

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

*IN THE NAME OF ALLAH, THE BENEFICENT
THE MERCIFUL*

*Read! And thy Lord is Most Honorable and Most Benevolent,
Who taught (to write) by pen, He taught man that which he knew not*

(Surah Al-Alaq 30: 3-5)

Al-Quran

**MOLECULAR EPIDEMIOLOGY OF ANTIBIOTIC RESISTANT
GENES IN PATHOGENIC BACTERIA, (A CASE STUDY OF
KHYBER PUKHTUNKHWA, PAKISTAN)**



**By
Johar Jamil**

**CENTRE FOR BIOTECHNOLOGY AND MICROBIOLOGY
UNIVERSITY OF PESHAWAR
2013**

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GENES IN PATHOGENIC BACTERIA, (A CASE STUDY OF
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Johar Jamil

A thesis submitted to the University of Peshawar in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology and Microbiology

**CENTRE FOR BIOTECHNOLOGY AND MICROBIOLOGY
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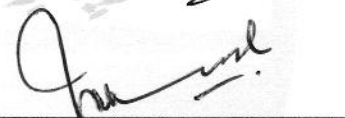
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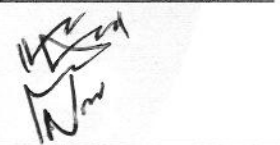
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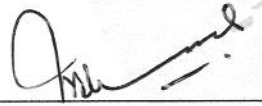
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Dedication

Dedicated To My Loving Parents

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LIST OF ABBREVIATIONS

PBPs	Penicillin binding proteins
PABA	p-aminobenzoic acid
MDR	Multidrug resistant
AMEs	Aminoglycosides-modifying enzymes
MRSA	Methicillin resistant <i>S. aureus</i>
MSSA	Methicillin sensitive <i>S. aureus</i>
SCC _{mec}	<i>Staphylococcal</i> Cassette Chromosome
HA-MRSA	Hospital Acquired Methicillin resistant <i>S. aureus</i>
CA-MRSA	Community acquired methicillin resistant <i>S. aureus</i>
PCMX	Chloroxynol (4-chloro-3,5-dimethy phenol):p-chloro-m-xynol
SEs	<i>S. aureus</i> Enterotoxins
TSST-1	Toxic Shock Syndrome
PVL	Penton Valentine Leukocidenes
CPE	Carbapenemase Producing <i>Enterobacteriaceae</i>
MBL	Metallo- β -lactamases
CRE	Carbapenems Resistant <i>Enterobacteriaceae</i>
ESBLs	Extended spectrum- β -lactamases
AIDs	Acquired immune deficiency syndrome
UTIs	Urinary tract infections
ETEC	Enterotoxigenic <i>E. coli</i>
EPEC	Enteropathogenic <i>E. coli</i>
EIEC	Enteroinvasive <i>E. coli</i>
EHEC	Enterohemorrhagic
UPEC	Uropathogenic <i>E. coli</i>

KPC	<i>Klebsiella pneumoniae</i> Carbapenemase
NADM-1	NewDalhi Metallo- β -lactamses-1
PCR	Ploymrersae Chain Reaction
KTH	Khyber teaching hospital
LRH	Lady Reading Hospital
CLED	Cysteine lacytose electrolyte deficient agar
MSA	Menitol Salt Agar
MR-VP	Methyl Red Voges-Proskauer Test
TSI	t ripple Sugar Iron test
TSA	Tryptic soya Agar
TSB	Tryptic soya broth
ATCC	American type culture collection
NCTC	National collection of type cultures
MIC	Minimum Inhibitory concentration
TAE	t ris –acetic acis –EDTA
ETDA	Ethylenediaminetetraacetic Acid
ETBr	Ethedium bromide
FOX	Cefoxitin
OX	Oxacillin
BSAC	British Society of Antimicrobial Chemotherapy
CLSI	Clinical Laboratory and Standards Institute
ISA	Iso-Sensitest Agar
TE	Tris-EDTA
CDT	Combined disc test
PMQR	Plasmid–mediated Quinolones
16S rDNA	16S Ribosomal DNA

ABSTRACT

This study was conducted from 2009 to 2012 on isolates collected from two tertiary hospitals of Peshawar, Khyber Pakhtunkhwa, Pakistan. The aim of this study was to find out the resistance profile of different antibiotics and biocides, prevalence of extended-spectrum β -lactamases (ESBLs) and carbapenemases in *S. aureus*, *E. coli*, *Klebsiella* spp. and *P. aeruginosa*, respectively.

Among 133 *S. aureus*, 47% were Methicillin resistant *S. aureus* (MRSA). Cefoxitin was found to be the most efficient and effective method for the detection of MRSA. In *S. aureus*, vancomycin (VAN) and linezolid (LZD) showed best activity against both MRSA and MSSA. High level of antibiotic resistance was found in MRSA isolates. Most of our *S. aureus* isolates were susceptible to biocides. The genes including *sea* and *pvl* were the most dominant genes in *S. aureus* isolates.

Carbapenems including meropenem and imipenem showed very good activity against *E. coli* and *Klebsiella* spp. and *P. aeruginosa*. ESBL and carbapenemase producers were also detected in *E. coli* and *Klebsiella* spp.

Results of the present study indicate that in Pakistan most of the infections are caused by antibiotic resistant bacteria. The identification of these bacteria should be done properly. Brilliance ESBL, CRE and MRSA agars were found best for the phenotypic detection of β -lactamases, carbapenemases and MRSA.

Chapter 1

Introduction

The chemicals which are used for the cure or prevention of diseases are called the chemotherapeutic agents. Most of these agents are produced naturally or synthesized in laboratory. These agents may be used orally or systematically for the treatment of infections in both animals and humans. These agents include antibiotics, disinfectants, antiseptics, and preservatives which are used in cosmetics, foods and pharmaceuticals. The most important are the antibiotics, which are used for their antibacterial, antiviral, and antifungal properties. "Antibiotic" is chemical agent, which is produced by one microorganism against the other microorganism by killing or inhibiting its growth. In modern era of medicine, antibiotics are the most beneficial form of chemotherapy [1]. Antibacterial can be divided in two groups on the basis of their action. Those which kill the bacteria are called bactericidal (organisms killed) and those which inhibit the growth or keep bacteria in a stationary phase are called bacteriostatic. Selman in (1942) used the term antibiotic for the first time [2].

In 1877 Louis Pasteur and Robert Koch observed that an airborne bacillus could prevent the growth of *Bacillus anthracis* and for the first time antibiosis was described in late 1880. Paul Ehrlich (1854-1915) first found the synthetic compound with antimicrobial activity and introduced synthetic antimicrobial chemotherapy as a science. He observed that some dyes would color the living cells like human, animal and bacterial cells. In 1904 he found that trypan red dye was active against the parasite called trypanosome, which causes African sleeping sickness. Arsphenamine was discovered by Ehrlich which showed a activity against the syphilis spirochete. Salvarsan was the commercial name of

that compound. Alexander in 1927 for the first time found the antimicrobial activity against bacteria of a fungus of genus penicillium and suggested that this property was because of antimicrobial substance named penicillin. Gerhard Domagk in 1927 discovered prontosil red dye which had an antimicrobial activity against streptococci and staphylococci. He also discovered the sulfa drugs and got Nobel Prize for it. The antibiotics era began from the discovery of sulfa drugs. Gramicidin was first derived from *Bacillus. brevis* in 1939, by Rene Dubos and was used in World War II for the treatment of wounds and ulcers of war victims. In 1940 Howard Florey, Ernst chain and Norman Heatly extracted penicillin from penicillium and used it against streptococci and staphylococci and got Nobel Prize for their efforts. In 1944 Selman discovered streptomycin. Similarly in 1953 chloramphenicol, neomycin, teramycin were isolated from microorganisms. The modern era of medicine has completely changed with the discoveries of new and effective antimicrobial drugs which have minimized the sufferings of mankind to a great extent [2].

1.1 Mechanisms of Action of Antibiotics

On the basis of effectiveness, antibiotics can be classified into two categories. Broad spectrum antibiotics are active against several types of bacteria while narrow spectrum antibiotics are active against a few. There are 20 main antibiotic classes which are divided on the basis of their source of origin, chemical composition and their mode and site of action [2]. But clinically six families i.e. penicillins, cephalosporins, tetracyclines, aminoglycosides, macrolides and fluoroquinolones are the most important [3]. The mechanisms of action of antibiotics are the following:

1.2 Antibacterial activity through inhibition of cell wall synthesis (Bacitracin, cephalosporins, cycloserine, penicillin, vancomycin, carbapenems)

Bacteria have a rigid protective outer layer, the cell wall. It gives shape to the bacterium cell. The bacterial cell wall has chemically distinct complex polymer, peptidoglycan (murein, mucopeptide) consisting of polysaccharides and a highly cross linked polypeptides. Penicillin binding proteins (PBPs) catalyze the transpeptidase reaction that removes the terminal alanine to form crosslink with a nearby peptide and natural D-alanyl-D-alanine substrate. They are covalently bound by PBPs at the active site which inhibits peptidoglycan synthesis and the cell dies because the weaker cell wall as a result cannot maintain integrity of the cell. Among the antibacterial, the β -lactams such as penicillin and cephalosporins, have their functional portions called β -lactam rings. The β -lactams inhibit peptidoglycan formation by irreversible binding to the enzyme that cross-link NAM subunit [4].

