

	Strains collection	No of Specimens	Number of isolates
Samples collected from patients	Khyber Teaching Hospital LAB Samples collected from the clinical lab (KLS).	8,975	167
	Khyber Teaching Hospital Laboratory Blood Samples (KLBS).	1161	15
	Samples collected from Khyber Teaching Hospital Burn ward (KBRN).	400	89
	Samples collected from orthopedic ward of Hayat Abad Medical Complex (HMC)	250	92
Environmental samples	Khyber Teaching Hospital environmental samples (KE).	200	64
	Strains taken from currency notes (CRN) collected from the cafeteria of Khyber Teaching Hospital (KTH)	150	18
	Total number	11,136	445

3.2 Culture sensitivity data by disc diffusion test

A total of 8975 samples received for culture and sensitivity were processed. One sixty seven isolated were found *S. aureus*. Among the isolated *S. aureus*, 100 (59.88 %) were confirmed as MRSA as they showed resistance to ceftazidime. A total of 21 antibiotics were used against *S. aureus* belonging to various groups (Table 3.2). Vancomycin showed the highest activity of 94.01%. Chloramphenicol showed 90.42% activity, fusidic acid 88.02%, rifampicin 80.24% and linezolid 78.44% activity. Among the beta lactams used, meropenem had the highest activity i.e. 59.88%. This was followed by ceftazidime and cefepime each showing 40.12% activity, ceftiofur showing 38.92%, cefaclor showing 37.13% activity. The least activity recorded among beta lactams was of ampicillin (4.19%). The aminoglycosides amikacin and gentamicin were equally effective having 56.89% activities. Doxycycline, clarithromycin, ciprofloxacin and SXT showed 62.87, 51.5, 43.71 and 29.94% respectively.

Table 3.2: Susceptibility data of *S. aureus* in KLS (n=167)

Antimicrobial Agents	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	100 (59.88)	67 (40.12)	0 (0)
Amoxicillin + Clualinic acid	101 (60.48)	59 (35.33)	7 (4.19)
Ampicillin	160 (95.81)	7 (4.19)	0 (0)
Cephadrine	96 (57.49)	66 (39.52)	5 (2.99)
Cefaclor	91 (54.49)	62 (37.13)	14 (8.38)
Ceftazdime	141 (84.43)	13 (7.78)	13 (7.78)
Cefixime	135 (80.84)	23 (13.77)	9 (5.39)
Cefepime	89 (53.29)	68 (40.72)	10 (5.99)
Cefpirome	96 (57.49)	65 (38.92)	6 (3.59)
Ciprofloxacin	74 (44.31)	73 (43.71)	20 (11.98)
Clarithromycin	63 (37.72)	86 (51.5)	18 (10.78)
Meropenem	47 (28.14)	100 (59.88)	20 (11.98)
Gentamicin	67 (40.12)	95 (56.89)	5 (2.99)
Amikacin	47 (28.14)	95 (56.89)	25 (14.97)
Doxycycline	37 (22.16)	105 (62.87)	25 (14.97)
Trimethoprim+Sulphamethoxazole	104 (62.28)	50 (29.94)	13 (7.78)
Vancommycin	0 (0)	156 (93.41)	11 (6.56)
Linezolid	36 (21.56)	131 (78.44)	0 (0)
Fusidic acid	20 (11.98)	147 (88.02)	0 (0)
Rifampicin	26 (15.57)	134 (80.24)	7 (4.19)
Chloamphenicol	11 (6.59)	151 (90.42)	5 (2.99)

To find the differences in the culture sensitivity data of MRSA and MSSA, the data was divided and separately observed in **table 3.3** and **3.4**.

No resistance was noted to vancomycin in the MRSA isolated in the KTH microbiology laboratory i.e. all MRSA were susceptible to vancomycin (**Table 3.3**). Among the 100 MRSA isolates, 98 were resistant to cefoxitin. Next to vancomycin was chloramphenicol, killing 89% MRSA. Fusidic acid had 84% activity, RD 75% while linezolid had 71% activity. Out of the 100 MRSA, 44 were susceptible to doxycycline, 40 to meropenem, 36 to amikacin and 32 to gentamicin. Clarithromycin, ciprofloxacin and SXT showed low activity. The beta lactams had very low activity against the MRSA isolates.

Table 3.3: Susceptibility data of MRSA in KLS isolates (n=100)

Antimicrobial Agents	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	100 (100)	0 (0)	0 (0)
Amoxicillin + Clualinic acid	91 (91)	9 (9)	0 (0)
Ampicillin	98 (98)	2 (2)	0 (0)
Cephadrine	96 (96)	4 (4)	0 (0)
Cefaclor	88 (88)	5 (5)	7 (7)
Ceftazdime	94 (94)	1 (1)	5 (5)
Cefixime	96 (96)	1 (1)	3 (3)
Cefepime	78 (78)	19 (19)	3 (3)
Cefpirome	92 (92)	7 (7)	1 (1)
Ciprofloxacin	65 (65)	20 (20)	15 (15)
Clarithromycin	56(56)	30 (30)	14 (14)
Meropenem	42 (42)	40 (40)	18 (18)
Gentamicin	63 (63)	32 (32)	5 (5)
Amikacin	43 (43)	36 (36)	21 (21)
Doxycycline	35 (35)	44 (44)	21 (21)
Trimethoprim+Sulphamethoxazole	73 (73)	20 (20)	7 (7)
Vancomycin	0 (0)	95 (95)	5 (5)
Linezolid	29 (29)	71 (71)	0 (0)
Fusidic acid	16 (16)	84 (84)	0 (0)
Rifampicin	19 (19)	75 (75)	6 (6)
Chloramphenicol	10 (10)	89 (89)	1 (1)

Among 167 *S. aureus* collected from KHT lab, 67 were MSSA (Table 3.4). All of these were susceptible to cefoxitin. Generally, the MSSA isolates were susceptible to most of the antibiotics used. All the isolates were inhibited by vancomycin and linezolid (100% activity). Cephadrine, a first generation cephalosporin, inhibited 98.51% MSSA while Fusidic acid and gentamicin inhibited 94.03% isolates. Chloramphenicol, doxycycline, meropenem, RD, amikacin, cefpodoxime and cefaclor also exhibited good activities being 92.54%, 91.04%, 89.55%, 88.06%, 86.57% and 85.07% respectively. The least active antibiotic against MSSA was ampicillin with only 7.46% activity.

Table 3.4: Susceptibility data of MSSA in KLS isolates (n=67)

Antimicrobial Agents	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	0 (0)	67 (100)	0 (0)
Amoxicillin + Clualinic acid	14 (20.9)	50 (74.63)	3 (4.48)
Ampicillin	62(92.54)	5 (7.46)	0 (0)
Cephadrine	0(0)	66 (98.51)	1 (1.49)
Cefaclor	3(4.48)	57 (85.07)	7 (10.45)
Ceftazdime	47 (70.15)	12 (17.91)	8 (11.94)
Cefixime	39 (58.21)	22 (32.84)	6 (8.96)
Cefepime	11 (16.42)	49 (73.13)	7 (10.45)
Cefpirome	4 (5.97)	58 (86.57)	5 (7.46)
Ciprofloxacin	9 (13.43)	53 (79.1)	5 (7.46)
Clarithromycin	7 (10.45)	56 (83.58)	4 (5.97)
Meropenem	5 (7.46)	60 (89.55)	2 (2.99)
Gentamicin	4 (5.97)	63 (94.03)	0 (0)
Amikacin	4 (5.97)	59 (88.06)	4 (5.97)
Doxycycline	2 (2.99)	61 (91.04)	4 (5.97)
Trimethoprim+Sulphamethoxazole	31 (46.27)	30 (44.78)	6 (8.96)
Vancommycin	0 (0)	61 (91.04)	6 (8.96)
Linezolid	7 (10.45)	67 (100)	0 (0)
Fusidic acid	4 (5.97)	63 (94.03)	0 (0)
Rifampicin	7 (10.45)	59 (88.06)	1 (1.49)
Chloamphenicol	1 (1.49)	62 (92.54)	4 (5.97)

3.3 Minimum inhibitory concentration (MIC) of KLS isolates

The MIC value of the following seven antibiotics was determined for *S. aureus* isolates by broth micro dilution method. The MIC₅₀ and MIC₉₀ are given in table 3.5. The antistaphylococcal agents were found with high activity. The MIC₅₀ and MIC₉₀ of MRSA were noted more than that of MSSA.

Table 3.5: MIC₅₀ and MIC₉₀ values of antibiotics against *S. aureus* (n=167), MRSA (n=100) and MSSA (n=67) of KLS isolates in µg/ml

Antimicrobial Agents	<i>S. aureus</i>		MRSA		MSSA	
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
Cefoxitin	64	256	128	256	1	2
Cephadrine	64	256	128	256	4	8
Ciprofloxacin	4	128	64	128	1	16
Erythromycin	2	128	32	128	0.5	32
Vancomycin	1	2	1	2	1	2
Linezolid	2	16	2	32	2	4
Fusidic acid	1	8	1	16	1	2

3.4 Distribution of genes in KLS

All (167) of the *S. aureus* in KLS isolates were *nuc* positive and 100 were *mecA* positive i.e. MRSA. Out of 167 *S. aureus*, 102 (61.07%) contained *pvl* gene, 77 (46.10%) *sea* gene, 51 (30.53%) *seb* gene, 16 (9.58%) *sec* gene and 6 (3.59%). No *sed* gene was detected. *Pvl* was highly prevalent in MRSA (80.59%) as compared to MSSA (48%). Prevalence of *sea*, *seb*, *sec* and *tsst* genes was more in MRSA (49, 31, 10 and 4%) as compared to 41.79, 29.85, 8.95 and 2.98%). Overall prevalence of *erm* genes was, *ermA* 40 (23.95%), *ermC* 39 (23.35) and *ermB* 35 (20.95%) being more in MRSA.

Isolates possessing more than one toxin gene were found both in MRSA and MSSA. One MRSA strain contained *pvl*, *sea*, *seb* and *sec* toxin genes. PCR gels are shown in figure 3.1, 3.2 and 3.3 while the result of brilliance MRSA agar 2 is shown in figure 3.4. Distributions of various genes in these samples are shown in Tables 3.6-3.8.

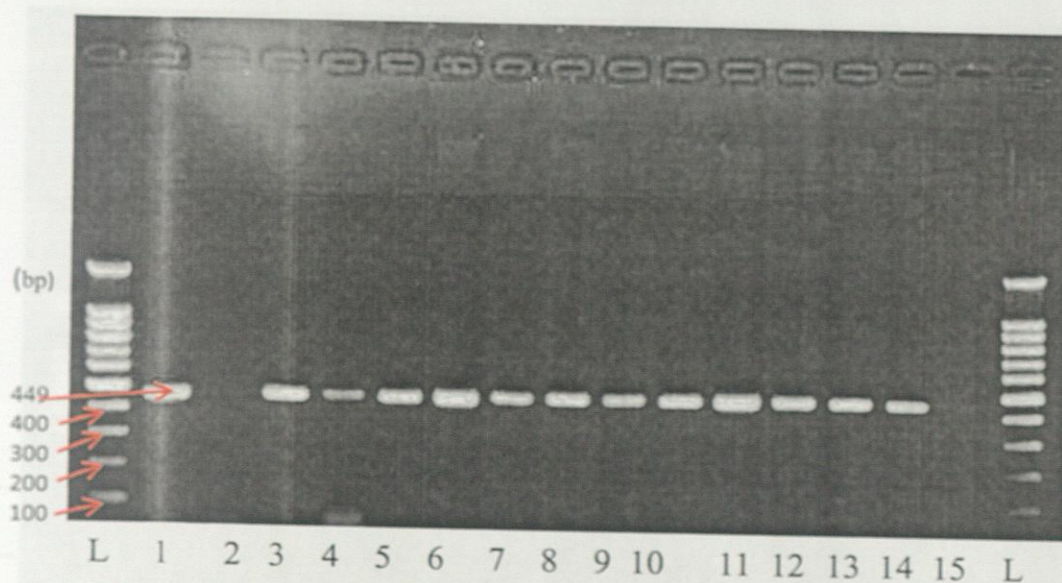


Fig 3.1: Gel electrophoresis of PCR product for *mecA* gene in *S. aureus* (KLS) isolates; L: 100 bp ladder, Lane 1: *S. aureus* ATCC 43300. 2: *S. aureus* ATCC 259234, 3: Isolate named as KLS 2, 4: Isolate KLS 3, 5: Isolate KLS 4, 6: Isolate KLS 5, 7: Isolate KLS 8, 8: Isolate 14, 9: Isolate 15, 10: Isolate 16, 11: Isolate KLS 18, 12: Isolate KLS 20, 13: Isolate KLS 21, 14: Isolate KLS 22, 15: Isolate KLS 23.

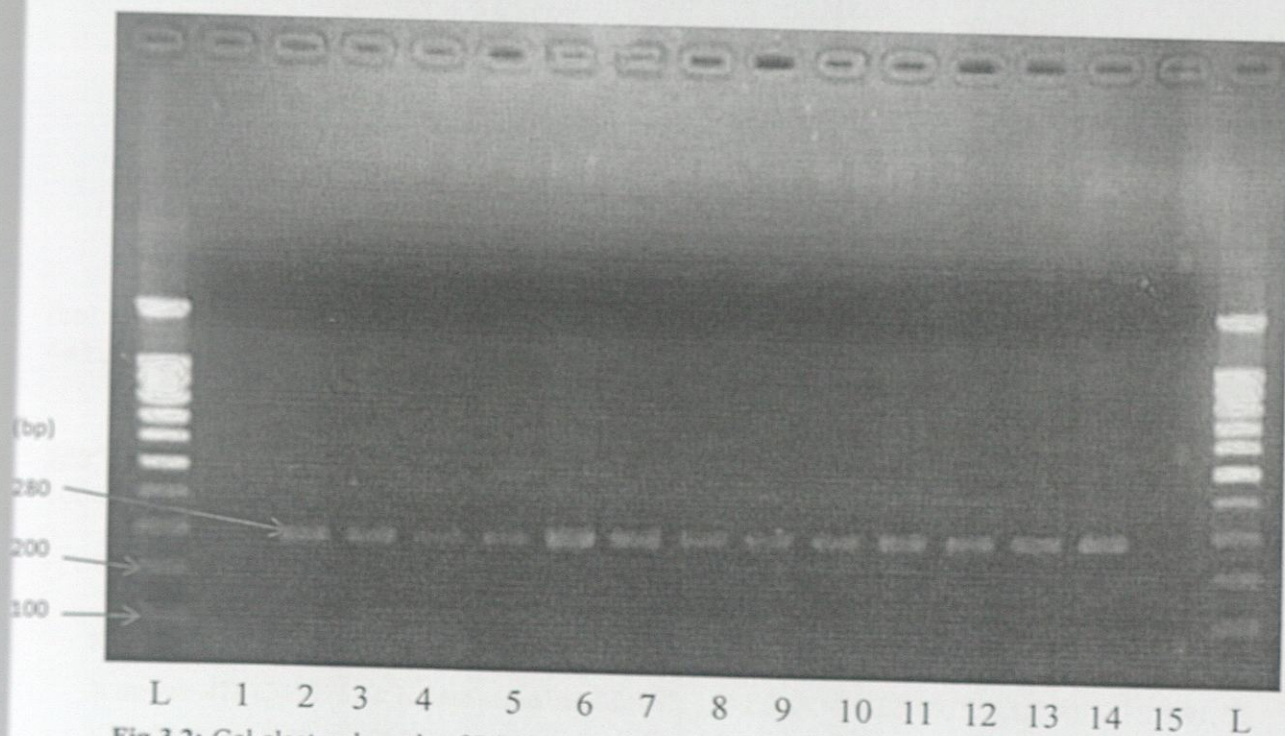


Fig 3.2: Gel electrophoresis of PCR product for *nuc* gene in *S. aureus* (KLS) isolates; L: 100bp ladder, 1: PCR mixture without template DNA, 2: *S. aureus* ATCC 259234, 3 Isolate named as KLS 1, 4: Isolate KLS 2, 5: Isolate KLS 3, 6: Isolate KLS 4, 7: Isolate KLS 5, 8: Isolate 6, 9: Isolate 7, 10: Isolate 8, 11: Isolate KLS 9, 12: Isolate KLS 10, 13: Isolate KLS 11, 14: Isolate KLS 12, 15: *S. epidermidis*.

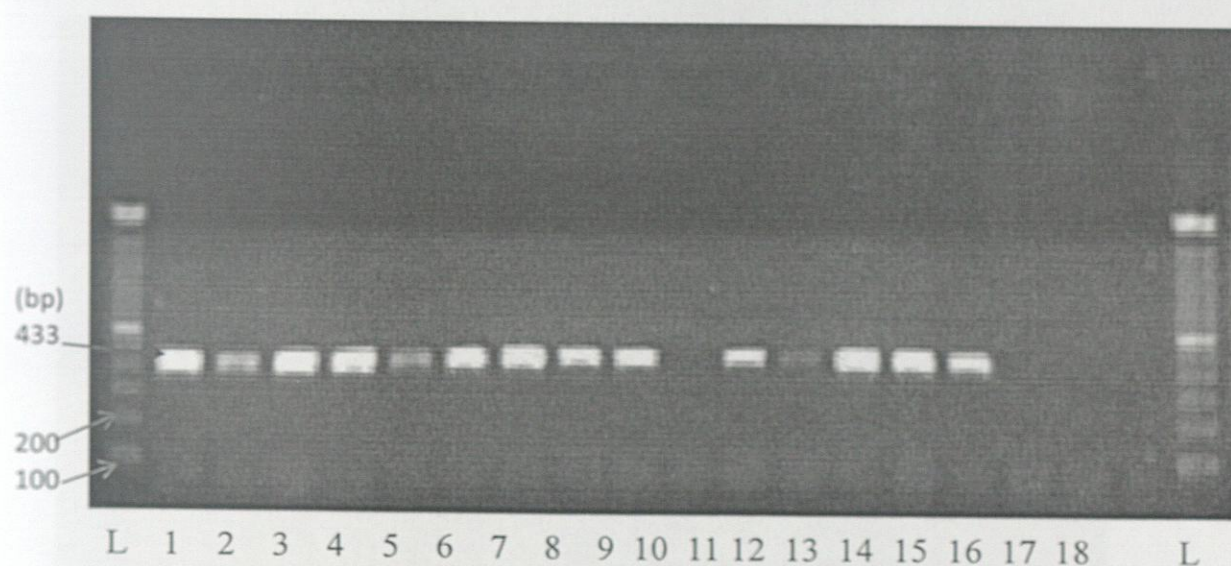


Fig 3.3: Gel image of PCR product for *pvl* in *S. aureus* (KLS) isolates. L: 100bp ladder, 1: *S. aureus* ATCC 49775, 2: Isolate named as KLS 87, 3: KLS 88, 4: KLS 89, 5: KLS 90, 6: KLS 91, 7: KLS 92, 8: KLS 93, 9: KLS 94, 10: KLS 95, 11: KLS 96, 12: KLS 97, 13: KLS 98, 14: KLS 99, 15: KLS 100, 16: KLS 101, 17: KLS 102, 18: PCR mixture without template DNA.

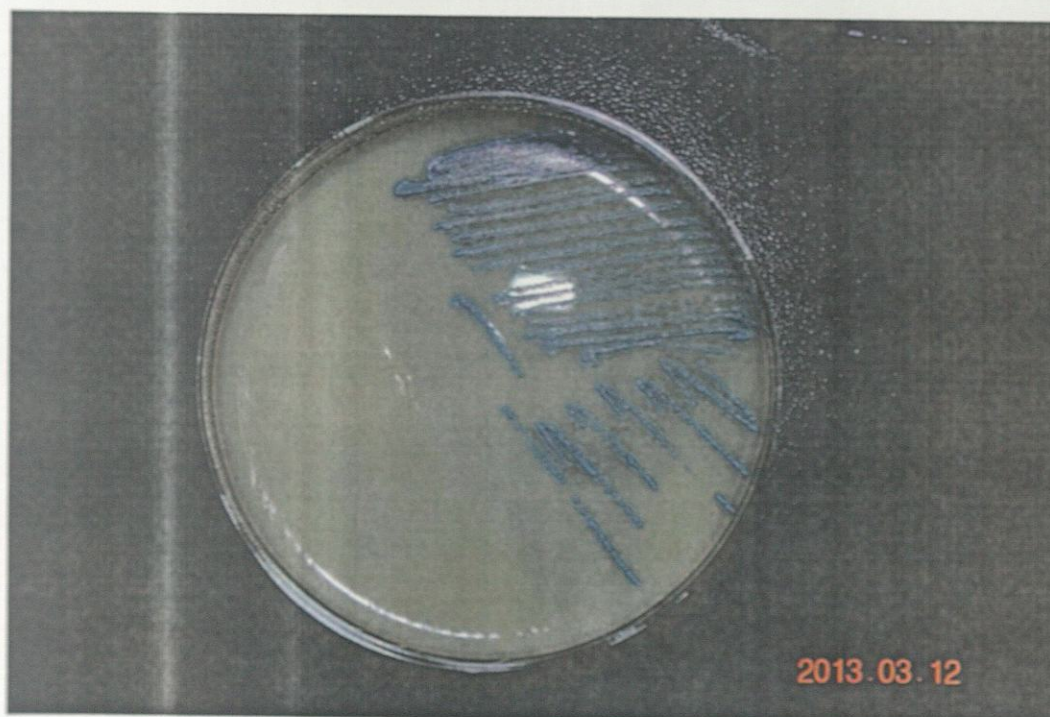


Fig. 3.4: Denim blue color colonies of MRSA culture on *Brilliance MRSA 2 AGAR* medium.

Table 3.6: Distribution of different genes in *S. aureus* of KLS isolates (n=167)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	100 (59.88)	67 (40.12)
<i>nuc</i>	167 (100.00)	0 (0.00)
<i>pvl</i>	102 (61.08)	65 (38.92)
<i>ermA</i>	40 (23.95)	127 (76.05)
<i>ermB</i>	35 (20.95)	132 (79.04)
<i>ermC</i>	39 (23.35)	128 (76.65)
<i>sea</i>	77 (46.11)	90 (53.89)
<i>seb</i>	51 (30.54)	116 (69.46)
<i>sec</i>	16 (9.58)	151 (90.42)
<i>sed</i>	0 (0.00)	167(100.00)
<i>tst</i>	6 (3.59)	161 (96.41)

mecA: Gene located on the staphylococcal cassette chromosome *mec* for the confirmation of MRSA; *nuc*: Thermonuclease gene in *S. aureus*, *pvl*:Panton Valentine Leukocidin; *erm*: erythromycin ribosomal methylase including *ermA*, *ermB* and *ermC*; *Se*: Staphylococcal enterotoxins including *sea*, *seb*, *sec*, *sed* and *tst*: Toxic shock syndrome toxin.

Table 3.7: Distribution of different genes in MRSA of KLS isolates (n=100)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	100 (100.00)	0 (0.00)
<i>Nuc</i>	100 (100.00)	0 (0.00)
<i>Pvl</i>	48 (48.00)	52 (52.00)
<i>ermA</i>	35 (35.00)	65 (65.00)
<i>ermB</i>	21 (21.00)	79 (79.00)
<i>ermC</i>	28 (28.00)	72 (72.00)
<i>Sea</i>	49 (49.00)	51 (51.00)
<i>Seb</i>	31 (31.00)	69 (69.00)
<i>Sec</i>	10 (10.00)	90 (90.00)
<i>Sed</i>	0 (0.00)	100 (100.00)
<i>Tst</i>	4 (4.00)	96 (96.00)

mecA: Gene located on the staphylococcal cassette chromosome *mec* for the confirmation of MRSA; *nuc*: Thermonuclease gene in *S. aureus*, *pvl*:Panton Valentine Leukocidin; *erm*: erythromycin ribosomal methylase including *ermA*, *ermB* and *ermC*; *Se*: Staphylococcal enterotoxins including *sea*, *seb*, *sec*, *sed* and *tst*: Toxic shock syndrome toxin.

Table 3.8: Distribution of different genes in MSSA of KLS isolates (n=67)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	0 (0.00)	67 (100.00)
<i>nuc</i>	67 (100.00)	0 (0.00)
<i>pvl</i>	54 (80.59)	13 (19.40)
<i>ermA</i>	5 (7.46)	62 (92.53)
<i>ermB</i>	14 (20.89)	53 (79.10)
<i>ermC</i>	11 (16.42)	56 (83.58)
<i>sea</i>	28 (41.79)	39 (58.21)
<i>seb</i>	20 (29.85)	47 (70.15)
<i>sec</i>	6 (8.95)	61 (91.04)
<i>sed</i>	0 (0.00)	67 (100.00)
<i>tst</i>	2 (2.98)	65 (97.01)

mecA: Gene located on the staphylococcal cassette chromosome *mec* for the confirmation of MRSA; *nuc*: Thermonuclease gene in *S. aureus*, *pvl*:Panton Valentine Leukocidin; *erm*: erythromycin ribosomal methylase including *ermA*, *ermB* and *ermC*; *Se*: Staphylococcal enterotoxins including *sea*, *seb*, *sec*, *sed* and *tst*: Toxic shock syndrome toxin.

3.5 Khyber Teaching Hospital Laboratory blood sample (KLBS)

Isolates collected from the blood specimens that were received in the KTH lab for culture sensitivity were tested. A total of 15 *S. aureus* strains were obtained from blood samples cultured. All these isolates were resistant to cefoxitin and no strain was resistant to vancomycin. The most active antibiotics were linezolid and RD inhibiting 93.33% isolates. Chloramphenicol showed 86.67% activity while 80% isolates were susceptible to fusidic acid, 73.33% to amikacin and 60% to gentamicin. The other antibiotics used had less than 50% activities (Table 3.9).

Table 3.9: Susceptibility data of total *S. aureus* in KLBS isolates (n=15)

Antimicrobial Agent	Resistance n (%)	Susceptibility n(%)	Intermediate n (%)
Cefoxitin	13 (86.67)	2 (13.33)	0 (0)
Amoxicillin + Clualinic acid	13 (86.67)	2 (13.33)	0 (0)
Ampicillin	15 (100.00)	0 (0.00)	0 (0)
Cephadrine	12 (80.00)	2 (13.33)	1 (6.67)
Cefaclor	13 (86.67)	2 (13.33)	0 (0.00)
Ceftazdime	13 (86.67)	0 (0.00)	2 (13.33)
Cefixime	15 (100.00)	0 (0.00)	0 (0.00)
Cefepime	14 (93.33)	0 (0.00)	1 (6.67)
Cefpirome	12 (80.00)	2 (13.33)	1 (6.67)
Ciprofloxacin	12 (80.00)	2 (13.33)	1 (6.67)
Clarithromycin	8 (53.33)	6 (40.00)	1 (6.67)
Meropenem	9 (60.00)	5 (33.33)	1 (6.67)
Gentamicin	10 (66.67)	4 (26.67)	1 (6.67)
Amikacin	3 (20.00)	9 (60.00)	3 (20.00)
Doxycycline	0 (0.00)	15 (100.00)	0 (0.00)
Trimethoprim+Sulphamethoxazole	10 (66.67)	5 (33.33)	0 (0.00)
Vancomycin	0 (0.00)	15 (100.00)	0 (0.00)
Linezolid	0 (0.00)	15 (100.00)	0 (0.00)
Fusidic acid	6 (40.00)	9 (60.00)	0 (0.00)
Rifampicin	1 (6.67)	13 (86.67)	1 (6.67)
Chloramphenicol	5 (33.33)	9 (60.00)	1 (6.67)

To look into the antibiotic susceptibility of MRSA and MSSA, we have grouped them in separate tables (Table 3.10 and 3.11). Among the 15 *S. aureus* recovered from blood samples, 13 were MRSA (86.67%). None of the isolates were resistant to vancomycin and linezolid (100% activity). There were 11 (84.62%) isolates susceptible to RD and 7 (53.85%) to chloramphenicol, fusidic acid and amikacin. Rest of the antibiotics had less than 40% activities (Table 3.10).

There were only two MSSA strains isolated from Blood samples. Their data is shown in table 3.11.

Table 3.10: Susceptibility data of MRSA in KLBS isolates (n=13)

Antimicrobial Agent	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	13 (100)	0 (0.00)	0 (0.00)
Amoxicillin + Clualinic acid	12 (92.31)	1 (7.69)	0 (0.00)
Ampicillin	13 (100.00)	0 (0.00)	0 (0.00)
Cephadrine	12(92.31)	0 (0.00)	1 (7.69)
Cefaclor	13 (100.00)	0 (0.00)	0 (0.00)
Ceftazdime	11 (84.62)	0 (0.00)	2 (15.38)
Cefixime	13 (100.00)	0 (0.00)	0 (0.00)
Cefepime	12 (92.31)	0 (0.00)	1 (7.69)
Cefpirome	11 (84.62)	1 (7.69)	1 (7.69)
Ciprofloxacin	11 (84.62)	1 (7.69)	1 (7.69)
Clarithromycin	7 (53.85)	5 (38.46)	1 (7.69)
Meropenem	8 (61.54)	4 (30.77)	1 (7.69)
Gentamicin	9 (69.23)	3 (23.08)	1 (7.69)
Amikacin	3 (23.08)	7 (53.85)	3 (23.08)
Doxycycline	0 (0.00)	13 (100.00)	0 (0.00)
Trimethoprim+Sulphamethoxazole	9 (69.23)	4 (30.77)	0 (0.00)
Vancomycin	0 (0.00)	13 (100.00)	0 (0.00)
Linezolid	0 (0.00)	13 (100.00)	0 (0.00)
Fusidic acid	6 (46.15)	7 (53.85)	0 (0.00)
Rifampicin	1 (7.69)	11 (84.62)	1 (7.69)
Chloramphenicol	5 (38.46)	7 (53.85)	1 (7.69)

3.6 MIC of KLBS isolates

Out of the total *S. aureus* isolates in the KLBS samples 7 (47.00%) were detected to be resistant to fusidic acid by broth dilution method. The MIC₉₀ is therefore high for these *S. aureus* isolated from blood specimens of patients. These isolates were mostly MRSA.

Table 3.12: MIC₅₀ and MIC₉₀ values of antibiotics against *S. aureus* (n=15), MRSA (n=13) and MSSA (n=2) of KLBS isolates in µg/ml.

Antimicrobial Agents	<i>S. aureus</i>		MRSA		MSSA	
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
Cefoxitin	128	256	128	256	1	2
Cephadrine	128	256	128	256	4	8
Ciprofloxacin	64	128	64	128	128	128
Erythromycin	64	128	64	128	32	64
Vancomycin	0.25	1	0.25	1	0.125	0.5
Linezolid	0.5	4	0.5	4	0.25	0.25
Fusidic acid	1	64	1	64	1	16

3.7 Distribution of various genes in *S. aureus* of KLBS

Presence of *nuc* gene was positive in the 15 KLBS samples. The *mecA* gene was found in 13 isolates. Out of 15 samples, *pvl* gene was found in 10 (66.67%) isolates, *seb* in 7 (46.67%), *sea* in 5 (33.33%), *sec* in 2 (13.33%) and *tsst* in 1 (6.67%) isolates. No *sed* gene was detected in any isolate. Only 2 isolates were resistant to erythromycin carrying *ermC* genes (Table 3.13, 3.14, 3.15).

Table 3.13: Gene distribution in *S. aureus* of KLBS isolates (n=15)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	13 (86.67)	2 (13.33)
<i>nuc</i>	15 (100.00)	0 (0.00)
<i>pvl</i>	10 (66.67)	5 (33.33)
<i>ermA</i>	0 (0.00)	15 (100.00)
<i>ermB</i>	0 (0.00)	15 (100.00)
<i>ermC</i>	2 (13.33)	13 (86.67)
<i>sea</i>	5 (33.33)	10 (66.67)
<i>seb</i>	7 (46.67)	8 (53.33)
<i>sec</i>	2 (13.33)	13 (86.67)
<i>sed</i>	0 (0.00)	15 (100.00)
<i>tst</i>	1 (6.67)	14 (93.33)

Table 3.14: Distribution of different genes in MRSA of KLBS isolates

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	13 (100.00)	0 (0.00)
<i>nuc</i>	13 (100.00)	0 (0.00)
<i>pvl</i>	9 (69.23)	4 (30.77)
<i>ermA</i>	0 (0.00)	13 (100.00)
<i>ermB</i>	0 (0.00)	13 (100.00)
<i>ermC</i>	2 (15.38)	11 (84.62)
<i>sea</i>	5 (38.46)	8 (61.54)
<i>seb</i>	6 (46.15)	7 (53.85)
<i>sec</i>	2 (15.38)	11 (84.62)
<i>sed</i>	0 (0.00)	13 (100.00)
<i>tst</i>	1 (7.69)	12 (92.31)

Table 3.15: Distribution of different genes in MSSA of KLBS isolates (n=2)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	0 (0.00)	2 (100.00)
<i>nuc</i>	2 (100.00)	0 (0.00)
<i>pvl</i>	1 (50.00)	1 (50.00)
<i>ermA</i>	0 (0.00)	2 (100.00)
<i>ermB</i>	0 (0.00)	2 (100.00)
<i>ermC</i>	0 (0.00)	2 (100.00)
<i>sea</i>	1 (50.00)	1 (50.00)
<i>seb</i>	1 (50.00)	1 (50.00)
<i>sec</i>	0 (0.00)	2 (100.00)
<i>sed</i>	0 (0.00)	2 (100.00)
<i>tst</i>	0 (0.00)	2 (100.00)

mecA: Gene located on the staphylococcal cassette chromosome *mec* for the confirmation of MRSA; *nuc*: Thermonuclease gene in *S. aureus*, *pvl*:Panton Valentine Leukocidin; *erm*: erythromycin ribosomal methylase including *ermA*, *ermB* and *ermC*; *Se*: Staphylococcal enterotoxins including *sea*, *seb*, *sec*, *sed* and *tst*: Toxic shock syndrome toxin.

3.8 Khyber Teaching Hospital environmental samples KE

To check contamination in the wards, environmental samples were also processed (Table 3.16). There were 64 *S. aureus* samples collected from tables, chairs, bed sheets etc. of various wards of KTH. These samples were then subjected to susceptibility testing. There was no isolate resistant to vancomycin. A total of 52 isolates (81.25%) were susceptible to linezolid and amikacin, 51 (79.69%) to fusidic acid, 49 (76.56%) to chloramphenicol, 47 (73.44%) to doxycycline and 44 (68.75%) to meropenem. The cephalosporins were not much effective.

3.8 Khyber Teaching Hospital environmental samples KE

To check contamination in the wards, environmental samples were also processed (Table 3.16). There were 64 *S. aureus* samples collected from tables, chairs, bed sheets etc. of various wards of KTH. These samples were then subjected to susceptibility testing. There was no isolate resistant to vancomycin. A total of 52 isolates (81.25%) were susceptible to linezolid and amikacin, 51 (79.69%) to fusidic acid, 49 (76.56%) to chloramphenicol, 47 (73.44%) to doxycycline and 44 (68.75%) to meropenem. The cephalosporins were not much effective.

Table 3.16: Susceptibility of *S. aureus* in KE isolates (n=64)

Antimicrobial Agent	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	37 (57.81)	27 (42.19)	0 (0)
Amoxicillin + Clualinic acid	35 (54.69)	27 (42.19)	2 (3.13)
Ampicillin	64 (100.00)	0 (0.00)	0 (0)
Cephadrine	30 (46.88)	33 (51.56)	1 (1.56)
Cefaclor	29 (45.31)	29 (45.31)	6 (9.38)
Ceftazdime	62 (96.88)	0 (0.00)	2 (3.13)
Cefixime	63 (98.44)	0 (0.00)	1 (1.56)
Cefepime	32 (50.00)	26 (40.63)	6 (9.38)
Cefpirome	31 (48.44)	28 (43.75)	5 (7.81)
Ciprofloxacin	22 (34.38)	38 (59.38)	4 (6.25)
Clarithromycin	20 (31.25)	34 (53.13)	10 (15.63)
Meropenem	15 (23.44)	44 (68.75)	5 (7.81)
Gentamicin	21 (32.81)	40 (62.50)	3 (4.69)
Amikacin	5 (7.81)	52 (81.25)	7 (10.94)
Doxycycline	2 (3.13)	47 (73.44)	15 (23.44)
Trimethoprim+Sulphamethoxazole	35 (54.69)	23 (35.94)	6 (9.38)
Vancomycin	0 (0.00)	56 (87.50)	8 (12.5)
Linezolid	12 (18.75)	52 (81.25)	0 (0.00)
Fusidic acid	13 (20.31)	51 (79.69)	0 (0.00)
Rifampicin	15 (23.44)	48 (75.00)	1 (1.56)
Chloramphenicol	11 (17.19)	49 (76.56)	4 (6.25)

Among the 37 MRSA isolated from environment of KTH, 89.19% were susceptible to vancomycin and 86.47% to linezolid. FD and chloramphenicol also showed good activities (81.08% each). These were followed by RD, doxycycline, amikacin, meropenem, gentamicin, ciprofloxacin, clarithromycin showing 75.68%, 67.57%, 54.05%, 40.54% and 35.14% activities.