Cycloserine stops formation of basic peptidoglycan subunits and glycopeptides like vancomycin prevents the elongation of the glycoprotein. Another glycopeptide teicoplanin prevents the polymerization of peptidoglycan by interacting with D-alanyl-D-alanine terminal of the muramylpentapeptide which fits into a cleft inside the antibiotic molecule [5].

1.3 Inhibition of Protein Synthesis

Some antibiotics prevent synthesis of protein by interacting with ribosome of bacteria. There are two subunits of ribosome in bacteria i.e. 30S and 70S. These two subunits play an important role in initiation of protein synthesis [6]. Most of the antibiotics inhibit the translation or protein synthesis by reacting with the ribosomal mRNA. Aminoglycosides

cause misreading of mRNA, which results in the formation of abnormal proteins. Tetracyclines inhibit the building of peptide bonds by blocking the attachment of tRNA on 50S subunit of rRNA (chloramphenicol) or erythromycin prevents the translocation of subunit during translation [2].

1.4 Inhibition of Nucleic acid Synthesis (RNA and DNA)

Some antimicrobial have diverse effects on the nucleic acid of a bacterial cell. Quinolones including nalidixic acid and its fluorinated derivatives i.e. norfloxacin, ciprofloxacin, and ofloxacin etc are synthetic antibacterial which prevent the activity of both bacterial enzymes DNA gyrase and topoisomerase IV. Both of these enzymes play an essential role in super coiling of DNA and are also important for DNA replication in the intact cell. The bacterial cell dies if their essential activities are stopped [7].

These antibiotics have two sites for their action in the bacterial cell. In gram negative bacilli its target is DNA gyrase, while topoisomerase IV is the secondary but in gram positive bacteria the mode of action is reversed [8]. Quinolones prevent the replication of bacterial DNA by disturbing the action of DNA gyrase, topoisomerase II, bacterial growth and reproduction. A complex is formed by binding of quinolones with both these enzymes and DNA, which prevents the rejoining step, and thus induces cleavage in DNA which may lead to cell death [3].

1.5 Inhibition of Metabolic Pathways

The folic acid synthesis is inhibited by the antimetabolites which are the synthetic compounds. The synthesis pathway of folic acid acts as coenzymes for one carbon transfer reactions which are important for formation of thymidine, all purines, and many other amino acids. Bacterial cell growth is ceased by the inhibition of folate synthesis and

causes cell death [7]. There are many antibiotics which inhibit the metabolic pathway by acting as antimetabolites which completely stop the use of metabolites by enzymes in a bacterial cell. Sulfonamides were used as first antimetabolites. These drugs are structurally similar to sulfanilamide analog of p-aminobenzoic acid, or PABA which is used in the formation of co-factor folic acid (folate). By entering in the bacterial cell these antibiotics compete with PABA for active site which results in low concentration of folic acid. This lowering of concentrations of folic acid results in damaging of cell because it serves as important component in the formation of purines and pyrimidines. These are the important bases which are used in building of DNA, RNA and other important components of bacterial cell. Therefore the bacterial cell cannot synthesize purines and pyrimidines which results in cell death [4]. Trimethoprim is a diaminopyrimidine, which is a structural analogue of the pteridine moiety of folic acid. This is a competitive inhibitor of dihydrofolate reductase. This enzyme reduces the dihydrofolic acid into tetrahydrofolic acid which is the important final component in synthesis of the folic acid. Similarly, trimethoprim acts like both bactericidal and bacteriostatic depending on absence of thymine and the presence of pyrimidines in high concentration respectively [7].

1.6 Disruption of Cell Membrane

Some antibiotics like polyenes break the cytoplasmic membrane of the bacterial cells, and incorporate into the membrane by destroying its integrity [6]. Polymyxins increase the permeability of bacterial membrane which results in the leakage of membrane [9].

1.7 Antibiotic Resistance

“Antibiotic resistance is defined as that an organism is resistant when it is inhibited in vitro by an antibiotic concentration that is greater than that achievable in vivo” [8].

Over the last 60 years, antibiotics are used in modern medicine. Since then, the problem of resistance in bacteria has started and increased in the last few years. Many original antibiotics are becoming useless because of resistant bacteria. If the problem of increasing resistance among the pathogens is not controlled, the golden era of antibiotics will soon end [10]. So it is found in almost all the bacteria which cause serious infections both in human and animal. Thus the problem of resistance to antibiotics is increasing all over the world. The increasing problem of antimicrobial resistance shows the adaptability of bacteria and limited effect of all novel drugs. Many genes and process are involved in antibiotic resistance in both gram negative and gram positive bacteria. These include the exchange of heredity information by mutation, transformation and conjugation [1]. The sulfonamide resistance which was caused by the single step mutation of chromosomal in origin was noticed very early. Similarly the Oxford group noticed the activity of an enzyme produced by *E. coli* which inactivates the penicillin G in 1944. Treptomycin resistance was also noticed. The use of antibiotics are main factor for development of both types of chromosomal and plasmid bacterial resistance. The use of antibiotics in community and health care will decreases the susceptibility of pathogen to them. The resistance may be plasmid or chromosome mediated. The plasmid mediated resistance is more important from an epidemiological point of view, because of its easy transmission among the pathogens [5]. The abundance of diverse mobile genetic elements is the horizontal gene pool results in this transmission of gene in same species and also among

the different species which results in the formation of multidrug resistant (MDR) pathogens in both gram negative and gram positive bacteria. The problem of antibiotic resistance is because of the selective pressure of antibiotics use in both community and health care centers where the MDR can be observed [11].

The resistance to antimicrobial drugs remains the main public health concern [12]. In order to prevent the development of resistance the antibiotics should be used in a proper way [13]. In Asian countries the antibiotic resistance causes high medical cost and death rates because of high profile of resistance to many antibiotics. The public hygiene also plays an important role in the effectiveness of antibiotics in developing countries [14]. The resistance pattern of various bacteria may vary depending on the geographical condition of country, state, large hospital, small hospital and community. Like other developing countries Pakistan is also facing the increase in antibiotic resistance problem [15]. Antibiotic resistance in bacteria is becoming more important and needs more attention. Several different mechanisms of resistance are developed by bacteria against the antibiotics [1].

1.7.1 Mechanisms of Resistances

There are two main types of resistances i.e. intrinsic and acquired resistances. In some bacteria the resistance to antibiotics may be intrinsic or innate e.g. aminoglycosides resistance in obligate anaerobic bacteria and vancomycin resistance in gram negative bacteria (*E. coli*). But some bacteria acquire resistance firstly, by acquisition of exogenous genes by plasmids, transposons, integrons, bacteriophages and secondly, by gene mutation and thirdly by the combination of these process [8].

Bacteria that are ordinarily susceptible to antibiotics can acquire resistance which minimizes the effectiveness of antibiotic chemotherapy. The mutated genes or acquisition of new resistant genes can be transfer by horizontal gene transfer. The resistance caused by acquisition of genes has bad effect on the curing value of many available antibiotics [7, 8]. The genes which are present on plasmid can be easily transferred within the genus and other different genus as compared to the resistance associated with the genes on chromosome [1].

So the most frequent type of resistance is acquired resistance which is the most prevalent type of resistance and which can be spread by horizontal gene transfer. But nowadays a very dangerous mechanism of resistance results in MDR bacteria or “super bugs” which are resistant to most of the available antibiotics. The genes for MDR are present in a DNA sequence that is moved from one plasmid to another or to the genomes, which are transposons or “jumping gene system” [1].

In both chromosomal and plasmid-mediated resistance, antibiotic usage plays a vital role. Because it destroys the susceptibility of sensitive bacterial strain in both health care and community settings and cause in spreading of such strains. The plasmid resistance is very important because this form of resistance is transmissible and can be related to many other properties that make a bacterium to colonize and get enter in a sensitive host [5].

The current situation of antibiotic resistance is very threatening to the whole world and may lead back to the pre-antibiotic era especially in treatment failure of certain infectious disease. In health care settings most of resistant strains of bacteria are produced which are now common in community settings as well. The factors including behavioral or environmental policy are contributing in emergence of bacterial resistance in community.

Behavioral factors are the improper use of antimicrobials and failure of infection control and hygiene practices. While the regular use of antibiotics in agriculture, livestock and the lack of new drug development lies under the heading of environmental policy. The government should contribute in the better policies in behavioral strategies in the community and such regulatory decisions will help to minimize the problem of antibiotic resistance throughout the world [16]. There are different types of resistance mechanisms which are mostly found in clinically important pathogenic bacteria.

1.7.2 Antibiotic Inactivation or Modification

The bacteria produce a lot of enzymes which are responsible for the inactivation or modification of antibiotics. But the most important enzymes that play an important role in the inactivation of antibiotics are β -lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferases [17].