Table 3.17: Susceptibility of MRSA in KE isolates (n=37)

Antimicrobial Agent	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	37 (100.00)	0 (0.00)	0 (0.00)
Amoxicillin + Clualinic acid	31 (83.78)	4 (10.81)	2 (5.41)
Ampicillin	37 (100.00)	0 (0.00)	0 (0.00)
Cephadrine	28 (75.68)	9 (24.32)	0 (0.00)
Cefaclor	23 (62.16)	9 (24.32)	5 (13.51)
Ceftazdime	35 (94.59)	0 (0.00)	2 (5.41)
Cefixime	36 (97.30)	0 (0.00)	1 (2.70)
Cefepime	24 (64.86)	9 (24.32)	4 (10.81)
Cefpirome	24 (64.86)	9 (24.32)	4 (10.81)
Ciprofloxacin	18 (48.65)	15 (40.54)	4 (10.81)
Clarithromycin	14 (37.84)	13 (35.14)	10 (27.03)
Meropenem	13 (35.14)	20 (54.05)	4 (10.81)
Gentamicin	15 (40.54)	20 (54.05)	2 (5.41)
Amikacin	5 (13.51)	25 (67.57)	7 (18.92)
Doxycycline	2 (5.41)	25 (67.57)	10 (27.03)
Trimethoprim+Sulphamethoxazole	23 (62.16)	10 (27.03)	4 (10.81)
Vancomycin	0 (0.00)	34 (91.89)	3 (8.11)
Linezolid	5 (13.51)	32 (86.49)	0 (0.00)
Fusidic acid	7 (18.92)	30 (81.08)	0 (0.00)
Rifampicin	9 (24.32)	28 (75.68)	0 (0.00)
Chloramphenicol	4 (10.81)	30 (81.08)	3 (8.11)

There were 27 MSSA isolates obtained from the environment of KTH. A total of 26 (96.3%) isolates were susceptible to ceftazidime. Cephadrine and meropenem showed 88.89% activities each. Next were AMC, ciprofloxacin and vancomycin which inhibited 85.19% isolates each. Doxycycline inhibited 81.48% isolates, clarithromycin and fusidic acid inhibited 77.78% isolates, cefaclor, gentamicin, linezolid and RD inhibited 74.07% isolates each. CPO and chloramphenicol both showed 70.37% activities. Ceftazidime and CFM were completely inactive against the isolated MSSA showing 0% activities.

Table 3.18: Susceptibility of MSSA isolates in KE isolates (n=27)

Antimicrobial Agent	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	0 (0.00)	27 (100.00)	0 (0.00)
Amoxicillin + Clualinic acid	4 (14.81)	23 (85.19)	0 (0.00)
Ampicillin	27 (100.00)	0 (0.00)	0 (0.00)
Cephadrine	2 (7.41)	24 (88.89)	1 (3.70)
Cefaclor	6 (22.22)	20 (74.07)	1 (3.70)
Ceftazdime	27 (100.00)	0 (0.00)	0 (0.00)
Cefixime	27 (100.00)	0 (0.00)	0 (0.00)
Cefepime	8 (29.63)	17 (62.96)	2 (7.41)
Cefpirome	7 (25.93)	19 (70.37)	1 (3.70)
Ciprofloxacin	4 (14.81)	23 (85.19)	0 (0.00)
Clarithromycin	6 (22.22)	21 (77.78)	0 (0.00)
Meropenem	2 (7.41)	24 (88.89)	1 (3.70)
Gentamicin	6 (22.22)	20 (74.07)	1 (3.70)
Amikacin	0 (0.00)	27 (100.00)	0 (0.00)
Doxycycline	0 (0.00)	22 (81.48)	5 (18.52)
Trimethoprim+Sulphamethoxazole	12 (44.44)	13 (48.15)	2 (7.41)
Vancomycin	0 (0.00)	22 (81.48)	5 (18.52)
Linezolid	7 (25.93)	20 (74.07)	0 (0.00)
Fusidic acid	6 (22.22)	21 (77.78)	0 (0.00)
Rifampicin	6 (22.22)	20 (74.07)	1(3.70)
Chloramphenicol	7 (25.93)	19 (70.37)	1 (3.70)

3.9 MIC of KE isolates

Good activity of ciprofloxacin was recorded against *S. aureus* isolates. Surprisingly the MIC₉₀ for linezolid was found high in the isolates collected from the environmental samples.

Table 3.19: MIC₅₀ and MIC₉₀ values of antibiotics against *S. aureus*, MRSA and MSSA of KE isolates in µg/ml

Antimicrobial Agents	<i>S. aureus</i>		MRSA		MSSA	
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
Cefoxitin	64	256	128	256	1	2
Cephadrine	64	256	128	256	2	4
Ciprofloxacin	1	64	16	64	1	4
Erythromycin	0.5	128	32	128	0.5	64
Vancomycin	2	4	2	2	1	4
Linezolid	2	32	2	4	4	16
Fusidic acid	1	8	1	8	1	4

3.10 Distribution of various genes in *S. aureus* of KE isolates

Out of 64 *S aureus* isolates, 37 (57.81%) were positive for *meca* gene. *Pvl* gene was detected in 17 (26.56%) isolates, with a distribution of 9 (33.33%) in MSSA and 8 (21.62%) in MRSA. The *sea* gene was present in 36 (56.25%) *S. aureus* samples, 22 (59.45%) in MRSA and 14 (51.85%) in MSSA. *Seb* gene was detected in 28 (43.75%), being 44.44% in MSSA and 43.24% in MRSA. *Sec* was found in only 8 (12.5%) isolates. *Sed* gene was not present in any environmental sample. Presence of *ermc* gene was detected in 18 (28.12%) *S. aureus* isolates (Table 3.20, 3.21, 3.22).

Table 3.20: Distribution of various genes in *S. aureus* of KE isolates (n=64)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	37 (57.81)	27 (42.19)
<i>nuc</i>	64 (100.00)	0 (0.00)
<i>pvl</i>	17 (26.56)	47 (73.44)
<i>ermA</i>	11 (17.19)	53 (82.82)
<i>ermB</i>	11 (17.19)	53 (82.81)
<i>ermC</i>	18 (28.12)	46 (71.87)
<i>sea</i>	36 (56.25)	28 (43.75)
<i>seb</i>	28 (43.75)	36 (56.25)
<i>sec</i>	8 (12.50)	56 (87.50)
<i>sed</i>	0 (0.00)	64 (100.00)
<i>tst</i>	3 (4.6875)	61 (95.31)

Table 3.21: Distribution of various genes in MRSA of KE isolates (n=37)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	37 (100.00)	0 (0.00)
<i>nuc</i>	37 (100.00)	0 (0.00)
<i>pvl</i>	8 (21.62)	29 (78.38)
<i>ermA</i>	7 (18.92)	30 (81.08)
<i>ermB</i>	8 (21.62)	29 (78.38)
<i>ermC</i>	10 (27.03)	27 (72.97)
<i>sea</i>	22 (59.46)	15 (40.54)
<i>seb</i>	16 (43.24)	21 (56.76)
<i>sec</i>	6 (16.22)	31 (83.78)
<i>sed</i>	0 (0.00)	37 (100.00)
<i>tst</i>	0 (0.00)	37 (100.00)

Table 3.22: Distribution of various genes in MSSA of KE isolates (n=27)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	0 (0.00)	27 (100.00)
<i>nuc</i>	27 (100.00)	0 (0.00)
<i>pvl</i>	9 (33.33)	18 (66.67)
<i>ermA</i>	4 (14.81)	23 (85.18)
<i>ermB</i>	3 (11.11)	24 (88.89)
<i>ermC</i>	8 (29.63)	19 (70.37)
<i>sea</i>	14 (51.85)	13 (48.15)
<i>seb</i>	12 (44.44)	15 (55.55)
<i>sec</i>	2 (7.40)	25 (92.59)
<i>sed</i>	0 (0.00)	27 (100.00)
<i>tst</i>	3 (11.11)	24 (88.89)

mecA: Gene located on the staphylococcal cassette chromosome *mec* for the confirmation of MRSA; *nuc*: Thermonuclease gene in *S. aureus*, *pvl*:Panton Valentine Leukocidin; *erm*: erythromycin ribosomal methylase including *ermA*, *ermB* and *ermC*; Se: Staphylococcal enterotoxins including *sea*, *seb*, *sec*, *sed* and *tst*: Toxic shock syndrome toxin.

3.11 Isolates of *S. aureus* collected from the currency notes (CRN)

The samples collected from the currency notes were processed for culture sensitivity. 9 of the total 18 isolates were MRSA. All the isolates were susceptible to vancomycin while 94.44% were susceptible to both linezolid and fusidic acid. Doxycycline and chloramphenicol were equally effective inhibiting 88.89% *S. aureus* isolates. RD inhibited 83.33% isolates, amikacin 72.22%, meropenem 66.67%, FEP 61.11%, CPO 55.56% and clarithromycin inhibited 50% isolates. Other antibiotics had below 50% activities.

Table 3.23: Susceptibility data of *S. aureus* in CRN isolates (n=18)

Antimicrobial Agent	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	9 (50.00)	9 (50.00)	0 (0.00)
Amoxicillin + Clualinic acid	9 (50.00)	8 (44.44)	1 (5.56)
Ampicillin	18 (100.00)	0 (0.00)	0 (0.00)
Cephadrine	9 (50.00)	8 (44.44)	1 (5.56)
Cefaclor	12 (66.67)	5 (27.78)	1 (5.56)
Ceftazdime	15 (83.33)	1 (5.56)	2 (11.11)
Cefixime	15 (83.33)	2 (11.11)	1 (5.56)
Cefepime	6 (33.33)	11 (61.11)	1 (5.56)
Cefpirome	7 (38.89)	10 (55.56)	1 (5.56)
Ciprofloxacin	9 (50.00)	7 (38.89)	2 (11.11)
Clarithromycin	8 (44.44)	9 (50.00)	1 (5.56)
Meropenem	5 (27.78)	12 (66.67)	1 (5.56)
Gentamicin	7 (38.89)	9 (50.00)	2 (11.11)
Amikacin	3 (16.67)	13 (72.22)	2 (11.11)
Doxycycline	1 (5.56)	16 (88.89)	1 (5.56)
Trimethoprim+Sulphamethoxazole	9 (50.00)	8 (44.44)	1 (5.56)
Vancomycin	0 (0.00)	18 (100.00)	0 (0.00)
Linezolid	1 (5.56)	17 (94.44)	0 (0.00)
Fusidic acid	1 (5.56)	17 (94.44)	0 (0.00)
Rifampicin	2 (11.11)	15 (83.33)	1 (5.56)
Chloramphenicol	0 (0.00)	16 (88.89)	2 (11.11)

Nine MRSA were obtained from currency notes. All these isolates were susceptible to vancomycin, linezolid and fusidic acid. Eight isolates (88.89%) were susceptible to chloramphenicol, 77.78% to doxycycline, 66.67% to RD, 55.56% to amikacin and 33.33% to FEP, clarithromycin and meropenem each.

Table 3.24: Susceptibility data of MRSA in CRN isolates (n=9)

Antimicrobial Agent	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	9 (100.00)	0 (0.00)	0 (0.00)
Amoxicillin + Clualinic acid	9 (100.00)	0 (0.00)	0 (0.00)
Ampicillin	9 (100.00)	0 (0.00)	0 (0.00)
Cephadrine	7 (77.78)	1 (11.11)	1 (11.11)
Cefaclor	9 (100.00)	0 (0.00)	0 (0.00)
Ceftazdime	9 (100.00)	0 (0.00)	0 (0.00)
Cefixime	9 (100.00)	0 (0.00)	0 (0.00)
Cefepime	6 (66.67)	3 (33.33)	0 (0.00)
Cefpirome	7 (77.78)	1 (11.11)	1 (11.11)
Ciprofloxacin	8 (88.89)	0 (0.00)	1 (11.11)
Clarithromycin	5 (55.56)	3 (33.33)	1 (11.11)
Meropenem	5 (55.56)	3 (33.33)	1 (11.11)
Gentamicin	5 (55.56)	2 (22.22)	2 (22.22)
Amikacin	2 (22.22)	5 (55.56)	2 (22.22)
Doxycycline	1 (11.11)	7 (77.78)	1 (11.11)
Trimethoprim+Sulphamethoxazole	6 (66.67)	2 (22.22)	1 (11.11)
Vancomycin	0 (0.00)	9 (100.00)	0 (0.00)
Linezolid	0 (0.00)	9 (100.00)	0 (0.00)
Fusidic acid	0 (0.00)	9 (100.00)	0 (0.00)
Rifampicin	2 (22.22)	6 (66.67)	1 (11.11)
Chloramphenicol	0 (0.00)	8 (88.89)	1 (11.11)

All the 9 MSSA isolates obtained from currency notes were susceptible to cefoxitin, CPO, meropenem, doxycycline, vancomycin and RD. AMC, FEP, amikacin, linezolid, fusidic acid and chloramphenicol also showed good activities inhibiting 88.89% isolates each. Next were cephadrine, ciprofloxacin and gentamicin each inhibiting 77.78% isolates, followed by ciprofloxacin and SXT showing 77.78% activities.

Table 3.25: Susceptibility data of MSSA in CRN isolates (n=9)

Antimicrobial Agent	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	0 (0.00)	9 (100.00)	0 (0.00)
Amoxicillin + Clualinic acid	0 (0.00)	8 (88.89)	1 (11.11)
Ampicillin	9 (100.00)	0 (0.00)	0 (0.00)
Cephadrine	2 (22.22)	7 (77.78)	0 (0.00)
Cefaclor	3 (33.33)	5 (55.56)	1 (11.11)
Ceftazdime	6 (66.67)	1 (11.11)	2 (22.22)
Cefixime	6 (66.67)	2 (22.22)	1 (11.11)
Cefepime	0 (0.00)	8 (88.89)	1 (11.11)
Cefpirome	0 (0.00)	9 (100.00)	0 (0.00)
Ciprofloxacin	1 (11.11)	7 (77.78)	1 (11.11)
Clarithromycin	3 (33.33)	6 (66.67)	0 (0.00)
Meropenem	0 (0.00)	9 (100.00)	0 (0.00)
Gentamicin	2 (22.22)	7 (77.78)	0 (0.00)
Amikacin	1 (11.11)	8 (88.89)	0 (0.00)
Doxycycline	0 (0.00)	9 (100.00)	0 (0.00)
Trimethoprim+Sulphamethoxazole	3 (33.33)	6 (66.67)	0 (0.00)
Vancomycin	0 (0.00)	9 (100.00)	0 (0.00)
Linezolid	1 (11.11)	8 (88.89)	0 (0.00)
Fusidic acid	1 (11.11)	8 (88.89)	0 (0.00)
Rifampicin	0 (0.00)	9 (100.00)	0 (0.00)
Chloramphenicol	0 (0.00)	8 (88.89)	1 (11.11)

3.12 MIC of *S.aureus* isolated from currency notes (CRN)

The MIC₉₀ of cefoxitin (256µg/ml) is a much higher value for the *S. aureus* isolated from currency notes. Although anti-staphylococcal agents have shown good activity as the MIC₅₀ and MIC₉₀ values are low for these strains.

Table 3.26: MIC₅₀ and MIC₉₀ values of antibiotics against *S. aureus* (n=18), MRSA (n=9) and MSSA (n=9) of CRN isolates in µg/ml

Antimicrobial Agents	<i>S. aureus</i>		MRSA		MSSA	
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
Cefoxitin	4	256	128	256	2	2
Cephadrine	64	256	128	256	2	64
Ciprofloxacin	4	128	64	128	1	1
Erythromycin	1	64	16	64	0.5	64
Vancomycin	1	2	1	2	1	2
Linezolid	2	4	2	2	2	4
Fusidic acid	0.5	1	0.5	1	0.5	1

3.13 Distribution of various genes in *S. aureus* of isolates CRN samples

Isolates collected from the currency notes were also screened for the presence of above mentioned genes. Surprisingly 9 of the total 18 isolates were found *mecA* positive. In these isolates 12 (66.66%) were *pvl* positive while 6 (33.33%) were *sea* positive. No *sed* and *tsst* genes were detected in these isolates. Erythromycin resistant genes i.e. *ermA* (38.88%) and *ermC* (33.33%) were also highly prevailing genes in these isolates.

Comparatively MRSA isolates were harboring more *ermA* genes (66.66%) than (11.11%) in the MSSA isolates (Table 3.27, 3.28, 3.29).

Table 3.27: Distribution of various genes in *S. aureus* of CRN isolates (n=18)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	9 (50.00)	9 (50.00)
<i>Nuc</i>	18 (100.00)	0 (0.00)
<i>Pvl</i>	12 (66.67)	6 (33.33)
<i>ermA</i>	7 (38.89)	11 (61.11)
<i>ermB</i>	2 (11.11)	16 (88.89)
<i>ermC</i>	6 (33.33)	12 (66.67)
<i>Sea</i>	6 (33.33)	12 (66.67)
<i>Seb</i>	3 (16.67)	15 (83.33)
<i>Sec</i>	1 (5.55)	17 (94.44)
<i>Sed</i>	0 (0.00)	18 (100.00)
<i>Tst</i>	0 (0.00)	18 (100.00)

Table 3.28: Distribution of various genes in MRSA of CRN isolates (n=9)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	9 (100.00)	0 (0.00)
<i>nuc</i>	9 (100.00)	0 (0.00)
<i>pvl</i>	4 (44.44)	5 (55.55)
<i>ermA</i>	6 (66.67)	3 (33.33)
<i>ermB</i>	0 (0.00)	9 (100.00)
<i>ermC</i>	2 (22.22)	7 (77.78)
<i>sea</i>	4 (44.44)	5 (55.55)
<i>seb</i>	2 (22.22)	7 (77.78)
<i>sec</i>	1 (11.11)	8 (88.89)
<i>sed</i>	0 (0.00)	9 (100.00)
<i>tst</i>	0 (0.00)	9 (100.00)

Table 3.29: Distribution of various genes in MRSA of CRN isolates (n=9)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	0 (0.00)	9 (100.00)
<i>nuc</i>	9 (100.00)	0 (0.00)
<i>pvl</i>	8 (88.89)	1 (11.11)
<i>ermA</i>	1 (11.11)	8 (88.89)
<i>ermB</i>	2 (22.22)	7 (77.78)
<i>ermC</i>	4 (44.44)	5 (55.55)
<i>sea</i>	2 (22.22)	7 (77.79)
<i>seb</i>	1 (11.11)	8 (88.89)
<i>sec</i>	0 (0.00)	9 (100.00)
<i>sed</i>	0 (0.00)	9 (100)
<i>tst</i>	0 (0.00)	9 (100)

mecA: Gene located on the staphylococcal cassette chromosome *mec* for the confirmation of MRSA; *nuc*: Thermonuclease gene in *S. aureus*, *pvl*:Panton Valentine Leukocidin; *erm*: erythromycin ribosomal methylase including *ermA*, *ermB* and *ermC*; *Se*: Staphylococcal enterotoxins including *sea*, *seb*, *sec*, *sed* and *tst*: Toxic shock syndrome toxin.