1.7.3 Antibiotic Modification by Hydrolysis

β -lactamases are the most prevalent enzymes which are produced by the members of gram negative bacteria. These enzymes hydrolyze the cephalosporin, penicillin and other β -lactams [1]. About 300 different β -lactamases are discovered [18]. The genes for these enzymes are encoded on chromosomes and plasmids which can be easily transferred through transposons but some of these genes are also found in integrons [1]. ESBL producing bacteria are resistant to penicillins, third-generation oxyimin cephalosporins but they are sensitive to cephamycins and carbapenems, and can be inhibited by clavulanic acid, sulbactam, or tazobactam which are the ESBL inhibitors [19]. Bacteria producing ESBL are most often resistant to quinolones due to mutations in *gyrA* and *parC* genes [20].

A type of carbapenemase called Metallo- β -lactamases (MBLs) is common in *P. aeruginosa*, *K. pneumoniae*, *E. coli*, *Proteus mirabilis* and *Enterobacter* spp. Its mode of action is the same as serine β -lactamases. They cause resistance to most of the antibiotics by hydrolyzing e.g. imipenem, new-generations of cephalosporins and penicillins. The MBLs producing bacteria are resistant to inhibitors of β -lactamases but sensitive to aztreonam [21].

1.7.4 Antibiotic Inactivation by Transfer of Group

Transferases are the enzymes which inactivate the aminoglycosides, chloramphenicol, streptogramin, macrolides, or rifampicin. Antibiotic is inactivated by binding of adenylyl, phosphoryl, or acetyl groups to the antibiotic molecule. These changes are done by the process of transport across the cytoplasmic membrane [22]. Antibiotics like aminoglycosides are neutralized by special enzymes i.e. phosphoryltransferases, nucleotidyltransferases or adenylyltransferases and acetyltransferases. These enzymes are also called aminoglycoside-modifying enzymes (AMEs) which minimize the tendency of a modified molecule, and stop binding to the 30S ribosomal subunit. Such types of modified enzymes cause resistance to aminoglycosides [23]. Similarly another enzyme chloramphenicol transacetylase are responsible for the resistance to chloramphenicol that causes acetylation of hydroxyl groups in chloramphenicol. This enzyme is present in gram negative and gram positive bacteria which are resistant to chloramphenicol [24].

1.7.5 Antibiotic Inactivation by Redox Process

Pathogenic bacteria also use oxidation and reduction reactions as a resistance mechanism against some antibiotics [22].

1.7.6 Changes in Target Molecule:

Antibiotic and the target molecule have specific interaction. Even small change in a target molecule can affect the interaction between antibiotic and its target [22]. So bacteria develop a defensive mechanism by altering the target molecules which are responsible for the resistance to antibiotics [4].

β -lactams e.g., penicillins, cephalosporins, carbapenems, glycopeptides and monobactams inhibit the synthesis of cell wall. The β -lactam antibiotics binding ability is minimized by mutation in PBPs. The resistance of ampicillin in *E. faecium* and resistance of penicillin in *S. pneumoniae* are because of these mechanisms. The PBP2a causes high resistance to β -lactam, penicillins and cephalosporins [4]. In gram positive bacteria the cell wall synthesis is inhibited by glycopeptides, e.g., vancomycin or teicoplanin. These antibiotics bind to acyl-D-alanyl-D-alanine (acyl-D-Ala-D-Ala) residues of peptidoglycan precursors. Resistance to glycopeptides resistance may be intrinsic or acquired [22].

Similarly, aminoglycosides get bind to the 30S ribosomal subunit, while chloramphenicol and macrolides bind to the 50S ribosomal subunit and cause interference in protein synthesis [2]. Resistance to kanamycin and sparsomycin are caused by the non-methylated 23S rRNA and 16S rRNA at U2584 position in some other bacteria. While resistance to clindamycin, linezolid, and tiamulin caused by *rluC* gene [25].

Quinolone resistance is normally caused by the chromosomal mutations in topoisomerase genes [26]. So changes in two enzymes because of mutation i.e. in DNA gyrase which is also known as topoisomerase II and topoisomerase IV. The replication process is ceased by the mutations in genes which are responsible for production of DNA gyrase and this mutation cannot allow the quinolones/fluoroquinolones to bind [25].

1.7.7 Permeability of Outer Membrane and Efflux pump

Some bacteria use membrane proteins which take out antimicrobial agent from the cell and also retain their low intracellular concentrations called efflux pumps. The reduction in the permeability of the outer membrane leads to reduced uptake of drug. The detection of efflux pumps is important in antimicrobial agent's resistance process. Various types of efflux pumps have been found. The single component efflux systems transfer their substrates out of the cytoplasmic membrane while multicomponent pumps along with the periplasmic membrane synthesized a protein component and that protein component transfers substrates across the cell envelope. The multi component efflux pump is found in gram negative bacteria [22]. Tetracycline resistant bacteria mostly produce membrane proteins in large amount which are encoded by *tet* (A) gene, which are used as efflux pumps for antibiotic [27]. *P. aeruginosa* use efflux pumps to get rid of toxic chemical compounds from both cytoplasm and periplasm. It has more than four MDR efflux pumps which are responsible for resistance to ethidium bromide, erythromycin, chloramphenicol, fluoroquinolones, acriflavine and tetracycline [28]. Similarly, *msrA* gene encodes the efflux pump which is responsible to get rid of erythromycin from bacterial cell. This minimizes the amount of drug inside the cell and makes the drug less effective or non effective on bacterial cell [29]. In gram negative bacteria, the outer membrane has an inner layer of phospholipids and an outer layer of lipid A. This composition of outer membrane reduces antibiotic uptake to a cell and transfer it through porin proteins, e.g., *ompF* in *E. coli* and *oprD* in *P. aeruginosa* [22].

1.7.8 Bypass of Antibiotic Inhibition

Some bacterial resistance is antibiotic specific. Bacteria produce an alternative target that is resistant to inhibition of antibiotic. The alternative target allows bacteria to survive by adopting the role of a native protein. Trimethoprim and sulfonamides resistance is caused by reduced sensitivity and affinity of altered enzymes dihydropteroate synthetase and dihydropteroate reductase to trimethoprim and sulphanamides [19].

1.8 *Staphylococcus aureus*

Staphylococcus aureus belongs to gram-positive bacteria. It causes many infections in both health care and community settings all over the world. It is a part of normal microbiota of both humans and animals which makes it a successful pathogen of them. It can live without any harm on skin surfaces like around the mouth, nose, genitalia and rectum. The colonized individuals are more prone to *S. aureus* infections [2]. It causes infections like folliculitis and food borne intoxications and life threatening infections like endocarditis, osteomyelitis, or septicemia and bacteremia [30].

The *S. aureus* genome is a circular chromosome having about 2800 base pairs with additional prophages, plasmids, and transposons. Both the chromosomes and plasmids have genes ruling virulence, antibiotic resistance and virulence gene. The transference of these genes within the *S. aureus* strains and other bacteria takes place by plasmid [31].

The last 50 years could be named as “half century of MRSA” [32]. Penicillin was the first line drug against all strains of *S. aureus* but now most of its strains are resistant to it [33]. In 1959, methicillin first came into use by the physicians. But only after two years the first MRSA had been evolved and from that time it is now increasing globally [34]. So in 1960 MRSA was emerged as a cause of infection in hospitals [35]. MRSA have the *mecA*

gene which is present on mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*) codes for the altered PBP2a [36]. Four main types of SCC*mec* have been discovered i.e. I-IV. Among these four, I-III are present mostly in hospital acquired MRSA (HA-MRSA) while IV is prevalent in community acquired (CA-MRSA) [37]. In health care centers many infections are caused by MRSA. Such infections are called hospital acquired infections or HA-MRSA infections. These types of infections are mostly ported in hospital settings which can be transmitted from one patient to another by health services providers when they are dealing patients without sanitizing their hands [38].

However, MRSA after its emergence was associated with the hospital settings. But this statement has been changed in the last 15 years and now the infections caused by CA-MRSA are reported in individuals in community settings [37]. The first CA-MRSA infection case was reported in 1990 in Australia [39]. The out breaks of community acquired MRSA (CA-MRSA) infections have mostly found in military trainees, homosexual men, intravenous drug users [40]. CA-MRSA not only causes disease in soft skin tissues but it can be also linked with necrotizing pneumonia and sepsis [41]. In Pakistan the prevalence of MRSA is little studied. But according to some reports in Pakistan about (42-51%) most of hospital acquired infections are caused by MRSA [42].

1.8.1 Biocides

Biocide is a term used for chemical agents (disinfectants and antiseptics) used to kill bacteria, virus and moulds [43]. Triclosan is a bisphenol biocide which is used for the control of various gram negative and gram positive bacteria. It is bacteriostatic at lower concentrations, while bactericidal at higher concentrations [44]. Its use began in the US

in the 1970s in soaps, 76% of 395 soaps commercially available in the US contained it [45]. Since then its use has risen dramatically [46]. Apart from its use in the soaps, it is also used in mouth washes, in preservation of food products and in many hospital used products [47]. In 1998 its use was also recommended for the control of MRSA [48]. Similarly in Europe, 350 tons of Triclosan is produced every year for use in commercial products [49]. TCC, 3,4,4-trichlorocarbanilide (Triclorcarban) is the member of the most widely studied anilides group which are used as antiseptics. This is used in commercial soaps and deodorants. It has good activity against gram positive bacteria as compared to gram negative bacteria and fungi [50].

Quaternary ammonium compounds (QACs) are another type of biocides which are used for the control of microorganisms both in the hospitals and in the community settings. These are the most important and beneficial antiseptics/disinfectants [51]. Some of these compounds are known as cationic detergents, used as preoperative disinfectants on unbroken skin and also used for many other purposes. It is also used in cleaning hard surfaces and for neutralization of odours [43].

Benzalkonium Chloride is a QAC. This compound is mainly active against gram positive organisms and is used to disinfect surfaces in healthcare environments. It is also used as a preservative for contact lenses and as a veterinary disinfectant [52]. Cetrimide is a halogenated QAC. It has antiseptic as well as detergent properties with a wide spectrum activity against bacteria and fungi [53]. Chloroxylenol (4-chloro-3, 5-dimethylphenol; *p*-chloro-*m*-xylenol) (PCMX) is a bactericidal halophenol which is used in the preparation of antiseptics and disinfectants. Some bacteria like *Pseudomonas* spp. and moulds are resistant to it. Although it has been used worldwide for many years, but its mode of

action is not well understood. However, it is thought that it affects membrane of the microbes [43].

Chlorhexidine is a bactericidal which is used worldwide as antiseptic in different concentrations in topical solutions since 1954. It is present in hand washes, body washes, and mouth wash solutions, as preservative and in many hospitals used in antiseptic solutions. It acts by binding to negatively charged cell wall of bacteria thereby disturbing the osmotic equilibrium leading to death of the cell [54].

Metals in the form of ions alone or in combinations with other chemicals have been used as disinfectants for the decontamination of fluids, living tissues and solid surfaces for more than a century. In the 18th century, in the western world, copper was used extensively in hospitals for the treatment of various mental and lungs disorders. Being bacteriostatic, copper has many potential uses in the field of medicine being used as an antibacterial, antiviral and antifungal [55].

Silver has been used since 335 BC for the control of microbial growth and it is still used as coating in water tanks to prevent growth of bacteria [56]. Due to its biocidal characteristics, silver is used in a variety of products like wound dressings and clinical devices [57]. Silver ions are nowadays used for the control of bacterial growth in many medical procedures like dental work, catheterization etc. It is also used in nonmedical objects like the lining in toilet seats, refrigerators, dishwashers and laundry machines [58].

The activities of biocides are different in different types of organisms. Even in the same species it may be different. Favero et al., (1991) prepared a guide in which they grouped

the biocides on the basis of its appropriate application. But it could not explain the exact interaction of different organisms and biocides [59].

Researchers are now investigating resistance in bacteria to commonly used biocides. In UK and Australia, MSSA and MRSA isolates were found resistant to disinfectants [43]. However, resistance to biocides in bacteria is not very common as compared to antibiotics. This variation may be associated with different modes of action of biocides to kill microorganisms [60]. That is why resistance in bacteria to sanitizers is not well documented [61]. Some reports indicate that bacteria use similar tools in the development of resistance process against biocides like alteration of target sites, and efflux pump which have been documented for antimicrobial agents. However, level of resistance to biocides, whenever studied is low [52]. There are many studies which have described antibiotic and biocides resistance in bacteria which may be innate or acquired [62].

1.8.2 Enterotoxin Genes

S. aureus also has the virulence factors producing genes which enhance its pathogenicity. The exotoxins like toxic shock syndrome toxins and enterotoxins are also produced by some strains of *S. aureus* [63]. These enterotoxins and toxic shock syndrome are very important among the exoprotein [64]. The *S. aureus* enterotoxins (SEs) are the low molecular mass proteins, which have the same composition and biological activity but differ in antigenicity [65]. These are divided into different types i.e. from SEA to SEE and newly discovered SEG to SER and SEU [66]. But the role of some of these enterotoxin genes e.g. SEG, SEH, SEI and SEU is still unstudied in human diseases [67]. These can be detected by PCR and also by immunoassay [68]. These toxins play an important role in toxemia syndromes and food poisoning. Those strains of *S. aureus*

which cause food poisoning must have these genes [69]. Food poisoning caused by *S. aureus* is a common food borne disease in the world. This kind of food borne diseases is caused by taking that enterotoxin in food [70]. Its symptoms are abdominal, pains, cramps, and diarrhea after incubation [71]. If the food handlers are colonized with such strains, are mostly play an important role in transferring that strains of *S. aureus* into the food. If such foods are not stored properly for longer than 4hrs at 4-60⁰C than massive growth of *S. aureus* and production of enterotoxins will occur, which are the causes of staphyloenterotoxigenic [72]. Most of the strains produce one or more superantigenic toxins [73]. These toxins can bypass normal antigen presentation and have strong T cell mitogenic activity by direct binding to the Vb region of specific T cells and the major histocompatibility complex class II molecules of antigen presenting cells [77]. This leads to a massive release of pro inflammatory cytokines and contributes to the severity of *S. aureus* sepsis [75].

On the basis of sequence homologies 21 SEs have been found [76]. There are few studies on the SEs in the patients with *S. aureus* infections [77]. The different frequencies of enterotoxigenic genes in clinical *S. aureus* strains have been investigated in different parts of the world [76].

Todd et al (1978) reported *tsst* for the first time as multi system disease in a child [78]. Toxic shock syndrome 1 (TSST-1) is a superantigenic toxin produced by *S. aureus*. This virulence factor is encoded on *tsst* gene. TSST1 is a dangerous toxin which plays as virulence factor in many infections like staphylococcal scarlet fever, and neonatal toxic shock like exanthematous diseases (NTED). These types of infections are reported in Japan and France [79]. The main symptoms of the disease caused by this toxin are fever,

erthematous rash, mucosal hyperemia, hypotension and multi organ involvement and death [80]. In women it was first associates with tampon used by women but it was also reported in women with non-menstruation found in both health care and community settings [81].

1.8.3 Panton Valentine Leukocidin (PVL)

Panton Valentine Leukocidin (PVL) is also produced by *S. aureus*. This was discovered by Panton and Valentine in (1932). This is another virulence factor which can take part in the colonization, invading and evading the immune system. PVL is a pore forming virulence factor which is encoded by two genes *lukF-PV* and *lukS-PV*, where F is for fast and S is for slow elution in ion exchange column. It also contributes in the lysis of neutrophils, apoptosis and necrosis of tissues [82]. The term leukocidin means the toxic substance produce by *S. aureus* which destroys white blood cell of the host organism. This toxin is produced by *S. aureus* for its defense [83]. Provost et al., (1995) cloned the genes which are responsible for encoding the PVL. He also sequenced that genes, and named them as *lukF-PV* and *lukS-PV*. Both these genes encode proteins molecule which have a molecular weight of 34,000 Dalton by *lukF-PV* and 32,000 Dalton by *lukS-PV*. Within the genome of *S. aureus* both the proteins are produced from different loci. In *S. aureus* the PVL production is associated with severe cutaneous abscesses, furuncles and necrotic skin infections [84].

In human it also causes skin and soft tissues infections, necrotizing pneumonia bone and joint disease [85]. There are several studies that MSSA having *pvl* are responsible for the infections in skin and soft tissues and necrotizing pneumonia [86]. It is also reported in community required MRSA. It is the causes of high mortality rate [82]. The diseases

caused by the *S. aureus* having *pvl* can be more severe in patients suffering from respiratory tract disorders. A study reported by Aguilar et al., (2007) reported that the death rate in *pvl* positive MRSA were 100% as compared to the negative *pvl* MRSA which was 47% [87]. So *pvl* is the most common virulence factor related to CA-MRSA which causes the increased rate of mortality [82]. Less than 5% of *S. aureus* strains harbored *pvl* in Western Europe [84]. These clones have been isolated from many other countries [82]. During 2003, specific clones of *pvl* have been studied in different continents. These clones have been isolated from many other countries. CA-MRSA the presence of *pvl* can be related to its virulence but this has not been proved by using molecular methods [88]. However, different studies showed that the increase in the prevalence of *pvl* harboring gene are related to infections caused by CA-MRSA [89].

1.9 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram negative opportunistic pathogenic bacterium. It causes severe infections in skin, blood stream, lungs, meninges, urinary tract and burns [90]. It has innate resistance to many antimicrobial agents and can be changed to most resistant strains when exposed to drugs during therapy [91]. It is considered the main cause of hospital acquired infections because of its resistance to a wide range of antibiotics. It has capability to develop resistance against any antibiotics to which it is exposed previously because it has many resistance mechanisms [92]. This antibiotic resistance makes complications in the treatment of many severe infections [93]. The pathogenicity of *P. aeruginosa* depends on its resistance to wide range of antibiotics. As a result infection due to such multi drug resistant strains cause three times more deaths [94].

P. aeruginosa also developed a resistance mechanism against β -lactams by producing ESBLs like penicillinases, cephalosporinases and carbapenemases. The production of β -lactamases is the most common resistance mechanism found in members of enterobacteriaceae [95]. Along with it, another mechanism of resistance called the efflux pump is present in *P. aeruginosa*. The efflux pump has the ability to remove the drug from the inner cell environment of bacteria [96]. It has also intrinsic resistance to a wide range of antibiotics [92].