3.14 Samples collected from of Hayat Abad Medical Complex (HMC)

A total of 92 *S. aureus* isolates were obtained from various clinical samples at Hayatabad Medical Complex (HMC). Linezolid showed good activity inhibiting 92.39% isolates. A total of 88.04% isolates were susceptible to vancomycin, 85.87% to RD, 81.52% to chloramphenicol, 80.43% to fusidic acid, 72.83% to meropenem, 66.3% to amikacin and doxycycline each, 48.91% to gentamicin and 42.39% to SXT. Other antibiotics were less potent exhibiting below 40% activities (Table 3.30).

No isolate was sensitive or resistant to all of the antibiotics used. There was only one isolate that was resistant to all of the antibiotics except meropenem and vancomycin and one isolate was noted that was sensitive to all the isolates except AMC and ceftazidime. Beta lactam resistant isolates were generally co-resistant to ciprofloxacin (96.87%), clarithromycin (78.12%) and gentamicin (71.87%). The ciprofloxacin resistant isolates were mostly co-resistant to gentamicin and SXT (80%). All the 11 intermediately vancomycin resistant isolates were sensitive to linezolid while 9 (81.81%) among these were sensitive to RD and chloramphenicol each. The 7 linezolid resistant isolates were all sensitive to vancomycin and 6 (85.71%) were sensitive to both fusidic acid and chloramphenicol. Fusidic acid resistant isolates were mostly sensitive to linezolid (94.44%).

Table 3.30: Susceptibility data of *S. aureus* in HMC isolates (n=92)

Antimicrobial Agent	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	60 (65.22)	32 (34.78)	0 (0.00)
Amoxicillin + Clualinic acid	61 (66.30)	26 (28.26)	5 (5.43)
Ampicillin	90 (97.83)	2 (2.17)	0 (0.00)
Cephadrine	59 (64.13)	26 (28.26)	7 (7.61)
Cefaclor	54 (58.70)	26 (28.26)	12 (13.04)
Ceftazdime	86 (93.48)	2 (2.17)	4 (4.35)
Cefixime	88 (95.65)	1 (1.09)	3 (3.26)
Cefepime	50 (54.35)	31 (33.70)	11 (11.96)
Cefpirome	53 (57.61)	35 (38.04)	4 (4.35)
Ciprofloxacin	50 (54.35)	33 (35.87)	9 (9.78)
Clarithromycin	48 (52.17)	27 (29.35)	17 (18.48)
Meropenem	19(20.65)	67 (72.83)	6 (6.52)
Gentamicin	39 (42.39)	45 (48.91)	8 (8.70)
Amikacin	14 (15.22)	61 (66.30)	17 (18.48)
Doxycycline	15 (16.30)	61 (66.30)	16 (17.39)
Trimethoprim+Sulphamethoxazole	49 (53.26)	39 (42.39)	4 (4.35)
Vancomycin	0 (0.00)	85 (92.39)	7 (7.61)
Linezolid	7 (7.61)	85 (92.39)	0 (0.00)
Fusidic acid	18 (19.57)	74 (80.43)	0 (0.00)
Rifampicin	13 (14.13)	79 (85.87)	0 (0.00)
Chloramphenicol	12 (13.04)	75 (81.52)	5 (5.43)

A total of 60 MRSA isolates were collected from the HMC samples. Multidrug resistance was common among MRSA isolates. Along with beta lactam antibiotics these isolates were highly resistant to ciprofloxacin (CIP) i.e. 50 (83.33%) and clarithromycin (CLR) i.e. 39 (65 %). Still many of these strains were found susceptibility to LZD 53 (88.33 %), VA, 49 (81.67 %), RD 48 (80 %), FD and C 47 (78.33), DO 38 (63.33 %), MEM 35 (58.33 %) and AK 34 (56.67). 23 (38.33 %) isolates out 60 MRSA were found susceptible to the low cast oral drug SXT. Susceptibility data of MRSA isolated from HMC is shown in **table 3.31**.

Table 3.31: Susceptibility data of MRSA in HMC isolates (n=60)

Antimicrobial Agent	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	60 (100.00)	0 (0.00)	0 (0.00)
Amoxicillin + Clualinic acid	52 (86.67)	5 (8.33)	3 (5.00)
Ampicillin	60 (100.00)	0 (0.00)	0 (0.00)
Cephadrine	53 (88.33)	2 (3.33)	5 (8.33)
Cefaclor	49 (81.67)	4 (6.67)	7 (11.67)
Ceftazdime	58 (96.67)	1 (1.67)	1 (1.67)
Cefixime	60 (100.00)	0 (0.00)	0 (0.00)
Cefepime	43 (71.67)	12 (20.00)	5 (8.33)
Cefpirome	45 (75.00)	11 (18.33)	4 (6.67)
Ciprofloxacin	50 (83.33)	4 (6.67)	6 (10.00)
Clarithromycin	39 (65.00)	7 (11.67)	14 (23.33)
Meropenem	19 (31.67)	35 (58.33)	6 (10.00)
Gentamicin	35 (58.33)	19 (31.67)	6 (10.00)
Amikacin	11 (18.33)	34 (56.67)	15 (25.00)
Doxycycline	12 (20.00)	38 (63.33)	10 (16.67)
Trimethoprim+Sulphamethoxazole	34 (56.67)	23 (38.33)	3 (5.00)
Vancomycin	0 (0.00)	53 (88.33)	7 (11.67)
Linezolid	7 (11.67)	53 (88.33)	0 (0.00)
Fusidic acid	13 (21.67)	47 (78.33)	0 (0.00)
Rifampicin	12 (20.00)	48 (80.00)	0 (0.00)
Chloramphenicol	9 (15.00)	47 (78.33)	4 (6.67)

The MSSA strains were 32 out of the total 92 HMC isolates. These isolates shows low level of resistance to antibiotics tested except for AMP, CAZ and CFM as 30 (93.75 %) isolates were resistant to AMP and 28 (87.50 %) isolates were found resistant to CAZ and CFM. These strains were found to have a 46.88 % resistance to SXT which a little less than resistance to MRSA of the same group of samples i.e. 56.67 %.

Table 3.32: Susceptibility data of MSSA in HMC isolates (n=32)

Antimicrobial Agent	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	0 (0.00)	32 (100.00)	0 (0.00)
Amoxicillin + Clualinic acid	9 (28.13)	21 (65.63)	2 (6.25)
Ampicillin	30 (93.75)	2 (6.25)	0 (0.00)
Cephadrine	6 (18.75)	24 (75.00)	2 (6.25)
Cefaclor	5 (15.63)	22 (68.75)	5 (15.63)
Ceftazdime	28 (87.50)	1 (3.13)	3 (9.38)
Cefixime	28 (87.50)	1 (3.13)	3 (9.38)
Cefepime	7 (21.88)	19 (59.38)	6 (18.75)
Cefpirome	8 (25.00)	24 (75.00)	0 (0.00)
Ciprofloxacin	0 (0.00)	29 (90.63)	3 (9.38)
Clarithromycin	9 (28.13)	20 (62.50)	3 (9.38)
Meropenem	0 (0.00)	32 (100.00)	0 (0.00)
Gentamicin	4 (12.50)	26 (81.25)	2 (6.25)
Amikacin	3 (9.38)	27 (84.38)	2 (6.25)
Doxycycline	3 (9.38)	23 (71.88)	6 (18.75)
Trimethoprim+Sulphamethoxazole	15 (46.88)	16 (50.00)	1 (3.13)
Vancomycin	0 (0.00)	32 (100.00)	0 (0.00)
Linezolid	0 (0.00)	32 (100.00)	0 (0.00)
Fusidic acid	5 (15.63)	27 (84.38)	0 (0.00)
Rifampicin	1 (3.13)	31 (96.88)	0 (0.00)
Chloramphenicol	3 (9.38)	28 (87.50)	1 (3.13)

3.15 MIC of HMC isolates

The results of MIC of HMC isolates are given in **table 3.33**

Table 3.33: MIC₅₀ and MIC₉₀ values of antibiotics against *S. aureus* (n=93), MRSA (n=60) and MSSA (n=32) of HMC samples in µg/ml.

Antimicrobial Agent	<i>S. aureus</i>		MRSA		MSSA	
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
Cefoxitin	64	256	128	256	2	4
Cephadrine	64	256	128	256	2	64
Ciprofloxacin	32	128	64	128	0.5	1
Erythromycin	4	128	32	128	0.5	64
Vancomycin	1	2	1	4	0.5	2
Linezolid	2	4	2	4	2	4
Fusidic acid	1	16	1	32	1	16

3.16 Distribution of various genes in *S. aureus* of HMC samples

In the total 92 isolates which were collected from the HMC 60 (65.21%) were positive for *mecA* gene. In these isolates the prevalence of *pvl*, *seb*, *sea*, *sec*, were 39.13, 32.60, 31.52 and 11.95 percent respectively. In one sample *tst* genes was also detected. The *ermA* and *ermC* were 32.60 and 29.34 percent respectively while *ermB* prevalence were detected less (17.39%) in the HMC isolates (Table 3.34).

The prevalence of the above mentioned genes in the MRSA and MSSA isolates from the HMC samples were tabled separately. 18 out of 60 MRSA isolates and 18 out of 32 MSSA isolates were positive for *pvl* gene. Prevalence of the other toxic genes which were detected in this study was also less in MRSA as compared to MSSA in the HMC isolates. In MRSA *sea*, *seb* and *sec* were 30%, 23.33% and 13.33% respectively while in MSSA these were noted as 34.37%, 50% and 9.37% respectively. Prevalence of erythromycin resistant genes in MRSA isolates were noted to be more than MSSA isolates (Table 3.35).

In the HMC isolates 4 out of 92 were simultaneously positive for *pvl* and *sea* genes. Two MRSA isolates were positive for *pvl*, *seb* and *sec* while four isolates were simultaneously positive for *pvl* and *seb* genes. In the MSSA isolates one was positive for *pvl*, *seb* and *tst* genes concurrently. There were 8 MSSA isolates positive for *pvl* and *seb* at the same time. In these isolates one isolate was also positive for *sea* gene simultaneously (Table 3.36).

Table 3.34: Distribution of various genes in *S. aureus* of HMC isolates (n=92)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	60 (65.22)	32 (34.78)
<i>nuc</i>	92 (100.00)	0 (0.00)
<i>pvl</i>	36 (39.13)	56 (60.87)
<i>ermA</i>	30 (32.61)	62 (67.39)
<i>ermB</i>	16 (17.39)	76 (82.61)
<i>ermC</i>	27 (29.35)	65 (70.65)
<i>sea</i>	29 (31.52)	63 (68.48)
<i>seb</i>	30 (32.61)	62 (67.39)
<i>sec</i>	11 (11.96)	81 (88.04)
<i>sed</i>	0 (0.00)	92 (100.00)
<i>tst</i>	1 (1.08)	91 (98.91)

Table 3.35: Distribution of genes in MRSA of HMC isolates (n=60)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	60 (100.00)	0 (0.00)
<i>nuc</i>	60 (100.00)	0 (0.00)
<i>pvl</i>	18 (30.00)	42 (70.00)
<i>ermA</i>	22 (36.66)	38 (63.33)
<i>ermB</i>	9 (15.00)	51 (85.00)
<i>ermC</i>	16 (26.66)	44 (73.33)
<i>sea</i>	18 (30.00)	42 (70.00)
<i>seb</i>	14 (23.33)	46 (76.66)
<i>sec</i>	8 (13.33)	52 (86.66)
<i>sed</i>	0 (0.00)	60 (100.00)
<i>tst</i>	0 (0.00)	60 (100.00)

Table 3.36: Distribution of genes in MSSA of HMC isolates (n=32)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	32 (100.00)	0 (0.00)
<i>nuc</i>	0 (0.00)	32 (100.00)
<i>pvl</i>	18 (56.25)	14 (43.75)
<i>ermA</i>	8 (25.00)	24 (75.00)
<i>ermB</i>	7 (21.87)	25 (78.12)
<i>ermC</i>	11 (34.37)	21 (65.62)
<i>sea</i>	11 (34.37)	21 (65.62)
<i>seb</i>	16 (50.00)	16 (50.00)
<i>sec</i>	3 (9.37)	29 (90.62)
<i>sed</i>	0 (0.00)	32 (100)
<i>tst</i>	1 (3.125)	31 (96.87)

mecA: Gene located on the staphylococcal cassette chromosome *mec* for the confirmation of MRSA; *nuc*: Thermonuclease gene in *S. aureus*, *pvl*:Panton Valentine Leukocidin; *erm*: erythromycin ribosomal methylase including *ermA*, *ermB* and *ermC*; Se: Staphylococcal enterotoxins including *sea*, *seb*, *sec*, *sed* and *tst*: Toxic shock syndrome toxin.

3.17 Antimicrobial resistance pattern of *S. aureus* isolates of KBRN samples

A total of 89 *S. aureus* were collected from the burn ward of KTH. Among these isolates, 97.75% were susceptible to Vancomycin, 92.13% to RD, 91.01% to doxycycline, 89.89% to fusidic acid and 78.65% to linezolid. Ampicillin and FEP had very poor activities inhibiting only 4.49% of the isolates.

Table 3.37: Susceptibility data of *S. aureus* in KBRN isolates (n=89)

Antimicrobial Agent	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	54 (60.67)	35 (39.33)	0 (0.00)
Amoxicillin + Clualinic acid	57 (64.04)	30 (33.71)	2 (2.25)
Ampicillin	85 (95.51)	4 (4.49)	0 (0.00)
Cephadrine	55 (61.8)	34 (38.2)	0 (0.00)
Cefaclor	57 (64.04)	23 (25.84)	9 (10.11)
Ceftazdime	79 (88.76)	7 (7.87)	3 (3.37)
Cefixime	82 (92.13)	4 (4.49)	3 (3.37)
Cefepime	59 (66.29)	26 (29.21)	4 (4.49)
Cefpirome	51 (57.3)	30 (33.71)	8 (8.99)
Ciprofloxacin	49 (55.06)	32 (35.96)	8 (8.99)
Clarithromycin	44 (49.44)	34 (38.2)	11(12.36)
Meropenem	47 (52.81)	39 (43.82)	3 (3.37)
Gentamicin	41 (46.07)	47 (52.81)	1 (1.12)
Amikacin	27 (30.34)	48 (53.93)	14 (15.73)
Doxycycline	8 (8.99)	81 (91.01)	0 (0.00)
Trimethoprim+Sulphamethoxazole	63 (70.79)	22 (24.72)	4 (4.49)
Vancomycin	0 (0.00)	70 (78.65)	19 (21.35)
Linezolid	19 (21.35)	70 (78.65)	0 (0.00)
Fusidic acid	9 (10.11)	80 (89.89)	0 (0.00)
Rifampicin	6 (6.74)	82 (92.13)	1 (1.12)
Chloramphenicol	23 (25.84)	61 (68.54)	5 (5.62)

Among the 89 *S. aureus*, 54 were MRSA. These isolates were resistant. Among the 54 collected strains, none were found susceptible to all of the used antibiotics. The MRSA strains were found 100 % resistant to Ampicillin, Cefoxitin (Table 2) and Cephadrine (MIC 50=128 µg/ml; table 3) followed by Ceftazidime, Cefaclor and Cefexime (94%) and Cefepime, Cefpirome and Amoxicillin + Clavulanic acid (85%) while resistance to Meropenem was recorded as 50%.

Among aminoglycoside, Amikacin was very effective with 37% isolates being sensitive, followed by Gentamicin with 30% activity.

Noteworthy activity was exhibited by Rifampicin with 94% strains being susceptible to it. Decent activities were noted for Doxycycline (74%), and Chloramphenicol (56%). Among the macrolide, 41% activity was shown by clarithromycin, 19 % activity was shown by Trimethoprim+Sulphamethoxazole and the Ciprofloxacin exhibited 17% activity (Ciprofloxacin have a MIC 50=32 µg/ml tested on broth microdilution method).

Among the specific antistaphylococcal agents included in the study against MRSA, tested by broth microdilution technique, 17% of the total isolates exhibited resistance to Linezolid and Fusidic acid. Intermediate resistance to Vancomycin was noted in 28% of the strains with MIC in between 4-8 µg/ml (Table 3.38 and 3.40).

Table 3.38: Susceptibility data of MRSA in KBRN isolates (n=54)

Antimicrobial Agent	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	54 (100)	0 (0.00)	0 (0.00)
Amoxicillin + Clualinic acid	46 (85)	7 (13)	1 (2)
Ampicillin	54 (100)	0 (0)	0 (0)
Cephadrine	53 (98)	0 (0)	1 (2)
Cefaclor	51 (94)	1 (2)	2 (4)
Ceftazdime	51 (94)	2 (4)	1 (2)
Cefixime	51 (94)	3 (6)	0 (0)
Cefepime	46 (85)	4 (7)	4 (7)
Cefpirome	46 (85)	3 (6)	5 (9)
Ciprofloxacin	44 (81)	3 (6)	7 (13)
Clarithromycin	24 (44)	22 (41)	8 (15)
Meropenem	27 (50)	22 (41)	5 (9)
Gentamicin	37 (69)	16 (30)	1 (2)
Amikacin	25 (46)	20 (37)	9 (17)
Doxycycline	6 (11)	40 (74)	8 (15)
Trimethoprim+Sulphamethoxazole	41 (76)	10 (19)	3 (6)
Vancomycin	0 (0)	38 (70.37)	16 (29.63)
Linezolid	9 (17)	45 (83)	0 (0)
Fusidic acid	7 (13)	47 (87)	0 (0)
Rifampicin	3 (6)	51 (94)	0 (0)
Chloramphenicol	21 (39)	30 (56)	3 (6)

Thirty five MSSA strains were recovered from burn ward patients. All these MSSA were susceptible to cefoxitin and vancomycin. Doxycycline, cephadrine, gentamicin and chloramphenicol were also very potent antibiotics and inhibited more than 90% of the MSSA isolates. A total of 88.57% isolates were susceptible to RD, 82.86% to amikacin and 80% to meropenem. Prominent resistance was recorded to ampicillin and CFM showing only 11.43% activity each.