Among the gram negative bacteria, *P. aeruginosa* has become the most important pathogenic agent of causing infections in immunocompromized and hospitalized patients [91]. In specific situations the infection is more dangerous as in case of neutropenic patients with bacteremia and hospital acquired pneumonia in which the death rate is 30-50% and 45-70% respectively. Similarly *P. aeruginosa* is mainly responsible for bacteremia in patients suffering from neutropenic cancer who are undergoing chemotherapy [97]. The patients who are immunodeficient because of AIDS, severe burns and diabetes mellitus can be suffered from bacteremia and septicemia because of *P. aeruginosa* [98]. It also invades the respiratory tract milieu of the patients having chronic obstructive bronchopulmonary disease, in immunocompromized and in hospitalized intensive care patients [99]. Similarly in ventilated patients and cystic fibrosis the cause of nosocomial pneumonia and lung infections respectively are mostly caused by *P. aeruginosa* [100].

After *E. coli* which is the main and common cause of UTIs, *P. aeruginosa* is the third common cause of nosocomial (12%) of urinary tract infection [101]. Such UTIs infections occur mostly in urinary tract catheterization instrumentation or surgery by *P.*

aeruginosa [102]. It is also the main cause of “swimmer’s ear” in diabetic patients’ malignant otitis [103]. Although less frequent than other organisms, it rarely causes dangerous eye infection like keratitis in people using contact lens. Similarly *P. aeruginosa* also rarely causes infections in brain like meningitis and abscesses in patients having trauma, or surgical procedures of brain [104]. In intravenous drug users it causes endocarditis [105]. It causes skin and bone infections but may occur after wounds puncher [106]. Digestive tract infections caused by *P. aeruginosa* are rare, but peri-rectal infections, typical gastroenteritis and necrotising enterocolitis have been studied [107]. The MDR *P. aeruginosa* is associated with high morbidity and mortality [108].

1.10 *E. coli*

Escherichia coli (*E. coli*) belongs to gram-negative group of bacteria. It is rod-shaped, motile, flagellated and facultative anaerobe. It belongs to the family enterobacteriaceae. In 1885 Theoder Escherich discovered it [2]. It is genetically very important bacteria and it has many phage and plasmid encoded genes [109]. It is responsible for diarrhea and septicemia in both humans and animals. It has a lot of virulence factors which contributes in its pathogenicity which include invasiveness factors for invasions, heat labile and stable enterotoxins, verotoxins and or adhesion factors [110]. There are two groups of pathogenic *E. coli* which are enteropathogenic and uropathogenic. Pathogenic *E. coli* are further grouped into enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC), enterohemorrhagic *E. coli* (EHEC) [111]. The most common group of *E. coli* is uropathogenic *E. coli* (UPEC), which causes UTI. This group of *E. coli* is the resident of animal and man’s lower digestive tract [112].

Urinary tract infections (UTIs) caused by *E. coli* are the most common infection which are mostly seen in clinical practice with high incidence and medical cost. It causes 30-50% hospital acquired and 80-90% community associated UTIs [113]. Millions of people suffer from bladder infection every year with symptomatic infections like cystitis. The increase in the ratio of infections like recurrent cystitis caused by multi drug resistant bacteria is making the situation more worrisome for public health. Most of bladder infections are caused by the uropathogenic *Escherichia coli* (UPEC) [114]. It can be divided in to four phylogenetic groups (A, B1, B2 and D). Among these phylogenetic groups B2 was considered the most prominent group in pathogenic *E. coli* which is followed by group D, A and B1 [114]. The sequence type ST131 of *E. coli* is also member of group B2 which mostly carries the ESBL gene blaCTX-M-15. This is the most disseminated infectious bacteria all over the world [115].

The problem of emerging resistance in pathogenic bacteria is of global concern and its frequency is increasing. But this multidrug resistance varies from country to country, state to state and city to city [116]. According to Shifali et al. (2012) 46.87% of 96 urban respondents and 41.86% of 43 rural respondents were identified with UTI. Thirty-two (51.61%) of 62 females aged between 25 and 34 years and 15 (46.87%) of 32 females aged between 15 and 24 years were identified with UTI. Ten (40.0%) of 25 females between 35 and 44 years and 6 (30.0%) of 20 females above 45 years of age had UTI in Lucknow [117].

1.11 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a gram negative rod which is a member of family enterobacteriaceae. It is nonmotile and has both aerobic and anaerobic properties. It is a

lactose fermenter and oxidase negative and its size ranges from 0.3-6.0 μm [2]. The phagocytosis is prevented by polysaccharide capsule which surrounds the cell wall. It also form capsule which is the virulence factor and most of their pathogenic strains found in hospitals are capsulated [118]. In *K. pneumoniae* 77 capsular (K) serotypes have been reported. Some of these serotypes cause life threatening diseases in both animals and humans [119]. Human intestinal flora also has *K. pneumoniae* as a commensal like *E. coli*. It is also found in water and soil [120]. It is known as a common opportunistic bacterium. It causes many nosocomial infections and about 5-7% of hospital acquired infections are caused by this bacterium [121]. The infections caused by *Klebsiella* spp. which are epidemic and endemic hospital acquired are the main causes of morbidity and mortality [122]. It causes respiratory tract infections like pneumonia, rhinoscleroma, sinusitis otitis and causes infections of the digestive tract like enteritis, appendicitis and cholecystitis which are related to the infections of urinary tract, genital tract, and the eyes [123]. Several studies have been presented from various parts of world that *K. pneumoniae* causes severe diseases in different settings like hospitals, intensive care, nursing homes, and neonatal wards [124]. Some strains of *K. pneumoniae* also become resistant to β -lactams and cephalosporins by producing ESBLs. It causes serious problems in the nosocomial infections in neonatal intensive care units [125].

1.12 Extended beta-lactamases:

In family enterobacteriaceae, the plasmid mediated β -lactamases e.g. TEM1, TEM2 and SHV1 are responsible for the resistance to penicillin but are sensitive to extended-spectrum cephalosporins. But in 1980s a new group of enzymes called extended β -lactamases were found in some strains of bacteria. These enzymes were responsible for

the resistance to expanded-spectrum cephalosporin. Such enzymes were produced by mutated TEM1 and SHV1 genes present on plasmid [126].

ESBLs are plasmid-mediated enzymes. These enzymes hydrolyze broad-spectrum β -lactams. The genes responsible for ESBLs production are transferred from one bacterium to another through plasmids. Other resistant genes for antibiotics like trimethoprim-sulfamethoxazole, aminoglycosides and fluoroquinolones are mostly co-transferred on plasmid having ESBLs genes. This co-transference of genes result in multiple drug resistant strains of bacteria which is big problem for therapeutics [127]. Drugs like carbapenems are used in treating infections caused by ESBL producers [128].

ESBL producing strains are spreading worldwide very rapidly. Different studies on ESBL from different parts of the world have been reported. But the prevalence of ESBL varies widely from city to city, institute to institute and country to country. This change in the different values of prevalence of ESBL is because of difficulties in detecting the ESBL producers because of inconsistencies in testing and reporting [129]. Some of the prevalence data of ESBL percentage of *E. coli* and *Klebsiella* spp. from different countries are given in Table1.1.

Table 1.1: Prevalence of ESBLs in *E. coli* and *Klebsiella* spp. in different countries.

Country	ESBL%		Reference
	<i>E. coli</i>	<i>Klebsiella</i>	
Pak	57.4	71.7	[130]
India	58.5	41.5	[131]
Iraq	-	78.8	[132]
Iran	-	59.2	[127]
Spain	6.6	-	[133]
Asia Specific	62	75	[134]
Turkey	15.3	-	[135]
Bangladesh	47.83	57.89	[136]
Korea	10.2	-	[137]
France	48.5	-	[138]

1.13 Carbapenemase

Carbapenemase Producing Enterobacteriaceae (CPE) is the most prevalent problem in many countries of the world nowadays which is a major public health associated issue. Many members of Enterobacteriaceae are rapidly becoming antibiotic resistant to all available drugs. Even the last resort drugs such carbapenems which are used in life threatening diseases like bacteremia, septicemia and pyelonephritis which are caused by the most resistant strains are becoming less effective against these dangerous pathogens. These multidrug resistant pathogens are producing ESBLs and carbapenemases which make the β -lactams and carbapenems less effective in curing life threatening infections. There are many reports from different parts of the world on carbapenemase producing strains, which are considered as dangerous pathogen in hospital settings and are the major public health related problem [139].

Most of the β -lactams i.e. cephalosporins, monobactams, carbapenems and penicillin can be decomposed by carbapenemases [140]. Along with this most of CPE bacteria have also other resistant genes which are responsible for the resistance to other classes of antimicrobial drugs. These genes make these organisms multidrug resistant. Very small numbers of antibiotics are resistant to these CPE which are tigecycline, fosfomycin, polymyxins and nitrofurantoin. But there is no clinical evidence of their effectiveness against CPE. However, many studies showed clinical failure of these drugs and even resistant strains to these antibiotics [141]. There are three main molecular classes of β -lactamase (A, B and D) which lies in the heading of carbapenemases. The carbapenemases A and D have serine at their active site [140]. The plasmid encoded KPC is the most prevalent Class A carbapenemase. There are many variants of KPC ranging

from KPC2 to KPC13. These all variants are different from each other by mutated amino acid. These types of carbapenemase are very common in USA and also in other parts of the world [141].

Another important class of carbapenemase belongs to class B, which is also called metallo- β -lactamases (MBLs). These carbapenemases depends on zinc at their active site. Actually these were reported first in *Pseudomonas* spp. and *Acinetobacter* spp. But now they are also reported in Enterobacteriaceae [142]. New Delhi metallo- β -lactamase-1 (NDM-1) is the new type of carbapenemase. It is thought to be more prevalent in India. It is reported in people travelled from India and Pakistan. Such strains are studied in some cases of returned individuals from India and Pakistan, from indigenous cases or from infected people, or by secondary transmission in country. Another variant NDM-2 carbapenemase has been reported in North Africa [143].

The third Class D carbapenemases are the oxacillinases which include OXA-type carbapenemases. These are mostly found in *Acinetobacter* spp. and *P. aeruginosa*. In short the increasing frequencies of these strains occur by moving of people from one place to another place. The transmission between patients and hospital facilities is occurring locally and can also across the borders [144].

Aims and Objectives:

The aims of this study were to investigate:

1. The antibiotic resistance in the clinical isolates of two hospitals of Peshawar.
2. ESBL detection in *E. coli* and *Klebsiella* spp.
3. Carbapenemase detection in *E. coli* and *Klebsiella* spp.
4. MBL detection in *E. coli* and *P. aeruginosa*.
5. Phenotypic identification of MRSA
6. Enterotoxin gene detection by using PCR in *S. aureus*.

Chapter 2

Materials and Methods

All bacterial clinical isolates included in this study were collected from Khyber Teaching Hospital (KTH) Lady Reading Hospital (LRH) Peshawar Pakistan from 2009 to 2012. KTH and LRH are the two tertiary hospitals with a capacity of 1200 and 1500 beds, respectively. Both of these two hospitals serve as the major health care facilities in public sector for Peshawar and near cities, towns and villages of Khyber Pakhtunkhwa. In total 389 strains were collected from these two hospitals. The biochemical tests for the identification of all these strains were done at the Centre of Biotechnology and Microbiology, University of Peshawar, Pakistan. Some of the following experiments were done using these isolates at University of Bradford, Bradford, UK.

The study comprised of the following steps:

1. Collection of specimens
2. Isolation of pathogenic bacteria from the specimen
3. Identification of pathogenic bacteria from the specimens
4. Preservation of isolates
5. Antimicrobial susceptibility testing
6. Confirmation of MRSA
7. Detection of Toxic shock syndrome gene (*tsst*), enterotoxin genes in *S. aureus* and Panton Valentine Leukocidin (*pvl*).
8. Detection of non-specific Efflux pump in *S. aureus*
9. Detection of Extended spectrum- β -lactamases enzymes in Enterobacteriaceae.
10. Detection of Metallo- β -lactamases in Enterobacteriaceae.

2.1 Collection of specimen

The microbiology section of both hospitals provided the sterile containers for specimen collection. These were collected as:

1. Mid stream urine was collected in sterile containers.
2. In case of pus specimens sterile syringes were used for its collection while for open lesions, sterile cotton swabs were used. In this study only those specimens were included which were received within 15 minutes of its collection to the laboratory.

2.1.1 Isolation of pathogenic bacteria

Pathogenic bacteria were isolated from the specimens by inoculating them onto different media. Blood agar, MaConkey agar (Oxoid, England) agar, Eosin methylene blue agar (Merk) and Cystine lactose electrolyte deficient agar (CLED) (Oxoid, England) were used for the urine and pus samples. All the plates were observed for bacterial growth after 24 hours incubation at 37°C in aerobic conditions. The well grown bacterial colonies were then picked for identification.

Before using the prepared media were autoclaved for 15 minutes at 121°C and pressure of 15 lbs/inch² for sterilization. After autoclaving the media were allowed to cool down up to 50-55°C and were poured aseptically in petri dishes or test tubes which were then labeled with date and name of medium and stored at 4°C and were used in a week.

2.1.2 Bacterial isolates identification

The well grown and purely isolated bacterial colonies were then identified by their morphological appearance, Gram staining and different biochemical tests. The identification steps using biochemical profile are described below [145].

2.1.3 Gram staining

Gram staining was done for the identification of bacterial pathogens isolated from the clinical samples by their morphology and Gram staining reaction (Gram positive or Gram negative).

An isolated colony was picked from bacterial culture and was emulsified in a drop of distilled water on slide to make a thin layer of bacteria (smear). The smear was dried and fixed by gentle heat. The aim of fixation was to prevent smears being washed from slides during staining. Staining was started after the smear was cooled down.

2.1.3.1 Primary staining

- a. Crystal violet stain was first applied on fixed smear for 30-60 seconds and then rinsed with clean water.
- b. Gram's iodine (used as Mordant): Gram's iodine solution was then applied for 30-60sec on the fixed smear and washed with clean water.
- c. Decolorization: For decolorization of the smear, it was washed with acetone or alcohol for 2-3 seconds and then washed with gentle stream of water.
- d. Counter staining (secondary staining): Safranin was used as a secondary stain which was applied on the smear for 2 min and then washed with water.

The slide was then allowed to dry in air and examined under microscope with 100X objective lens for cell morphology.

2.1.4 Biochemical test for identification of *Staphylococcus aureus*

2.1.4.1 Catalase test

Catalase test was performed to differentiate, the catalase producing bacteria such as staphylococci and non-catalase producing bacteria such as streptococci. The catalase

enzyme acts as a catalyst which breaks down the hydrogen peroxide into oxygen and water. Two or three isolated colonies of the test organism were added to 3% hydrogen peroxide on slide and were observed for the immediate production of bubbles. The organism was considered as a catalase producer after in contact with 3% hydrogen peroxide, producing oxygen bubbles.

2.1.4.2 Coagulase test

This biochemical test was performed to differentiate between coagulase enzyme producers, like *S. aureus* from the non-coagulase producers. The coagulase enzyme clots the blood plasma by converting fibrinogen to fibrin, which causes the organism to agglutinate in small amount of plasma. The blood plasma was added to drop of saline on slide and then the isolated colonies of test organism were emulsified in the plasma. The organism was considered as positive when agglutination within five seconds was observed.

2.1.4.3 Mannitol Salt Agar test

Mannitol salt agar (MSA) is a selective medium. It has great nutritional value for gram positive bacteria including staphylococci. It contains peptones and beef extract. These ingredients provide important growth factors i.e. nitrogen, sulfur carbon and other nutrients. It also contains 7.5% NaCl which prevents the growth of bacteria other than staphylococci. Staphylococci also produce acid after growing on this medium which turns its colour from red to yellow.

2.1.4.5 DNase test

This test was used for the identification of *S. aureus*. This bacterium produces deoxyribonuclease (DNAase) enzyme which is the distinctive property of staphylococci.

Deoxyribonucleic acid (DNA) is hydrolyzed by deoxyribonuclease. The test isolates were inoculated on this medium and were incubated at 37°C for 24hrs. After incubation, the medium with bacterial growth was flooded with weak HCl. The clear zone around the DNAase producing colonies within 5 mins gave the positive test result. This clear zone was because of the hydrolysis of DNA.

2.1.3 Biochemical tests for Gram negative bacteria

2.1.3.1 Oxidase test

This biochemical test was used to identify *P. aeruginosa* which produces the enzyme cytochrome oxidase. A few drops of oxidase reagent (dimethyl-p-phenylenediamine hydrochloride) were added on piece of filter paper. A well isolated colony of the test organism was put on the filter paper with the help of a tooth pick. If the organism is oxidase- producing, the reagent will be oxidized turning color of filter paper to deep purple within 5-10 seconds. Purple color produced late was not significant for interpretation [145].

2.1.3.2 Citrate test

Citrate test depends on the utilization of citrate by the bacteria as a carbon source. Citrate is broken down into organic acid and carbon dioxide by bacteria. Sodium bicarbonate is formed by the combination of carbon dioxide and sodium from the conjugate base salt which results in change of color from green to blue. Slants of Simmons's citrate (Oxoid, England) were prepared using test tubes and were stored at 4°C. The test organism was first inoculated on slant by using a straight rod and then the butt was stabbed. The test tubes were then incubated on 37°C for 18-24 hrs. The bright blue color showed the positive test result while no color change was considered as negative test result [145].

2.1.3.3 Methyl Red Voges-proskauer (MR-VP) test

There are two groups of enteric bacteria on the basis of fermentation i.e mixed acid fermenters and 2, 3 butanediol fermenters. Large amounts of acids are produced by mixed acid fermenters group while acetoin (acetyl methylcarbinol) are produced by the butanediol fermenters group. Both these tests should be performed at the same time and same media. This test is called MR-VP test. The interpretation of the test depends on the addition of methyl red. If the organic acid is produced by the test organism, by adding methyl red, the medium will turn red (acidic medium) indicating positive result while medium turning yellow (pH increasing to 6 or above), shows negative test result. The negative result is due to the production of ethanol and acetoin.