Table 3.39: Susceptibility data of MSSA in KBRN isolates (n=35)

Antimicrobial Agent (Symbol)	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)
Cefoxitin	0 (0.00)	35 (100.00)	0 (0.00)
Amoxicillin + Clualinic acid	6 (17.14)	27 (77.14)	2 (5.71)
Ampicillin	31 (88.57)	4 (11.43)	0 (0.00)
Cephadrine	2 (5.71)	33 (94.29)	0 (0.00)
Cefaclor	6 (17.14)	22 (62.86)	7 (20.00)
Ceftazdime	27 (77.14)	6 (17.14)	2 (5.71)
Cefixime	29 (82.86)	4 (11.43)	2 (5.71)
Cefepime	10 (28.57)	22 (62.86)	3 (8.57)
Cefpirome	4 (11.43)	27 (77.14)	4 (11.43)
Ciprofloxacin	5 (14.29)	25 (71.43)	5 (14.29)
Clarithromycin	8 (22.86)	25 (71.43)	2 (5.71)
Meropenem	6 (17.14)	28 (80.00)	1 (2.86)
Gentamicin	3 (8.57)	32 (91.43)	0 (0.00)
Amikacin	2 (5.71)	29 (82.86)	4 (11.43)
Doxycycline	1 (2.86)	34 (97.14)	0 (0.00)
Trimethoprim+Sulphamethoxazole	23 (65.71)	10 (28.57)	2 (5.71)
Vancomycin	0 (0.00)	32 (91.42)	3 (8.57)
Linezolid	10 (28.57)	25 (71.43)	0 (0.00)
Fusidic acid	2 (5.71)	33 (94.29)	0 (0.00)
Rifampicin	3 (8.57)	31 (88.57)	1 (2.86)
Chloramphenicol	1 (2.86)	32 (91.43)	2 (5.71)

3.18 MIC of KBRN isolates

The MIC₅₀ and MIC₉₀ values of antibiotics against *S. aureus* (n=93), MRSA (n=60) and MSSA (n=32) of KBRN samples are given in table 3.40

Table 3.40: MIC₅₀ and MIC₉₀ values of antibiotics against *S. aureus* (n=93), MRSA (n=60) and MSSA (n=32) of KBRN isolates in µg/ml

Antimicrobial Agents	<i>S. aureus</i>		MRSA		MSSA	
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
Cefoxitin	64	256	128	>256	2	4
Cephadrine	64	256	128	>256	2	8
Ciprofloxacin	16	128	32	128	0.5	4
Erythromycin	8	128	32	128	1	64
Vancomycin	2	4	2	4	1	2
Linezolid	2	8	4	8	2	8
Fusidic acid	0.5	4	1	4	0.5	2

3.19 Distribution of various genes in *S. aureus* isolates of KBRN samples

Gene specific for MRSA, *mecA* was detected for confirmation. The *nuc* gene was also detected. Out of 89 *S. aureus* isolates, 54 were positive for presence of *mecA*, while all contained the *nuc* gene. These samples were also screened for presence of various toxin genes like *pvl*, *sea*, *seb*, *sec*, *sed* and *tsst*. Prevalence of *seb* gene was high i.e. 34 (38.20%) followed by *pvl* 23 (25.84), *sea* (17.97%) and *sec* 9 (10.11%). No isolate contained *sed* and *tsst*. *Seb* was found in 23 (42.59%) while *sea* in 10 (18.52) MRSA. Ten MRSA were positive for *sea* and five for *sec*. Among the toxin genes, *pvl* was the most prevalent in MSSA. It was found in 16 (45.71%) of the MSSA isolates. *Seb* was detected in 11 (31.42%), *sea* in 6 (17.14%) and *sec* in 4 (11.42%) of the MSSA. The prevalence of *pvl* was found more in MSSA than in MRSA.

Three genes mediating resistance to erythromycin i.e. *ermA*, *ermB* and *ermC* were detected by PCR. *ErmC* was present in 19 (21.34%) of *S. aureus*, 11 (20.37%) in MRSA and 8 (22.85%) in MSSA. 12 (13.48%) of the *S. aureus* contained *ermA*, 6 (11.11%) in MRSA and 6 (17.14%) in MSSA. *ErmB* was low in the isolates (4), all in MSSA isolates.

Two MRSA isolates were simultaneously positive for *pvl*, *sea* and *sec*. Similarly three MSSA isolates were positive simultaneously for *pvl*, *sea* and *seb* (Table 3.41, Table 3.42 and Table 3.43).

Table 3.41: Distribution of various genes in *S. aureus* of KBRN isolates (n=89)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	54 (60.67)	35 (39.32)
<i>Nuc</i>	89 (100.00)	0 (0.00)
<i>Pvl</i>	23 (25.84)	66 (74.16)
<i>ermA</i>	12 (13.48)	77 (86.51)
<i>ermB</i>	4 (4.49)	85 (95.50)
<i>ermC</i>	19 (21.34)	70 (78.65)
<i>Sea</i>	16 (17.97)	73 (82.02)
<i>Seb</i>	34 (38.20)	55 (61.79)
<i>Sec</i>	9 (10.11)	80 (89.88)
<i>Sed</i>	0 (0.00)	89 (100.00)
<i>Tst</i>	0 (0.00)	89 (100.00)

Table 3.42: Distribution of various genes in MRSA of KBRN isolates (n=54)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	54 (100.00)	0 (0.00)
<i>nuc</i>	54 (100.00)	0 (0.00)
<i>Pvl</i>	7 (12.96)	47 (87.04)
<i>ermA</i>	6 (11.11)	48 (88.88)
<i>ermB</i>	0 (0.00)	54 (100.00)
<i>ermC</i>	11 (20.37)	43 (79.62)
<i>sea</i>	10 (18.51)	44 (81.48)
<i>seb</i>	23 (42.59)	31 (57.40)
<i>sec</i>	5 (9.25)	49 (90.74)
<i>sed</i>	0 (0.00)	54 (100.00)
<i>tst</i>	0 (0.00)	54 (100.00)

Table 3.43: Distribution of various genes in MSSA of KBRN isolates (n=35)

Genes	Strains positive for genes n (%)	Strains negative for genes n (%)
<i>mecA</i>	0 (0.00)	35 (100.00)
<i>nuc</i>	35 (100.00)	0 (0.00)
<i>pvl</i>	16 (45.71)	19 (54.28)
<i>ermA</i>	6 (17.14)	29 (82.85)
<i>ermB</i>	4 (11.42)	31 (88.57)
<i>ermC</i>	8 (22.85)	27 (77.14)
<i>sea</i>	6 (17.14)	29 (82.85)
<i>seb</i>	11 (31.42)	24 (68.57)
<i>sec</i>	4 (11.42)	31 (88.57)
<i>sed</i>	0 (0.00)	35 (100.00)
<i>tst</i>	0 (0.00)	35 (100.00)

mecA: Gene located on the staphylococcal cassette chromosome *mec* for the confirmation of MRSA; *nuc*: Thermonuclease gene in *S. aureus*, *pvl*:Panton Valentine Leukocidin; *erm*: erythromycin ribosomal methylase including *ermA*, *ermB* and *ermC*; *Se*: Staphylococcal enterotoxins including *sea*, *seb*, *sec*, *sed* and *tst*: Toxic shock syndrome toxin.

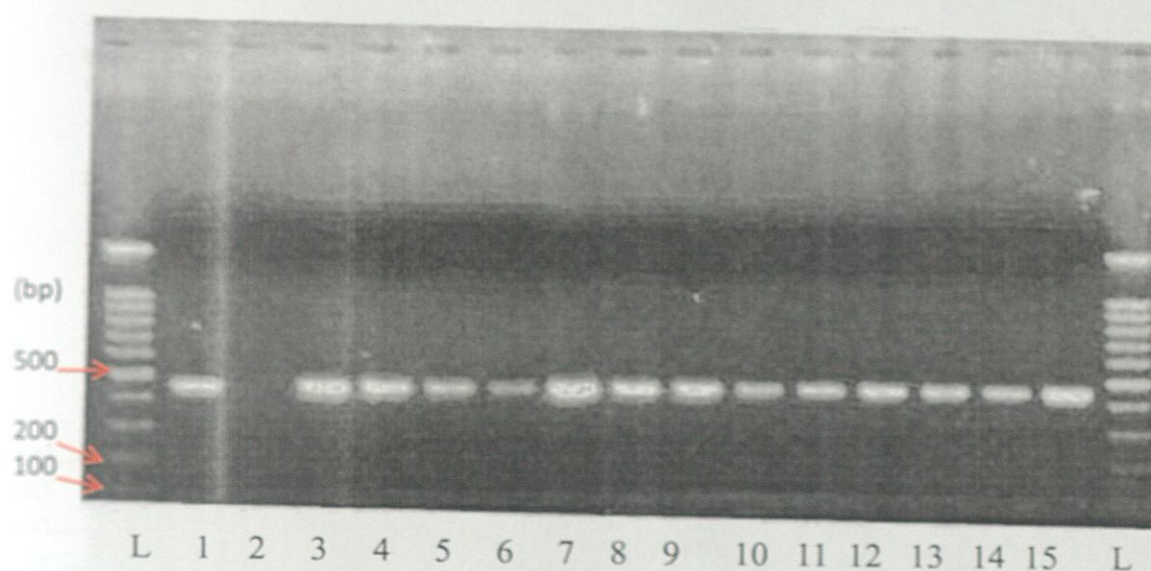


Fig. 3.5: Gel electrophoresis of PCR product for *mecA* gene in *S. aureus* (KBRN) isolates; L: 100 bp ladder, Lane 1: *S. aureus* ATCC 43300. 2: *S. aureus* ATCC 259234, 3: Isolate named as KBRN 1, 4: KBRN 2, 5: KBRN 3, 6: KBRN 4, 7: KBRN 5, 8: KBRN 6, 9: KBRN 7, 10: KBRN 8, 11: KBRN 9, 12: KBRN 10, 13: KBRN 11, 14: KBRN 12, 15: KBRN 13.

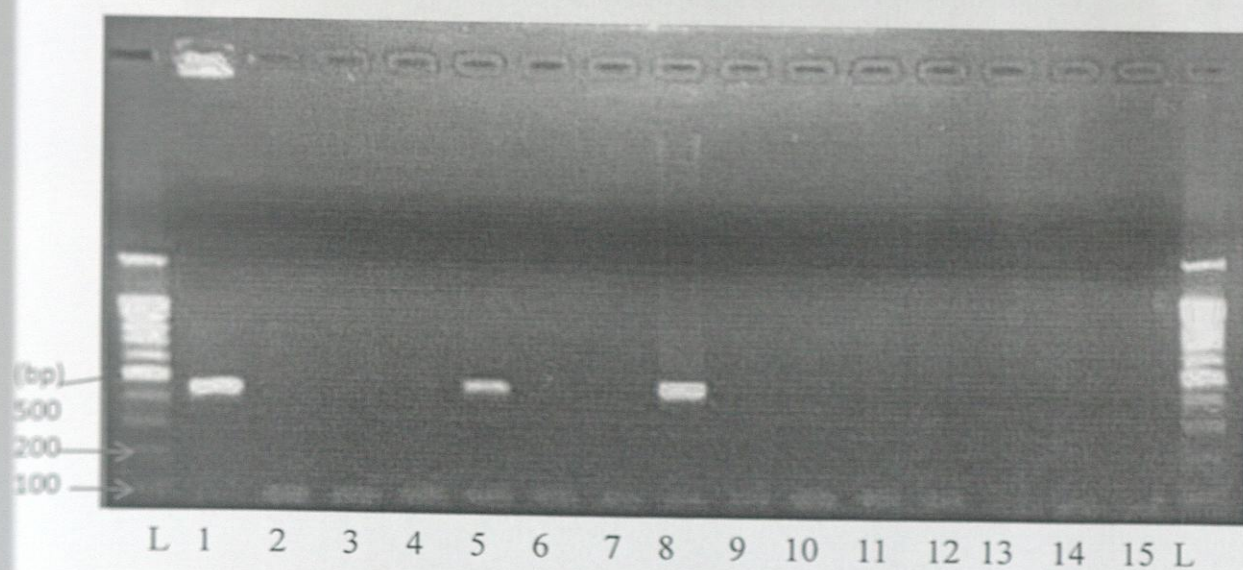


Fig. 3.6: Image of the gel electrophoresis for *pvl* gene in *S. aureus* (KBRN) isolates; L: 100bp ladder, 1: *S. aureus* ATCC 49775, 2: negative control (PCR mixture without template DNA), 3: Isolate named as KBRN 18, 4: KBRN 20, 5: KBRN 21, 6: KBRN 22, 7: KBRN 23, 8: KBRN 24, 9: KBRN 25, 10: KBRN 26, KBRN 27, 12: KBRN 28, 13: KBRN 29, 14: KBRN 31, 15: KBRN 32

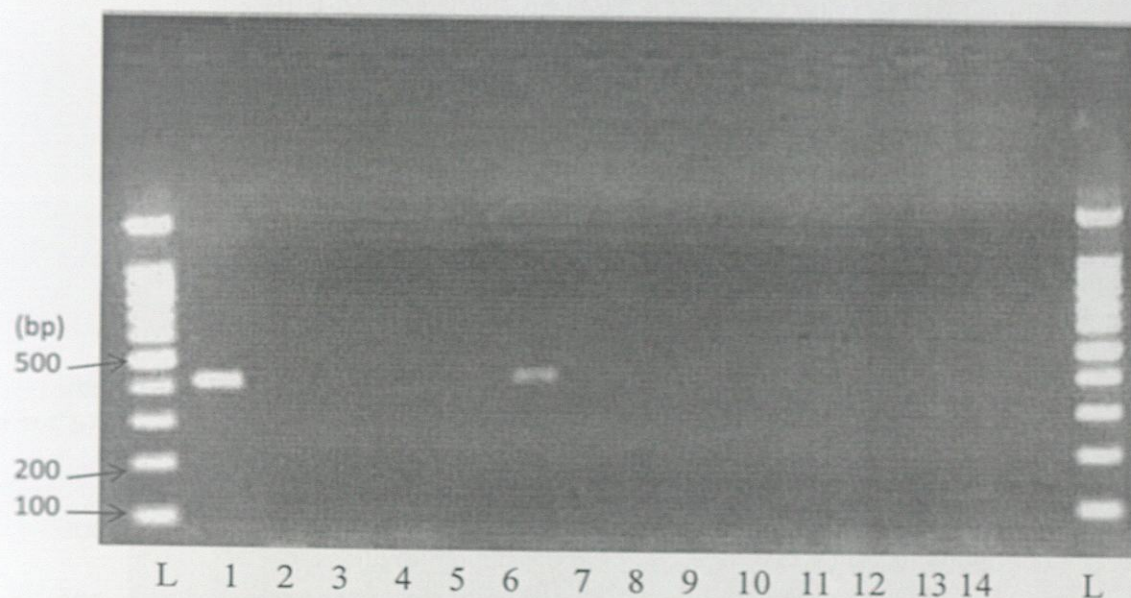


Fig. 3.7: Image of the gel electrophoresis for *ermA* gene in *S. aureus* (KBRN) isolates; L: 100 bp ladder, 1: Positive control (known *ermA* *S. aureus*), 2: Negative control (PCR mixture without template DNA), 3: Isolate named as KBRN 49, 4: KBRN 50, 5: KBRN 51, 6: KBRN 52, 7: KBRN 53, 8: KBRN 54, 9: KBRN 55, 10: KBRN 56, KBRN 57, 12: KBRN 58, 13: KBRN 59, 14: KBRN 60.

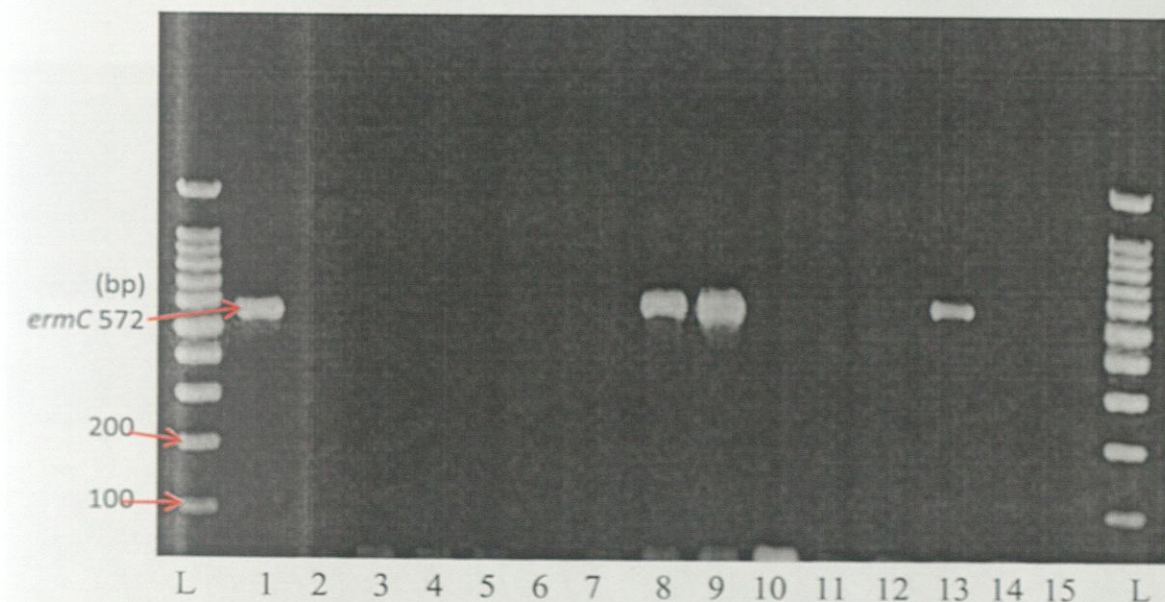


Fig. 3.8: Image of the gel electrophoresis for *ermC* gene in *S. aureus* (KBRN) isolates; L: 100 bp ladder, 1: Positive control (known *ermC* *S. aureus*), 2: Negative control (PCR mixture without template DNA), 3: Isolate named as KBRN 46, 4: KBRN 47, 5: KBRN 48, 6: KBRN 49, 7: KBRN 50, 8: KBRN 51, 9: KBRN 52, 10: KBRN 53, 11: KBRN 54, 12: KBRN 55, 13: KBRN 56, 14: KBRN 57, 15: KBRN 58.