The test organism was inoculated in two MR-VP broth tubes and was allowed to incubate for 48 hrs at 37°C. In each tube after incubation 5ml of methyl red was added as a indicator. In one tube 5% α -naphthol dissolved in absolute alcohol (VP1) and 2-3 drops of 40% Potassium hydroxide solution (VP2) were added in another tube and shaken gently and observed for the color change [145].

2.1.3.4 Triple sugar iron test (TSI)

The medium used for this test was, triple sugar iron agar. The main ingredients of this medium are ferrous sulfate and the three sugars, glucose, lactose and sucrose. It has red color. Slants of this medium were prepared. The test interpretation was carried out by change in colour of the medium after inoculation and incubation. The test organisms like *E. coli*, *K. pneumoniae* turned the color of medium yellow (acidic) while in the case of *P. aeruginosa* no color change was observed.

2.1.3.5 Indole test

This test is used for the detection of enzyme tryptophanase in bacteria. For this test tryptone water broth and Kovac's reagent containing 4(p)-dimethylaminobenzaldehyde were used. This reagent was used for the detection of indole production in the tested organism cultured in tryptophan containing medium. This reagent reacts with indole forming a red color ring on the surface. In a test tube containing 3ml of sterile tryptone water was inoculated with test organism and was incubated at 37°C for 48 hrs. After incubation 0.5ml of Kovac's reagent was added to the tube, was observed for the red ring at surface within 10 mins. Formation of red ring was indication of positive test result.

2.1.3.6 Urease test

The test organism should be cultured in a medium containing urea and phenol red indicator. The bacteria producing urease enzyme will break down the urea into ammonia and carbon dioxide. Ammonia makes the medium alkaline and gives red color to the medium [145].

All the test isolates were inoculated on the medium containing urea and the indicator phenol red and were incubated at 37°C for 2-12 hrs. The pink color was considered as a positive result.

2.2 Preservation of Isolates

The isolates were sub cultured on nutrient agar plates every week and placed at 4°C for short term preservation. For long term preservation more than a month, bacterial isolates were preserved on nutrient agar (Oxoid, England) slants and maintained at 4°C because ultra-low temperature freezers like, -70°C was not available in Center of Biotechnology and Microbiology University of Peshawar, Pakistan. While in Bradford, UK where -70°C

facility was available, where isolates were preserved in -70°C freezer. The samples were preserved in 15% glycerol tryptic soya broth (TSB) (Oxoid, UK) to avoid breaking of bacterial cells due to formation of ice crystals at ultra-low temperature. The samples were grown overnight on tryptic soya agar (TSA) (Oxoid, UK). The growth from the plates was transferred to cryovials by wet sterile swabs aseptically and then placed in the freezer. For re growth of bacteria, a loop full suspension from cryovials was streaked on the dry surface of TSA plate from frozen stock cultures, streaked out for single colonies and incubated at 37°C overnight. To reduce stress on organisms care was taken not to repeatedly freeze and thaw the bacteria, by use of an ice bucket and only having a few strains out the freezer at a time. Plates were checked the following day for pure growth. Weak growth or mixed culture was repeated again on TSA. Subcultures were made on TSA each week, and plates were maintained at 4°C in the interim period.

Table: 2.1. Standard strains used as positive controls in this study

Bacterial strains	Stranded No ATCC/NCTC
Oxford Staph	ATCC 9144
<i>Sea, Seb</i>	NCTC 10657
<i>Sec</i>	NCTC 10655
<i>Sed</i>	NCTC 10656
<i>tsst</i>	NCTC 11962
<i>pvl</i>	MSSA 62 Collected from Peshawar in 2005
Big MRSA	Collected in Horrogate Hospital UK.
<i>K. pneumoniae</i>	NCTC 700603
<i>E. coli</i>	NCTC 10418
<i>P. aeruginosa</i>	NCTC 10662
<i>B. capacia</i>	NCTC 10744

2.3 Antimicrobial Susceptibility Testing

The antimicrobial susceptibility test was performed for all identified bacterial isolates by using:

- a. Disc Diffusion (Bauer- Kirby 1966) Susceptibility test method
- b. Minimum inhibitory concentration (MIC) method

2.3.1 Antimicrobial Susceptibility testing disc diffusion method

This test was performed for all the bacterial isolates used in this study. Muller Hinton agar (MHA) was used as a growth medium. This medium was prepared according to the manufacturer's instructions. All the antibiotic discs were purchased from Oxoid, UK. The antibiotic discs were placed on the MHA plates which were already inoculated with test organism. The growth of the microorganism is inhibited by the diffusion of antibiotic into the agar.

2.1.2 Inoculum preparation

The inoculum for the disc diffusion method was prepared by using TSB (Oxoid, UK) which was made according to the manufacturer's instructions. The screw capped test tubes were poured with 5ml of broth medium and were sterilized by autoclaving at 121°C for 15 min at 15psi. The medium was allowed to cool down up to room temperature and an isolated colony of the tested organism was inoculated in the sterilized test tube. This test tube was incubated for 2-6 hours at 35°C. The turbidity of broth cultures were adjusted according to 0.5 McFarland standards by adding sterile saline against a white background with contrasting black lines.

The bacterial suspension was then applied on the MHA plates by using a sterile cotton swab by dipping in the bacterial culture. The excess culture was removed by turning the

swab against the inner side of test tube. The inoculum was spread evenly by swabbing back and forth on MHA plate in three directions for even lawn. The plate was then left for 15 mins for drying. The antibiotics discs of the specific potencies were then placed with help of sterile syringe needle. The plates were then allowed for incubation and were placed in inverted position. The zone of inhibition diameters were then measured with a ruler after 18 hrs incubation at 37°C.

2.1.3 Determination of minimum inhibitory concentration (MIC's) by agar dilution method

The agar dilution method (CLSI, M100-S20) [146] was used to determine inhibitory concentration. Serial two fold dilutions of antibiotics were prepared and incorporated into molten agar at around 50°C. Antibiotics were obtained from Sigma–Aldrich along with the information such as potency, manufacturing/expiry dates.

2.1.4 Inoculum preparation

The inoculum used for MIC was prepared by taking two or three well isolated colonies of the test organism in plastic universals containing 5ml of distal water. In each well of sterile plate; only 0.5 microlitre of the bacterial inoculum was transferred with the help of micropipette. The plate was then placed on its proper place in the multipoint inculcator. A serial dilution of antibiotics was prepared depending upon antimicrobial agent and species in sterile universals. Muller Hinton agar was used as a growth medium. Nineteen ml of agar was made in a universal, and steamed twice in a streamer to avoid loss of water, for a couple of hours. After steaming these universals were shifted to the water bath to avoid solidification. After cooling to 50°C, 1ml of antibiotic solution was added to the agar mixed and then poured in petri plate. After solidification of media, the plates

were dried in a drier for further use. A 37 pin head Denley–Tech Multipoint Inoculator (Denly Industries, Sussex, UK) was used to deliver approximately 10 µl of each isolate to the surface of each plate. After inoculation, plates were incubated at 37 °C for 18 hrs. The plates were examined and MIC's determined.

MIC is the lowest concentration of antibiotic inhibiting the visible growth of microorganism. The MIC of the specific antibiotic for the tested organism was considered when no growth was observed on the inoculum spot.

Table: 2.2 List of antimicrobials used in this study and their details

S.NO	Antimicrobial Agent	Antibiotic Group	Code	Disc potency (µg)
1	Amoxicillin+Clavulanic acid	Penicillin	AMC	30
2	Ampicillin	Penicillin	AMP	30
3	Cephradine	Cephalosporin	CE	30
4	Cefaclor	Cephalosporin	CEC	30
5	Ciproflaxacin	Fluoroquinolones	CIP	5
6	Enoxacin	Fluoroquinolones	ENX	30
7	Ethromycin	Macrolide	E	15
8	Imipenem	Carbapenem	IMP	10
9	Gentamicin	Aminoglycoside	CN	10
10	Doxycycline	Tetracycline	DO	30
11	Trimethoprim +Sulphamethoxazole	Sulpha Drug	STX	1.25/23.75
12	Vancomycin	Glycopeptide	VA	30
13	Ceftazidime	Cephalosporin	CAZ	30
14	Aztreonam	Monobactam	ATM	30
15	Cefoxitin	Cephalosporin	FOX	30/10
16	Ceftriaxone	Cephalosporin	CRO	30
17	Linezolid	Oxazolidinones	LZD	30
20	Norflaxacin	Quinolones	NOR	10
21	Kanamycin	Aminoglycosides	K	30
22	Amikacin	Aminoglycosides	AK	30
23	Gentamicine	Aminoglycosides	CN	10
24	Oxacillin	Penicillin	OX	1
25	Minocycline	Tetracyclines	MH	30
26	Neomycine	Aminoglycoside	N	10
27	Mupiracin	Monoxycarbolic acid	MUP	5
28	Rifampicin	Rifampicin	RD	2
29	Chloramphenicol	Chloramphenicol	C	10
30	Tetracycline	Tetracycline	TE	10
31	Clindamycin	Lincosamide	DA	2
32	Pencillin G	Penicillin	PG	1
33	Fusidic Acid		FD	10
34	Amoxiellin	Penicillin	AML	30

2.4 Detection of Extended spectrum β -lactamases (ESBLs) in Enterobacteriaceae (*E. coli* and *K. pneumoniae*) by Brilliance Chromogenic agar (Oxoid, UK)

Chromogenic Brilliance ESBL (Oxoid, UK) agar plates were used for the detection of ESBL producing Gram negative isolates i.e. *E. coli* and *K. pneumoniae*. The main ingredient of this medium is cefpodoxime along with other antimicrobial drugs which prevent the growth of non ESBL producers. *K. pneumoniae* and *E. coli* are two most important ESBL producers which can be phenotypically identified by this medium. The chromogenic material of this medium targets the enzymes which are expressed by both ESBL producers, *K. pneumoniae* and *E. coli*. The enzyme expressed by *K. pneumoniae* galactosidase gives green color colonies and *E. coli* expresses galactosidase and glucuronidase which result in blue color colonies.