3.36 Pulse Field Gel Electrophoresis (PFGE) results of MRSA isolates of KBRN samples. A preliminary picture was derived by PFGE typing technique as the MRSA isolates were tested (Fig. 3.7). This study identified 14 clusters (A-H) with 14 different PFGE patterns and 11 PFGE types were grouped two or more isolates. The major cluster found was C (10 isolates) followed by A (8 isolates), B (7 isolates), D (6 isolates), E (5 isolates), F (4 isolates), G (3 isolates) and H (2 isolates).

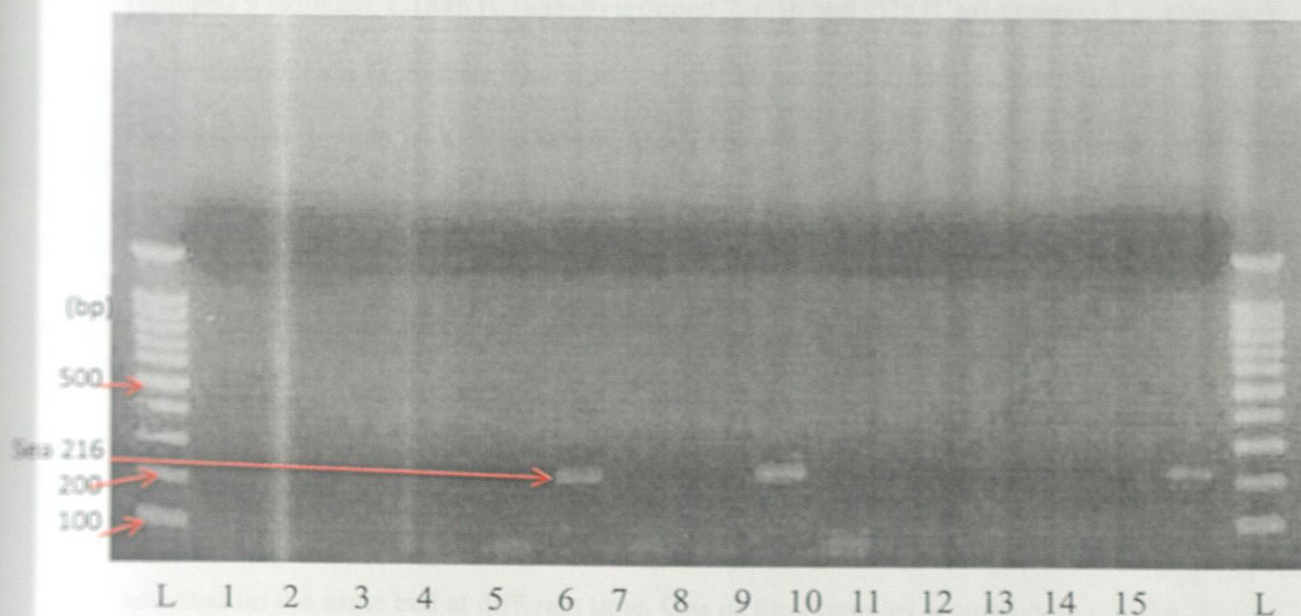


Fig. 3.9: Image of the gel electrophoresis for *sea* gene in *S. aureus* (KBRN) isolates. L: 100 bp ladder, 1: Isolate named as KBRN 25, 2: KBRN 26, 3: KBRN 27, 4: KBRN 28, 5: KBRN 29, 6: KBRN 31, 7: KBRN 32, 8: KBRN 33, 9: KBRN 34, 10: KBRN 35, 11: KBRN 36, 12: KBRN 37, 13: KBRN 38. 14: Negative control (PCR mixture without template DNA) and 15: Positive control (known *sea* positive *S. aureus*).

3.20 Pulse Field Gel Electrophoresis (PFGE) results of MRSA isolates of KBRN samples

A polyclonal picture was derived by PFGE typing technique as the MRSA strains were tested (Fig. 3.2). This study identified 14 clusters (A-N) with 29 different PFGE pulso-types, out of which 11 PFGE types were grouped two or more isolates. The major clone found was C1aaba (grouped 9 isolates), C1a4b (C1aaaab) grouped 7, C1b and C2a (Caa) grouped 3 isolates each while clone C1a5, D, E1aaa, E1aab, G1, H and I1 grouped 2 isolates each. Similar alphabet indicates that there exist a close association among the groups but are calculated as different pulso-types by BioNumerics software Gel Compar II (version 6.5; Applied Maths).

All of the MRSA were found multi-drug resistant (MDR). Pulso-types C1a5, C1a4b, C1a3b, C1aaba and C1aabb grouped 20 strains. These groups have 94.8 % similarities as shown in the dendrogram (Fig 3.2). These strains were isolated from patients admitted in the burn unit for adults and patients admitted in the burn unit located in pediatric surgery ward. Similar clones were isolated from patients admitted on different beds at the same time and patients admitted on the same bed at different time. One of the examples is pulso-type C1aaba. Strains (Key: kbrn old 1 and kbrn old 3) grouped in this pulso-type were collected from patients admitted on different beds (b. no PSW/6 and PSW/24) but specimen were taken on the same day. Similarly patients admitted to the same bed at different time, during the study duration acquired similar pulso-type strains (Key: kbrn 37 and kbrn kbrn 41). From the burn ward we have isolated the pulso-type C1a3b from one patient in July 2011 and pulso-type C1a4b from two other patients on 19 October 2011 and 22 November 2011 subsequently. These two types are 96.8 % similar with each other that suggest that subtype have also evolved. We observed that these outbreak strains were found during the whole study duration from July 2011 until December 2011. In another incident three strain of the same pulso-type C2a were isolated

from three different patients on the same day. Two of them were admitted in the burn ward (BW) on two adjacent beds while the third patients were admitted in burn unit pediatric surgical ward (PSW). Pulso-type C1b is another example of strains that are isolated from patients admitted in the burn ward at different times during the study.

The susceptibility pattern of antibiotics of the same type of clones is given in (Table 3.44). The results shows that some similarities exist in the antibiotic susceptibility pattern of the same pulso-type strains even though it is not uniform for all strains grouped in the same pulso-type. The minimum inhibitory concentration values were found different for some strains that were grouped in the same pulso-type clone.

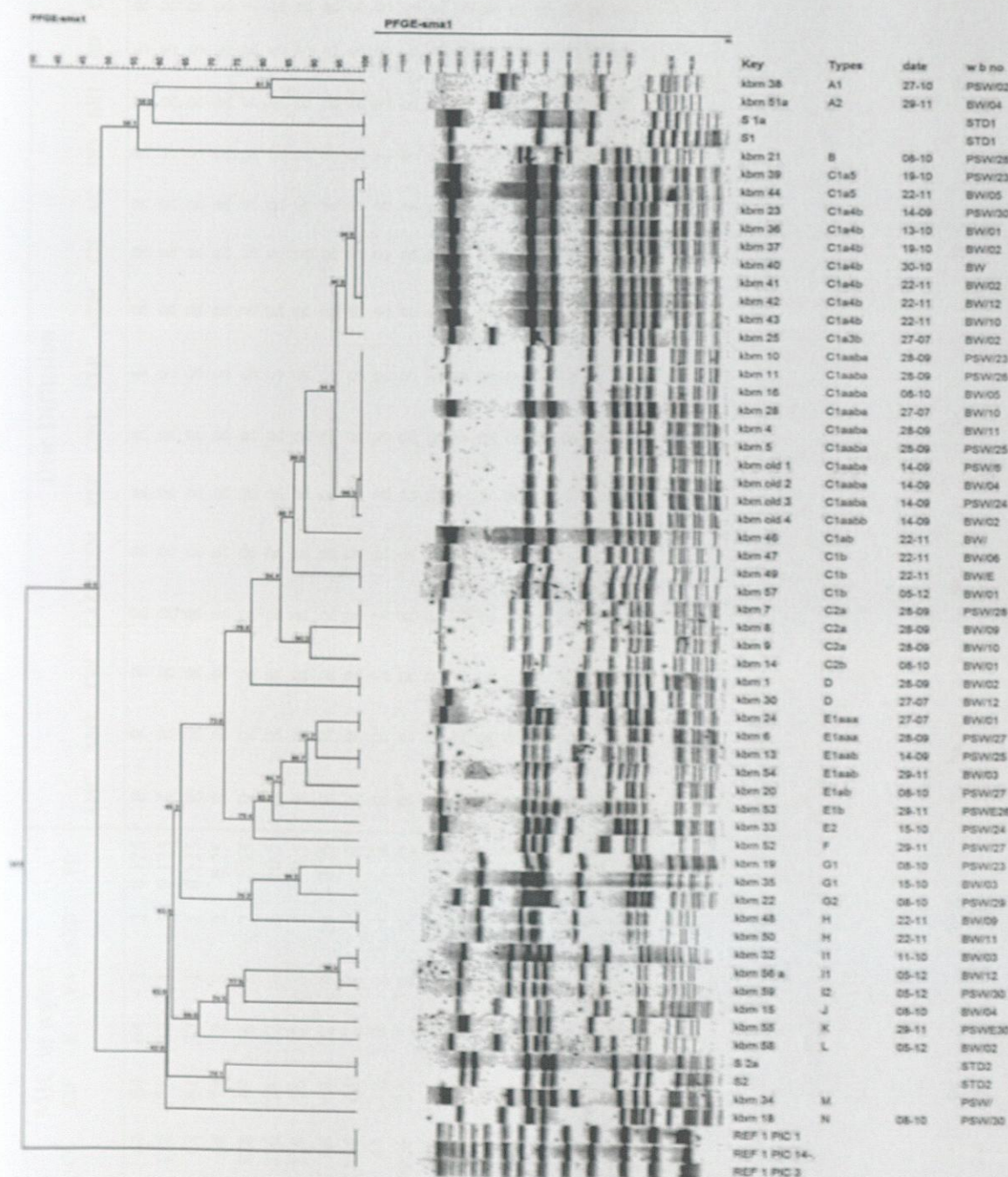


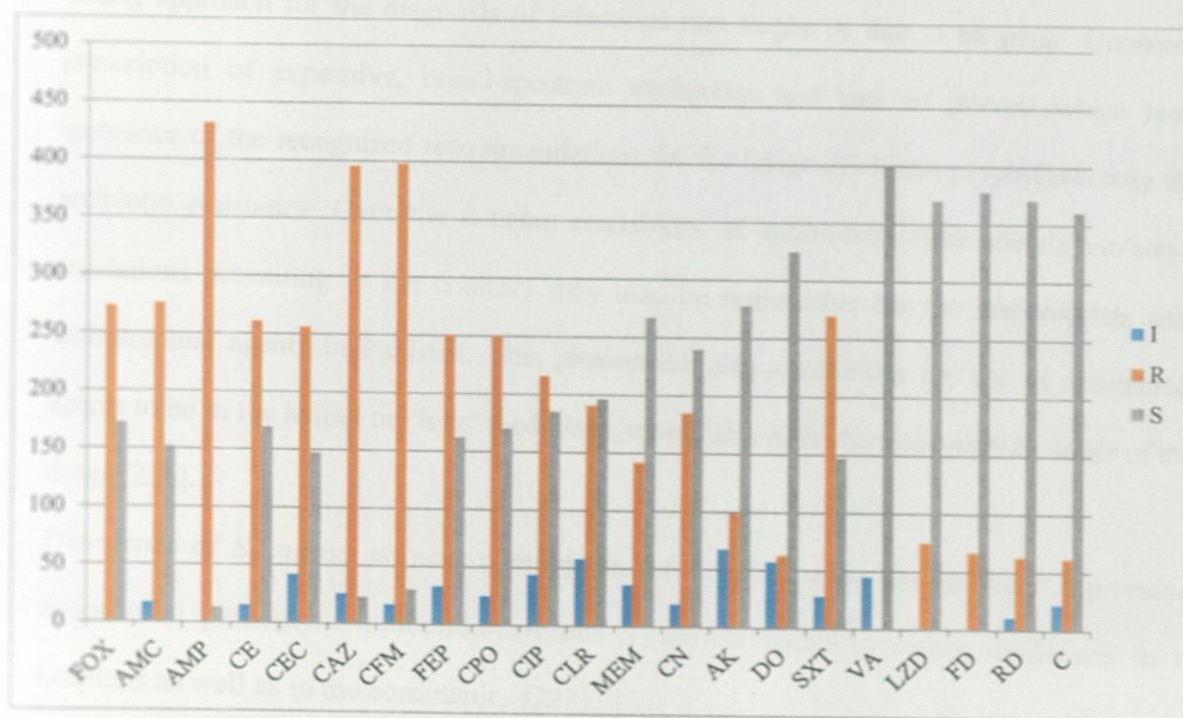
Fig 3.10: A part of dendrogram which shows relatedness of MRSA Isolates. Key: Strain number, Types: PFGE Types, date (day-month- 2011) W b no: ward and bed number (BW: Burn Ward, PSW: Pediatric Surgical Ward). S1 and S1a: ATCC 43300, S2 and S2a: ATCC 29213. REF: Lambda Ladder 50-1000kb in different gels was used.

Table 3.44: Antimicrobial resistance of related PFGE types strains

Strain's Key	PFGE type	FOX	MIC in µg/ml					Disc Diffusion															
			CE	CIP	E	VA	LZD	FD	AMC	AMP	CEC	CAZ	CFM	FEP	CPO	CLR	MEM	CN	AK	DO	SXT	RD	C
Kbrn old1	Cl aaba	128	64	64	64	2	2	0.125	R	R	R	R	R	R	R	I	R	R	R	S	R	S	R
Kbrn old2	Cl aaba	128	128	128	32	1	1	0.125	I	R	R	R	R	R	R	S	R	R	R	S	R	S	R
Kbrn old3	Cl aaba	128	64	64	32	1	1	0.125	R	R	R	R	R	R	R	S	R	R	R	S	R	S	R
Kbrn004	Cl aaba	128	64	128	32	2	2	0.25	R	R	R	R	R	R	R	S	R	R	R	S	R	S	R
Kbrn005	Cl aaba	128	128	64	16	2	2	0.25	R	R	R	R	R	R	R	S	S	S	S	S	R	I	R
Kbrn010	Cl aaba	128	256	128	32	4	4	0.5	R	R	R	R	R	R	R	S	R	R	R	S	R	S	R
Kbrn011	Cl aaba	128	128	16	32	0.5	4	0.5	S	R	R	R	R	R	R	S	R	R	S	S	R	S	R
Kbrn016	Cl aaba	64	64	64	32	4	16	0.25	R	R	R	R	R	R	R	S	R	R	I	S	R	S	R
SKbrn028	Cl aaba	256	256	64	2	4	4	2	R	R	R	R	R	R	R	R	R	S	S	S	R	S	S
Kbrn023	Cl a4b	64	64	32	2	2	4	1	R	R	S	I	R	S	S	R	S	S	S	I	S	S	S
Kbrn036	Cl a4b	>256	>256	32	2	2	4	2	R	R	R	R	R	R	R	S	S	R	I	S	R	S	I
Kbrn037	Cl a4b	256	256	16	2	2	4	1	R	R	R	R	R	R	R	I	S	R	R	S	R	S	R
Kbrn040	Cl a4b	256	256	16	2	2	8	1	S	R	I	S	R	I	S	S	S	S	S	I	S	S	S
Kbrn041	Cl a4b	>256	>256	64	1	2	2	0.25	R	R	R	R	R	R	R	S	S	R	R	S	R	S	R
Kbrn042	Cl a4b	256	256	64	1	2	4	0.5	R	R	R	R	R	R	R	S	S	R	R	S	R	S	R
Kbrn043	Cl a4b	256	256	32	4	2	8	1	R	R	R	R	R	R	R	S	S	R	R	S	R	S	R
Kbrn007	C2a	128	256	16	16	2	1	2	R	R	R	R	R	R	R	I	R	R	R	R	I	S	R
Kbrn008	C2a	>256	>256	32	16	4	2	1	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
Kbrn009	C2a	>256	>256	32	128	2	2	2	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
Kbrn047	C1b	128	128	0.5	16	4	4	4	S	R	R	R	R	I	I	R	R	S	S	S	S	S	S
Kbrn049	C1b	>256	>256	2	8	2	4	1	R	R	R	R	R	R	R	R	R	I	S	I	I	S	R
Kbrn057	C1b	256	256	2	8	2	2	0.5	R	R	R	R	I	R	R	R	R	R	S	S	R	S	R

Note: Abbreviations for antibiotics used in this table are the same as shown in Tables 2 & 3. R: Resistant, I:Intermediate, S: Susceptible

Overall, 445 *S. aureus* isolates were collected in this study. The most potent antibiotic was vancomycin to which 89.89% isolates were susceptible (**Figure 3.11**). Next to vancomycin were fusidic acid, rifampicin, linezolid and chloramphenicol inhibiting 84.94, 83.37, 83.15 and 81.12% of the isolates. Among the 445 isolates, 73.03% were susceptible to doxycycline, 62.47% to amikacin and 60% to meropenem. Gentamicin, clarithromycin and ciprofloxacin had average activity, i.e. 53.93, 44.04 and 41.57% respectively. Rest of the antibiotics had below 40% activities.

Fig. 3.11: Antibiotic susceptibility of *S. aureus* (n=445) strains.

3.21 Discussion

The inappropriate use of antimicrobial drugs by physicians against the viral infection and wrong approach for the diagnosis of infections that might be due to bacteria. Unnecessary prescription of expensive, broad-spectrum antibiotics and lack of interest which lead to ignorance of the recognized recommendations for the usage of chemo prophylaxis may cause antibiotic resistance. Over the counter availability of antibiotics which clearly violates the regulations prevailing in the contrary may also be responsible for the inappropriate use of antimicrobial agents in Pakistan. This phenomena also accelerates the use of antimicrobial agents to be in the herbal or "folk" medicines, which also rises the inappropriate usage of these drugs [226].

Prevalence of *S. aureus* is more common in the hospital setup and makes it a prominent nosocomial pathogen. Methicillin-resistant *S. aureus* (MRSA) causes epidemics in the hospitals as well as in the community [227].

MRSA can survive in dry environments and can reside in clinical places that are imperfectly cleaned [176]. The surveys of many epidemics have reported that MRSA can be easily spread by touch, predominantly in hospital setups [228; 229]. Aerial dissemination has also been assumed in numerous cases of nasal-MRSA carriage and upper-respiratory tract infections could lead to the spread of MRSA [179; 181]. MRSA also is known to be disseminated into air by the process of bed making [182]. Airborne transmission of MRSA from positive patients by respiratory droplets permits further study, not only for the sack of infection control in patients, but also gives clues for the use of personal protective devices by hospital personnel. More drug-resistant strains will make the treatment of infections cumbersome. Development of sophisticated techniques for prevention and treatment of

drug-resistant bugs will lead to better control of their spread. This aim can also be achieved by exploring their mechanism of virulence.

The aim of this study is to find out antibiotic susceptibility pattern of *S. aureus* collected in Peshawar from patients having wound, burn/skin infections. *Staphylococcus aureus* is the main pathogen causing blood stream infections, pneumonia and skin and soft tissue infections [230; 231; 232]. In this project, 445 *S. aureus* isolates were obtained. Post-operative wounds and burn infections yielded most of the isolates. These results are in accordance with observations of McCaig *et al.*, and Ali *et al.*, [233; 234].

The Food and Drug Administration (FDA) of USA approved vancomycin in 1958 for clinical use. It is the most potent antistaphylococcal agent. In our study no vancomycin resistance in *S. aureus* strains was found. Coagulase-negative staphylococci were collected in 1987 with intermediate resistance to vancomycin [235]. In 1996, a *S. aureus* isolate was obtained having MIC 8 mg/L from a child after going through heart surgery in Japan. This was called Vancomycin Intermediate *S. aureus* (VISA) [236]. In this way, resistance to vancomycin started rising in *S. aureus*. Highly vancomycin resistant *S. aureus* strain having MIC more than 128 mg/L was recovered in 2002 in Michigan which was also MRSA i.e. MIC > 16 mg/L [237]. Patients infected with *S. aureus* resistant to vancomycin are vulnerable to die as indicated by high mortality rate of 63% [125]. In our study we detected intermediate resistant isolates to vancomycin. We have found intermediate resistant *S. aureus* isolates in different groups, KLS (6.59%), KBLS (0%), KE (12.5%), CN (0%), HMC (7.61%) and KBRN (21.31%) by MIC method. The mean value is 10.11%. Other studies from Pakistan substantiate these results [212; 238; 239; 240]. Limited use of vancomycin in Staphylococcal infections due to high cost may be one of the reasons for this low resistance.

MIC of *S. aureus* increases after exposure to vancomycin [241]. Vancomycin resistance in *enterococci* is considered largely due to exposure to avoparcin (glycopeptide antimicrobial), which is used as growth promoter in animals. It is of great interest to find out the reason of this decrease level of resistance to vancomycin. Since no published data is available about avoparcin use in Pakistan, we cannot say that it is the only reason.

Among the antistaphylococcal agents, Fusidic acid is an active antibiotic [122; 242]. In a report from Poland it has been reported as 2.6%, while 6.3% resistance in *S. aureus* from France in 2008 [122; 243].

In our study, low resistance was found against Fusidic acid. Fusidic acid resistant *S. aureus* isolates in different groups of isolates were as KLS 11.98%, KBLS 40.00%, KE 20.31%, CN 5.56%, HMC 19.57% and KBRN 10.11%. The KBLS isolates were only 15 and 6 of these isolates were found resistant to Fusidic acid. This may be due to small sample size. The average resistance is 15.06%. In another study conducted by Hussain *et al.*, in 2008 in Rawalpindi, Pakistan 7.4% resistance was reported [244]. The use of fusidic acid in the form of topical application have increased in Pakistan which may lead to the subsequent increase in the resistance to fusidic acid [245].

Kedzierska *et al.*, reported 17% resistance to chloramphenicol from Poland in *S. aureus* in the year 2008 which were collected from skin infections [243]. We found resistance to chloramphenicol in KLS 6.59%, KBLS 33.33%, KE 17.19%, CN 0%, HMC 13.04% and KBRN 25.84 % with an average value of 13.93%. The lower resistance in *S. aureus* in the current study may be due to the limited use of this antibiotic as it is known to have potential harmful effects on bone marrow [246; 128].

Fluoroquinolones have good activity against *S. aureus* [247]. Denton *et al.*, in 2008 isolated

1390 *S. aureus* from the skin and soft tissue infections from Ireland, UK and France. They observed more than 95% susceptibility to ciprofloxacin. In this study ciprofloxacin was used which had 43.71% activity in KLS isolates, 13.33% in KBLS, 59.38% in KE, 38.89% in CN, 35.87% in HMC and 35.95% in KBRN. The mean of all these values is 41.57%. This study matches with the results conducted by Blandino *et al.*, in 2003, in Italy [248]. Tillotson *et al.*, have collected 380,000 isolates of *S. aureus* during 2005-2007 in USA and reported susceptibility data. Over 60% susceptibility of *S. aureus* isolates to fluoroquinolones have been recorded by them [249]. Reduced susceptibility to fluoroquinolones may be associated with overuse of this group of antibiotics and also available of the counter.

Aminoglycosides inhibits protein synthesis [128]. These antimicrobials agents show activity against *staphylococcal* infections [250; 251]. We have tested gentamicin (CN) and amikacin (AK) in this study. The percentage values of activity of CN against different isolates were KLS (56.89%), KBLS (26.67%), KE (62.50%), CRN (50%), HMC (48.91%) and KBRN (52.81 %) with a mean 53.93%. A much better activity was shown by AK against different *S. aureus* isolates as KLS (56.89%), KBLS (60.00%), KE (81.25%), CRN (72.22%), HMC (66.30%) and KBRN (53.93 %) were susceptible. The mean value of activity is 62.47%. Our results are consistent with Emaneuni *et al.*, (2009) from Iran [252].

Linezolid had good activity against the isolates of all the groups i.e. above 78%. In KBLS, it showed excellent activity i.e. 100%. Its mean activity in all the isolates was 83.15%. A study from China has reported 90.5% activity [253]. Previously linezolid was found with 100% activity [254]. This indicates an increase in the resistance to linezolid.

The susceptibility of RD to different groups of *S. aureus* isolates i.e. KLS, KBLS, KE, CRN,

HMC and KBRN was 80.24%, 86.67%, 75.00%, 83.33%, 85.87% and 92.13% respectively with a mean value 83.37%. Others researchers have reported 62% activity [255] and 95.2% activities [256].

Doxycycline was very effective in our study. This protein synthesis inhibitor is comparatively low cost. We recorded 73.03% activity of doxycycline in our overall isolates, which is just next to the costly anti-staphylococcal antibiotics. Another study conducted in this region has reported, 60.77% activity [214].

Macrolide-lincosamide-streptograminB (MLS_B) are very effective antibiotics for penicillin-allergic individuals suffering from *staphylococcal* infections [150]. Resistance to these antibiotics is rising due to extensive use, rapidly. The mechanisms of resistance which are the most common to MLS_B agents are:

- 1) 23S rRNA methylases, changes Ribosomal target encoded by *erm* genes [162].
- 2) Efflux pumps which is encoded by *msrA* gene [153].

Cross-resistance to macrolide by ribosomal methylation results, lincosamide and streptograminB (MLS_B phenotype) [257]. The *erm* genes are expressed constitutively (cMLS_B) or inducibly (iMLS_B) [159] which include (*ermA*, *ermB* and *ermC*). This leads to a kind of a reluctance to the use of clindamycin in the infections caused by strains resistance to erythromycin [160]. The principal genetic causes for ERY are *ermA* and *ermC* while *msrA* is rare [11], therefore erythromycin-resistant (ERY-R) *S. aureus* may be thought clindamycin resistant (CLI-R) in many geographical areas and in addition if an organism shows the iMLS_B phenotype, cautiously CLI should be used as there are chances that resistance may develop during the treatment [258; 159].

The use of ERY is extensive in Pakistan for throat and skin infections and is available as over the counter drug [259]. Still the surveillance work done on this agent is much less to assess the degree of resistance. At the same time, there is a lack of information about the MRSA types circulating. Determining the prevalence, MLS_B resistance and mechanisms of ERY resistance phenotypically amongst *S. aureus* and is one of the aims of this study was from different samples in Pakistan. Also the study aims to see if there were changes in the degree of ERY resistance between MSSA and MRSA and to examine the various toxins created by *S. aureus*.

We have included clarithromycin (CLR) in our study. Different group of *S. aureus* isolates i.e. KLS, KLBS, KE, CRN, HMC and KBRN showed 37.72%, 53.33%, 31.25%, 44.44%, 52.17% and 49.44% resistance to clarithromycin respectively with a mean 44.77% ERY-resistance, which is less than reported in the North America [260]. Mostly ERY-resistance was found in MRSA as compared to the MSSA which is in close agreement with a Colombian study [261].

Throughout the world regular investigation are carried out to know about the genes encoding resistance to macrolides and also to determine whether these are constitutively or inducibly expressed. Otsuka *et al.*, in 2007, from Japan stated 61.3% MLS_B constitutive and 38.7% MLS_B inducible resistance with *ermA* (95%) gene in MRSA being major, while in MSSA these were 1.3% and 94% respectively having *ermC* gene mainly (42%) [262]. In 2007a study conducted in Turkey revealed, MLS_B 58.3% constitutive, MLS_B 20.8% inducible resistance and MS phenotype 20.8% in *S. aureus*, while distribution of genes were as, *ermC* 62.5%, *ermA+ermC* 37.5%, *ermA* 50%, *ermB* 8.3%, and *msrA* 12.5%. One MRSA strain carrying four resistance genes, i.e., *ermA+ermB+ermC+msrA* were found by them [164].

ErmC is the major gene (96.5%) in Greece, conferring resistance to MLS_B antimicrobials and is expressed constitutively (94.7%) in most of the cases [165]. The SENTRY Antimicrobial Surveillance Programme conducted a large scale study on strains obtained from 24 European hospitals attached with university discovered *ermA* as the major resistance genes in MRSA (88%) was mostly in MLS_B-constitutive-resistance phenotype while *ermC* was predominantly observed in MSSA in strains with inducible-expression [166]. *ErmA* was found in majority (75.8%) of MRSA in Korea, in-comparison to 6.7% in MSSA [167].

The main genetic factors in *S. aureus* are *ErmA* and *ermC* [263; 262; 264]. The prevalence of *ermA* in different isolates i.e. KLS, KLBS, KE, CRN, HMC and KBRN was 23.29%, 0%, 17.18%, 38.88%, 32.60% and 13.48% respectively. The mean value is 20.91. The prevalence of *ermA* in MRSA isoaltes of each group of isolates i.e. KLS, KLBS, KE, CRN, HMC and KBRN was 35.00%, 0.00%, 18.91%, 66.66%, 36.66% and 11.11% respectively with a mean 28.05%. While in MSSA strains of different isolates group i.e. KLS, KLBS, KE, CRN, HMC and KBRN was 7.46%, 0.00%, 18.81%, 11.11%, 25.00% and 17.14% respectively with a mean value of 13.25%. This indicates that the prevalence of *ermA* was comparatively more in MRSA than MSSA.

The *ermB* prevalence in different groups of *S. aureus* isolates i.e. KLS, KLBS, KE, CRN, HMC and KBRN was 20.95%, 0.00%, 17.18%, 11.11%, 17.39% and 4.49% respectively with a mean value of 11.85%. To look into the differences in MRSA and MSSA strains with respect to the prevalence of *ermB* gene we had compared it. The *ermB* in MRSA in different sample groups i.e. KLS, KLBS, KE, CRN, HMC and KBRN was 21.00%, 0.00%, 21.62%, 0.00%, 15% and 0.00% respectively with a mean value of 9.60%. In MSSA strains of

different groups i.e. KLS, KLBS, KE, CRN, HMC and KBRN was 20.89%, 0.00%, 11.11%, 22.22%, 21.87% and 11.42% respectively with a mean 14.58%. *ermB* was found none in MSSA in comparison to MRSA. The *ermC* gene in different groups of *S. aureus* isolates i.e. KLS, KLBS, KE, CRN, HMC and KBRN was 23.35%, 13.33%, 28.12%, 33.33%, 29.34% and 21.34% respectively with a mean value of 24.80%. In the MRSA strain of different group of isolates i.e. KLS, KLBS, KE, CRN, HMC and KBRN was 28.00%, 15.38%, 27.02%, 22.22%, 26.66% and 20.37% with a mean value of 23.27%.

In the MSSA strains of different group of isolates i.e. KLS, KLBS, KE, CRN, HMC and KBRN was 16.41%, 0.00%, 29.62%, 44.44%, 34.37% and 22.85 respectively with a mean value of 24.60%. In MSSA the prevalence of *ermC* was found almost similar to MRSA strains.

The current study shows that ERY-resistance is more common in MRSA in-comparison to MSSA. Most of the ERY-resistance is due to *erm* genes and this result in the high level of resistance. This can also be expressed inducibly.

3.21.1 Methicillin resistant *staphylococcus aureus* (MRSA)

Infection caused by MRSA is a major problem throughout the world [265]. Therefore the cases of MRSA-infections have increased globally, as shown in different studies [266; 267; 232]. MRSA rate of prevalence is also high in Pakistan as is reported [233]. Still, its occurrence rate varies country to country. In the current study, rate of MRSA prevalence was found as 61.34% i.e. 273 out of 445 strains. This is higher than the previous studies reported from Pakistan [268; 212], but similar to the studies reported from the hospitals in Russia and Saudi Arabia [145; 269]. Multidrug-resistance in MRSA has also been reported frequently [143]. All of the MRSA isolates were found multidrug-resistant in the current

study. A study from Ireland by Galkowska *et al.*, in 2009 has reported that 70% MRSA were multidrug-resistance [270]. Similarly multidrug-resistant MRSA have also been isolated from animals. From China, Cui *et al.*, in 2009 have revealed that 70% of MRSA are multidrug-resistant isolated from animals [271]. Genes in the multidrug-resistance strains if located on plasmids can be transferred horizontally. Which can cause infections in humans too [272].

Against MRSA-infections, vancomycin is surely a drug of choice [273]. The MRSA collected in the current study were found susceptible to vancomycin which shows similarities with other the studies conducted [274; 275; 276; 212; 239; 277].

Previously chloramphenicol was found to have good activity against MRSA isolates reported from Pakistan [275]. In the current study MRSA showed 74% susceptibility to chloramphenicol. Similarly most of the MRSA isolates in this study are susceptible to fusidic acid, i.e. 80.76%. same results are published from the Agha Khan University Hospital, Karachi in which resistance to Fusidic acid was 18% [277]. In 2009, Askarian *et al.*, from Iran have published a study showing 100% susceptibility to fusidic acid by MRSA strains [278].

The aminoglycoside, amikacin, shows good activity against MRSA [279]. The aminoglycoside, activity of CN against different MRSA isolates i.e. KLS, KLBS, KE, CRN, HMC and KBRN are 32%, 23.08%, 54.05%, 22.22%, 31.67% and 30%, with a mean value 32.15%. AK showed good activity in comparison to CN against different isolates i.e. KLS, KLBS, KE, CRN, HMC and KBRN are 36%, 53.85%, 64.57%, 55.56%, 56.67% and 37%, with a mean 50.60%. Neela *et al.*, have reported lower rate (22%) of resistant from Malaysia but another study conducted in South Africa in 2009 (71%) have reported higher rate than

the current studies [280; 281].

Resistant MRSA is reported from England that has been transformed from the susceptible strains after therapy with Linezolid in a patient, by Peter Wilson *et al.*, in 2003 [282]. Our result showed good susceptibility to LZD by different MRSA isolates i.e. KLS, KLBS, KE, CRN, HMC and KBRN are 71%, 100%, 86.49%, 100%, 88% and 83% respectively with mean 88.08%. These results are similar to the study of John Weigelt published in 2004 [283]. A previous study from Pakistan have shown that all the tested isolates were susceptible to LZD. This shows that the resistance has been increased to LZD in the previous years. In the current study much higher resistance to Linezolid is reported than the previously reported data [284; 285; 286; 287].

Rifampin resistance in *Staphylococcus aureus* is chromosomally encoded, based mainly on mutations in the the *rpoB* gene [288].

Susceptibility to LZD of different MRSA isolates i.e. KLS, KLBS, KE, CRN, HMC and KBRN are 75%, 84.62%, 75.68%, 66.67%, 80% and 94% respectively with mean 79.33%. In other studies from Pakistan different susceptibility pattern have been reported for instance in one study all strains were found susceptible [256] while in another study 59% resistance was noted in MRSA [289]. A study from the Aga Khan hospital shows a 50% resistance [290] while an another report from Rawalpindi a 10.13% resistance was observed [291].

Susceptibility to DO of different MRSA isolates i.e. KLS, KLBS, KE, CRN, HMC and KBRN are 44%, 100%, 67.57%, 77.78%, 63.33% and 74% respectively with mean 71.11%. Previously 83.30 % susceptibility was reported by to doxycycline in MRSA isolates were reported by Ullah *et al.*, 2012 from the same hospital [214] which are similar to the current results.

MSSA are also a cause of post-surgical infections, necrotizing fasciitis, endocarditis, hospital-acquired infections [292; 293]. In-comparison to MRSA, infections of MSSA causes less mortality [294]. Mostly MSSA are highly susceptible to antibiotics. Similar results have been published from South Africa [295].

3.21.2 Toxins

Staphylococcal-infections with more severity have been found associated with the ability of the cell attachment to biomaterial. Recently the capabilities of *S. aureus* of forming biofilm have been extensively documented.

In the current study the *sea* gene was found the most common among the classical-staphylococcal-enterotoxin genes isolated from human strains. In various group of isolates that we have collected i.e. KLS, KLBS, HMC and KBRN the prevalence of *sea* was 46.35%, 33.33%, 31.52% and 17.97% respectively with a mean value of 32.29. While in environmental *S. aureus* isolates i.e. KE and CRN it is 56.25% and 33.33%. The MRSA isolates from human specimens in the above said groups showed that the prevalence of *sea* gene is 49.00%, 38.46%, 30.00% and 18.51% respectively with a mean value of 33.99%. The MRSA isolated from environmental specimens i.e. KE and CRN screened for *sea* gene gave results of 59.45% and 44.44% respectively. The MSSA strains isolated from the human specimens i.e. KLS, KLBS, HMC and KBRN showed 41.79%, 50%, 34.37% and 17.14% prevalence respectively. The mean value of these percentages is 35.85%. Similarly the MSSA strains isolated from environmental samples i.e. KE and CRN showed 51.85% and 22.22% prevalence of *sea* respectively. The findings of the current study demonstrated that the rate of occurrence of the *sea* is high in *S. aureus* strains collected from patients and in environmental samples. Previously reported data, regarding the prevalence of *sea* gene in

MSSA strains showed 23%, 17% and 32% in the invasive and nasal isolates, these values are lower than the current study [296; 297]. Similarly, it was reported that 17.5% of MSSA and 80% of MRSA from the clinical strains of *S. aureus* exhibit the *sea* gene [298].

In various groups of MRSA isolates that we have collected from human specimens i.e. KLS, KLBS, HMC and KBRN the prevalence of *seb* was 31%, 46.15%, 23.33% and 42.59% respectively with a mean value of 35.46%. While in MSSA isolates collected from human for the said groups of isolates it is 29.85%, 50.00%, 50.00% and 31.42% with a mean value of 40.31%. Similarly in environmental MRSA isolates i.e. KE and CRN the prevalence of *seb* gene is 43.24% and 22.22 %. While in environmental MSSA isolates i.e. KE and CRN it is 44.44% and 11.11%. In MSSA *seb* gene was found more prevalent than in MRSA strains. *Sec* gene was found both in MRSA and MSSA isolates collected in the current study but the prevalence rate was found low as compare to the *sea* and *seb* genes which is also suggested by other studies globally [299].

The *sed* gene was not found in the *S. aureus* strains. The overall rate of prevalence of the four classical *se* genes in the current study was 82.92% ($n = 369$). This shows that a larger number of the strains produce enterotoxins. Detection of enterotoxin-genes by PCR is not the sole proof for the production of enterotoxin. Bioassay done by immunologic methods should be performed to demonstrate the weightier of *S. aureus* strains are capable of producing enterotoxin-proteins [300]. Previously the prevalence rates of 73% and 86.2% for toxin genes were less than the current study in *S. aureus* but there were several genes that were not included in the earlier investigations compared to the four genes in the present study [79].

Chiang *et al.*, in 2008 [301] reported that 29.2%, 19.7%, 6.8% and 2.0% of the total 147 *S. aureus* strains from food poisoning epidemics the patients carried *sea*, *seb*, *sec* and *sed* genes respectively. The results are consistent with the current study but the prevalence of genes from the latter study were found less among food poisoning strains than that by Omoe *et al.*, [302]. There are some contradiction in a study to the current findings and that of the above three studies by Nashev *et al.*, (2004) [80] who reported that *seb* gene was (25%), the most prevalent, *sea* (15%) the second most and *sed* (5%) in the *S. aureus* strains from the nasal carriers with no symptoms. Prevalence of SE genes seems to vary from country to country. These variations could be due to nature of the strains and geographical differences.

A study conducted in 1995, in France reported the association between the skin infections and PVL production, especially in the cases of furuncles [59]. To explore the PVL production further in *S. aureus* strains, Lina *et al.*, conducted for the first time a comprehensive study in which 172 isolates were included. The study covered the whole France involving samples from pneumonia, skin infections and other infections. A PCR method was developed by them to find PVL (*lukS*-PV and *lukF*-PV) genes in the isolates collected. They detected 93% and 85% PVL genes distributed in the strains from necrotic pneumonia and furuncles, the common community acquired infections [50]. The PVL gene was detected by PCR in this study.

In the current study the prevalence of PVL in MRSA from different groups i.e. KLS, KLBS, KE, CRN, HMC and KBRN are 48.00%, 69.23%, 21.62%, 44.44%, 30.00% and 12.96% respectively. In the total MRSA isolated the prevalence is 37.71 %. In the MSSA isolated from different group of isolates i.e. KLS, KLBS, KE, CRN, HMC and KBRN the prevalence

of PVL is 80.59%, 50.00%, 33.33%, 88.88%, 56.25% and 45.71%. The prevalence of PVL in all MSSA isolated is 59.12%.

This suggests that the rate of occurrence of *S. aureus* strains positive for *pvl* in Pakistan is high in the MSSA as compared to MRSA. Same is also reported by Lina *et al.*, from France [50]. Low occurrence of *pvl*-positive MRSA detected in the current study proposes that the ability of these strains to cause diseases could be suggestively due to some other genes or features other than the *pvl* gene. The current results are much similar with Lina *et al.*, (1999) who have found that there was 85% sure association between the *pvl* gene and the detection of community-acquired-pneumonia [50]. Skiest *et al.*, however, have found some contrast results from both the present and the latter study [303]. In which he showed that, 97% MRSA isolates were positive for *pvl*. As the study was conducted in one hospital of an urban area, hence there is a chance that all of the strains might originated from one clones of MRSA. *Pvl* gene in *S. aureus* is considered to be associated with intense necrotising infections like skin abscesses and pneumonia [50]. PVL toxin is recognized to directly lyse/activate PMNs, consequently a central area of subcutaneous necrosis occurs as a result of furuncles. Cytotoxic and cytolytic variations in PMNs, macrophages and monocytes are induced by the production of PVL toxin in *S. aureus* infection is encoded by the *pvl* gene [51]. *Pvl*-positive *S. aureus* isolates are known to be contagious and virulent. The furuncles outbreaks in semi-closed communities like jails and sports teams are consider being associated with this gene [304; 305; 150]. Important point is that the attachment of *S. aureus* to the epithelial cells of the human is due to leukocidin induction of the expression of attachment proteins in the cell wall could be related to the widespread dissemination of potentially *pvl*-positive *S. aureus* [149]. Life threatening serious kind of diseases worldwide

have also been associated with the *S. aureus* strains that are *pvl*-positive with high mortality and morbidity rates although mainly they are a cause of simple soft and skin infections. *Pvl*-positive strains of *S. aureus* are not noteworthy causative agents of systemic infection in the UK but its occurrence at 20% rate in the USA are considered to be due to CA-MRSA *pvl*-positive, US 300 strain [306; 307].

The *tsst* gene prevalence in KLS and KLBS as 4.00% and 7.69% respectively. The prevalence of *tst* gene in all isolated MRSA is 1.94%. In the MSSA isolates *tst* gene was found in KLS (2.98%), KE (11.11%) and HMC (3.12%). In the total MSSA collected the prevalence of *tst* gene is 2.86%. Prevalence of the *tst* gene is reportedly low (7.5%) among *S. aureus* clinical isolates and 4.5% among stable *S. aureus* nasal carriers [308; 80]. Different studies are reported about the prevalence of *tst* gene as it is higher in MSSA than MRSA in the current and the results are in contrast with the earlier studies which state that the *tst* gene is more predominant in MRSA than in MSSA [132; 309]. The two MSSA strains positive for *tst* examined in the current research also harbored the *sea* gene. Our study reveals that MSSA strains which harbored both *tst* and *sea* genes are not common in this province of Pakistan compared to the other studies conducted in other parts where *S. aureus* clinical isolates carrying more than one genes are more prevailing i.e. *tst* and *sea* or *tst* and *sec* [310; 311; 312]. None of the isolate in the current study harbored both *sec* and *tst* genes. The *tst* gene along with certain superantigenic toxin genes, are related with mobile genetic factors for instance phages, plasmids and pathogenicity islands. A horizontal gene transfer is also known among isolates of *S. aureus* [302]. The isolates of *S. aureus* which harbor both *sea* and *tst* genes are related with MRSA enteritis as a consequence of the

creation of TSST 1 and SEA [313]. The production of cytokine is prompted by SEA and TSST 1 in mononuclear cells [314].

The incidence of *Staphylococcal* enterotoxin, *tst* and *pvl* genes in clinical isolates of *S. aureus* from Pakistan has not been frequently published. The consequence is that the data regarding these genes in clinical strains from Pakistan and adjacent countries are deficient. In the current study the finding of high discovery of these genes in Pakistan is much stimulating observation and the need of for further study to find the rate of occurrence of these and other prevailing genes in the strains from nasal carriage and colonization of *S. aureus* isolates. To detect these genes in *Staphylococcal* isolates the PCR techniques was used in this research. These genes gives the *S. aureus* isolates a capability to yield enterotoxins, PVL or TSST-1 toxins even though their presence does not essentially show that the corresponding toxin was formed.

3.21.3 Pulse Field Gel Electrophoresis (PFGE)

Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) are of rising concern in the Burn units due to the fact that the immunosuppressed patients with burn injuries are more readily prone to it. *Staphylococcus aureus* can reside on the skin surface, nasal cavity of patients or healthcare worker and inanimate objects including clothes and objects made of polyesters therefore its chance of dissemination and colonization on burn patients (Immunocompromised) is high [187; 198]. Previously MRSA positive patients can shed viable MRSA in the air through respiratory droplets that can infect newly admitted patients in the same room [197]. Similarly MRSA can be transmitted through contaminated air that acquires it from the colonized floors and walls of the hospital [186]. High susceptibility of burn patients [285; 183] and the extensive cross-transmission of isolates

[315] have also been emphasized by different researchers.

A poor hygienic environment in both the burn units for adult and children have been observed in the current study when it is compared against the guidelines adopted in the burn units worldwide [206]. There is no clean air filtration system installed. The routine cleaning of the surfaces including floors, walls, tables and bed for patients are not adequate and the exposure of patients to the surrounding environments is high due to the frequently visiting of relatives and health care personnel that are not properly trained to avoid cross-transmission. Due to these factors the occupancy and cross-transmission of MRSA clones could be understood easily. We have isolated same clones from different patients at different time who were admitted in the burn wards. As all of the 54 strains were classified in 14 clusters and named from A to N according to criteria set by McDougal, Steward et al. 2003 [225]. Cluster C and cluster E have grouped 28 and 7 strains respectively. In these clusters the groups and subgroups are more closer to each other in relatedness due to similarities in the band pattern of isolates. Pulso-type C1aaba, C1a4b have grouped 9, 7 isolates while C1b and C2a, have grouped 3 isolates each. Isolates grouped in every of the above mentioned pulso-type have the same band pattern. These clones are the most abundant clones in the burn units. Such a study conducted in a burn unit in Brazil shows that the same MRSA clones detected by PFGE were found in patients admitted at the same time in the same unit while same clones can be acquired to patients admitted later in the unit [316]. While in an another approach it was observed that the same clone of MRSA was transmitted to a neonate from the mother and subsequently the same clone, as identified by PFGE, was cross-transmitted horizontally to other neonates admitted at the same Neonatal Intensive Care Unit (NICU) [200].

Apart from the detection of similar pulso-type MRSA clones in this study, other clones with distinguishable band pattern have also been isolated. A total of 29 pulso-types out of 54 isolates were detected which suggest that new strain are harbored from other sources and this is understandable as the hygienic environment is poor in these units [197].

The prevalence of same pulso-type strains (same clones) in the burn wards were found common. This suggests that theses strains can easily transmit to the newly admitted patients in the same ward. Care should be taken to reduce the route of MRSA transmission. This will reduce the rate of morbidity and mortality among the burn patients. This is also important in the reduction of cost of medication and can reduce the length of hospitalization required.

Conclusions

- *S. aureus* was mostly found in the pus specimens.
- Among the tested antibiotics, Vancomycin was the most potent against *S. aureus*, followed by fusidic acid, rifampicin, linezolid, chloramphenicol and doxycycline.
- The prevalence of MRSA was noted to be 61.34%.
- The finding shows an agreement for the detection of methicillin-resistance in *S. aureus* among the different methods for instance cefoxitin disc, brilliance MRSA Agar 2 and *mecA* gene technique.
- In case of any non-agreement the results of *mecA* gene was considered as the final outcome.
- MRSA were mostly co-resistant to erythromycin, gentamicin, ciprofloxacin and tetracyclines.
- MSSA found in the study were generally prone to the other antimicrobials including cefoxitin and vancomycin.
- In comparison to MSSA the MIC₅₀ and MIC₉₀ were observed with high values to MRSA.
- In the erythromycin resistant *S. aureus* strains, *ermA* and *ermC* were found more common while *ermB* were found less.
- Strains resistant to erythromycin were also found positive for one erythromycin resistant gene.
- Most commonly, *sea* gene was detected among the classical enterotoxin genes in *S. aureus* strains followed by *seb* and *sec*.
- In the whole lot of our *S. aureus* isolates no *sed* enterotoxin gene was found.

- The MRSA strains negative for PVL gene were much less than the MSSA strains.
- Strains having more than one toxin gene were detected in the MSSA strains.
- PFGE detected 14 clusters which include 29 different pulso-types clones inhabiting in the burn units.
- Many of the same pulso-types clones were found in the burn units.
- Among these 29 pulso-types clones or types, 11 clustered two or more strains. For example C1aaba grouped 9 strains, C1a4b grouped 7, C1b and C2a grouped 3 strains each.
- Similarities up to 94.8 % were recorded for the groups designated as C1a5, C1a4b, C1a3b, C1aaba and C1aabb which suggest that these strains belong to the same-ancestor.
- On the other hand 29 pulso-types out of 54 strains were detected which suggest that a vast diversity occurs amongst the MRSA strain, which were collected from the burn patients.

RECOMMENDATIONS

- Culture sensitivity is necessary for the initiation of effective and specific therapy. This will help to achieve the goal of rational use of antibiotics. If the physicians are aware of the nature of pathogen will leads to a reduction in duration and cost of treatment. This system also reduces the risks associated with using irrational/irrelevant drugs.
- Susceptibility test for every infection should be stimulated to know what antibiotics can be the most suitable for the causative pathogen.
- Strict observation of the antimicrobial use and resistance records is necessary for encompassing rise of antibiotic resistance. Unceasing investigation is required for sustaining correct data of antibiogram patterns of all pathogens. Such statistical data found should be shared with other participants of health care system for instance pharmacists, physicians and nurses. With the help of this data they will be better informed about the recent antibiotic-susceptibility-patterns of microorganisms and will aid them to have better options in the treatment of patients. Infection Control Committee in a hospital might do this sort of investigation.
- For an overall image of susceptibility pattern in the country a reference laboratory should be formed. The organisms found if multidrug-resistant or have rare antibiotic-resistance-patterns must be sent to the reference laboratory by the Committee. The reference-laboratory will additionally describe this sort of pathogens and subject endorsements for handling diseases produced by such isolates.
- The Committee ought to create a plan for the country and should modify this time to time for infection control in the community based on antibiotic susceptibility pattern attained.

- *Staphylococcus aureus* if identified must be processed for MRSA identification.
- For the identification MRSA Cefoxitin (FOX) disc diffusion should be done. We also recommend brilliance MRSA agar2 (Oxoid, UK) for MRSA identification as it is much speedy procedure. All the MRSA obtained must be sent to the reference-laboratory for additional investigation. Infection due to MRSA should be reported to healthcare personnel and a flagging system must be familiarized. This system may help to recognize formerly MRSA positive patients, so that upon their subsequent admission these individuals are easily recognized and quarantined properly.
- Hospital staff and patients positive for MRSA and VRSA or carrier should be insulated until the test reveals them to be negative. For such isolates, "search and destroy" policy is very suitable.
- Attention should be given to reduce the MRSA transmission. This will reduce the rate of morbidity and mortality among the patients and will reduce the cost of drugs and can reduce stay in the hospital.
- Basic research for antimicrobial is also needed to discover new targets in microorganisms.
- Public awareness is important about the excessive use of antimicrobials that it can extinguish helpful bacteria.
- Compliance of individuals using antibiotics must be observed regularly.
- Antibiotics use as agent to promote growth in animals must be stopped.
- The pharmaceutical industry must be encouraged by different means as extended patent life, incentives to industry, rationalization registration procedure for products and

persuading them to initiate research ventures with the research institutes in the universities.

- The use of new-antibiotics should be restricted. This will decrease rise of resistance.
- Appropriate investigation is required to keep check on the emergence of resistance to new antibiotics after launch.
- Self-medication and over the Counter (OTC) sale of antimicrobials must be banned.
- Bacteria, fungi and plants are the industries of nature which produces a variety of agents. Research for the screening of bacteria, fungi and plants may help in finding novel antimicrobial agents.
- Research on vaccine production should be stimulated which may reduce the use of antibiotics and therefore antibiotic resistance in microorganisms.
- Antibiotic strategy must be applied strictly with significant features as given below:
 - i) Culture sensitivity tests must be performed and antibiotics must be used accordingly.
 - ii) In case of asymptomatic bacteremia the use of antibiotics must be avoided.
 - iii) A complete course of antibiotics is needed to eradicate the pathogen completely.
 - iv) Antibiotics use as a prophylactic agent should be in limit.
 - v) Viral infections should not be treated with antibiotics.
- Strains typing of MRSA by PFGE, MLST and *SCC mec* typing technique is needed to observe and overcome an epidemic in the hospital. This will certainly reduce the rate of morbidity and mortality.
- Adequate cleaning procedures must be started to reduce the spread of acquired-MRSA.

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Appendix 1

PATIENT PROFORMA FOR PhD PROJECT

No. _____ Lab. No. _____ Date _____

Name of patient _____ Age _____ Gender _____

In patient / outpatient _____ Ward/ bed No. _____

Clinical Features _____

Community acquired infection _____ Hospital acquired infection _____

Antibiotic history _____

Sample Pus HVS Blood Sputum

Test performed:

Gram Staining: Gram positive

Catalase _____

Coagulase _____

DNAasa _____

Novobiocin _____

MSA _____

Microorganism Identified _____

S.No	Antibiotic	Symbol	Strength of Antibiotic in μg	Zone of Inhibition	MIC	R-I-S	Remarks
1	Amoxicillin + Clualinic acid	AMC	20+10				
2	Ampicillin	AMP	10				
3	Cephadrine	CE	30				
4	Cefaclor	CEC	30				
5	Ceftazdime	CAZ	30				
6	Cefixime	CFM	5				
7	Cefepime	FEP	30				
8	Cefpirome	CPO	30				
9	Ciprofloxacin	CIP	5				
10	Clarithromycin	CLR	15				
11	Meropenem	MEM	10				
12	Gentamicin	CN	10				
13	Amikacin	AK	30				
14	Doxycycline	DO	30				
15	Trimethoprim +Sulphamethoxazole	SXT	25				
16	Vancommycin	VA	30				
17	Linezolid	LZD	30				
18	Fusidic acid	FD	10				
19	Rifampicin	RD	5				
20	Chloamphenicol	C	30				
21	Cefoxitin	FOX	30				