Few well isolated colonies of the of the test organism were suspended in sterile distal water and a loopful of 1 μ l of a 0.5 McFarland standard of that suspension was inoculated on the screening plates. *K. pneumoniae* NCTC 700603 and *E. coli* NCTC were used as positive and negative controls respectively. The plates were incubated at 37°C for 18-24 hours. With no growth plates were re-incubated for additional 18-24 hours.

2.5 Detection of Carbapenemases in Enterobacteriaceae by using Carbapenem resistant Enterobacteriaceae (CRE) Brilliance media (Oxoid, UK)

All strains of Gram negative bacteria including *E. coli* and *K. pneumoniae* were refreshed on TSA. Carbapenemase producing strains were detected by using CRE media. Before inoculation the CRE plates (Oxoid, UK) were kept at room temperature. All *E. coli* and *K. pneumoniae* strains were inoculated on this media according to the manufacturer's guidelines and incubated for 18 hrs at 37°C. The colonies appearing pale pink and blue in

color were considered as *E. coli* and *K. pneumoniae* carbapenemase producers respectively.

2.6 Phenotypic detection of Metallo- β -Lactamases (MBL) in Enterobacteriaceae by using Combined Disc test of imipenem and imipenem-Ethylenediaminetetraacetic acid (IMP and IMP-EDTA) Combined disc test (CDT) TEST

CDT was performed according to Galani et al., [147] by using only two discs imipenem 10 μ g (Oxoid, UK) and imipenem 10 μ g impregnated with 0.5 M EDTA (Sigma, UK). The 0.5M EDTA solution was prepared by dissolving 18.61g of disodium EDTA in 100ml of distilled water and pH was adjusted to 8.0 by sodium hydroxide (NaOH, Sigma Aldrich, UK) and autoclaved. Ten microliter of 0.5M EDTA was added to one disc of imipenem (10 μ g). The discs were placed on MHA plates inoculated with test organism. The inhibition zone of imipenem and imipenem/EDTA discs were compared after 18 hrs incubation at 35°C. The positive result for MBL, the difference of zone diameter of IMP disc containing EDTA compared to the IMP alone should be ≥ 7 mm.

2.7 Phenotypic identification of *Pseudomonas aeruginosa*

P. aeruginosa was identified by using Pseudomonas Cetrimide Agar (Oxoid, UK). This media is specific for *P. aeruginosa*. Cetrimide is a quaternary ammonium salt. This salt behaves as a cationic detergent that minimizes the surface tension at contact point. It precipitates and denatures the proteins of bacterial membrane. It inhibits the growth of many microbes including *Pseudomonas* spp. except *P. aeruginosa*. All the strains along with positive control strain *P. aeruginosa* NCTC 10662 were inoculated on Pseudomonas Cetrimide agar plates and were incubated at 37°C for 18 hrs. The strains which gave a green color on this media were considered *P. aeruginosa*.

2.8 Amplification of 16S ribosomal DNA (16S rDNA) gene in *P. aeruginosa* by polymerase chain reaction (PCR) for species specific

All the *Pseudomonas* strains were screened for *P. aeruginosa* species specific (16S rDNA) gene by PCR.

2.9 Extraction of DNA from Gram negative bacteria

DNA template preparation was performed as follows. The 2-3 well isolated colonies were suspended in distilled water and boiled for 5-10 mins and then centrifuged for 6 mins, the supernatant was used as source of template for amplification. Each reaction consisted of 20µl mixture and contained

- 1µl DNA template
- 1µl of each reverse and forward primers
- 7µl nuclease free water
- 10µl Taq polymerase master mix

P. aeruginosa NCTC 10662 was used as positive control while master mix without DNA template and *B. capaciae* NCTC 10744 was used as negative control. The screening for 16S rDNA was carried by PCR with the specific primers given in Table 2.3. The DNA amplification described by Spilker *et al.*, [148] was carried out in a thermocycler PCR Sprint Thermocycler (Thermo Electron Corporation UK) with the following thermal cycling profile. An initial denaturation for 2 minutes at 95°C, 25 cycles were completed each consisting of 20 seconds at 94°C, 20 seconds at annealing temperature 58°C and 40 seconds at 72°C. A final extension of 1 minute at 72°C was applied. The primers used in this study were purchased from Sigma, UK.

Table 2.3 Primers and conditions used for the detection of (16S rDNA) gene in *P.aeruginosa*

Gene	Primer	Nucleotide Sequence (5'-3')	PCR product size (bp)	References	Annealing temperature
16S rDNA	PA-SS-F	GGGGGATCTTCGGACCTTCA	956	[148]	20s at 58°C
	PA-SS-R	TCCTTAGAGTGCCCACCCG			

2.10 Buffer used in study for electrophoresis

10x TAE

TAE (Tris-Acetic acid-EDTA) buffer was prepared as

Chemical	Per/Litre	Final Construction
Tris base	48.44g	40mM
EDTA	7.44g	2mM

1x TE buffer was prepared

Chemical	Per/Litre	Final Construction
1M tris. HCl pH 8.0	10mL	10mM
0.5 EDTA pH 8.0	2mL	1mM

Net Buffer for acromopeptidase

100xTE buffer was mixed with 99mL PCR grade water and 200 μ L of 5M NaCl was added. The pH of buffer was adjusted to 7.9 with glacial acetic acid and then the volume was raised up to 1000ml with distilled water. Agarose gel was prepared in 1xTA buffer and this concentration of buffer was also used as running buffer.

2.11 Agarose gel preparation

PCR amplified products were resolved in 1.2% agarose gel (Invitrogen, UK) in 100ml 1x TAE buffer was dissolved and heated in a microwave (Tecnolec, Superwave 800) for 2-3 mins. Ethidium bromide (EtBr) was added in gel after cooling it down to 50°C. The melted warm agarose was then carefully poured into the centre of the running tray avoiding formation of any bubbles. A comb was added and it was allowed to set for about 40 minutes then comb was removed and enough amount of running buffer was added to fill the gel tank (Gene flow mini Electrophoresis tank, UK) and just cover the gel. Twelve

micro liters of each PCR reaction was loaded in each well. The gel was run in an electrophoresis tank for approximately 60 min at 100V. A 100 base pair (bp) DNA ladder (Norgen 100bp, UK) was run alongside the PCR products as a size marker. After running the gel, the DNA was then visualized under ultraviolet light in a Gel Documentation system (Uvitec) and photographed.

2.12 Phenotypic detection of MRSA by using three different type of methods

Three different methods were compared for the identification of MRSA and MSSA. The Oxford *Staph* ACTC 9144 was used as a negative control and MRSA BIG 0047 (collected at Harrogate District hospital, Harrogate UK, 2007) was used as a positive control. In total, 133 clinical isolates of *S. aureus* were tested.

All the *S. aureus* isolates were tested by using OX 1 μ g disc on Muller Hinton Agar (MHA) without the use of additional sodium chloride (NaCl). A suspension of each *S. aureus* isolate was prepared to a 0.5 McFarland standard. All the strains were inoculated on MHA and were incubated for 24 hrs at 30°C in air. According to CLSI (M100-S20) guidelines [146], if the zone diameter was ≤ 10 mm, the test strain was considered as MRSA. For FOX 30 μ g discs were used on MHA and incubated for 16-18 hrs at 33-35°C. If the zone diameter was ≤ 21 , then the test strain was considered as MRSA.

By using British Society of Antimicrobial Chemotherapy (BSAC) method [149], all the 133 clinical isolates of *S. aureus* were tested by using FOX 10 μ g on Iso-Sensitest Agar (ISA) (Oxoid, UK) and OX 1 μ g on MHA (Oxoid, UK) supplemented with 2% NaCl. All the strains were inoculated on ISA for FOX and on MHA for OX and incubated at 35°C and 30°C each for 18 hrs, respectively. According to BSAC recommendations if the zone

diameter was ≤ 21 mm for FOX and ≤ 14 mm for OX, then the test isolate was considered as MRSA.

BrillianceTM MRSA Agar (Oxoid, UK) has been used for the phenotypic identification of MRSA. It is an opaque medium which contains new chromogen. Many staphylococci including *S. aureus* have phosphatase activity which gives blue color on this chromogenic medium. The growth of MSSA is inhibited by the cocktail of antibiotics present in this medium. It also contains many compounds that inhibit the phosphatase activity in other Staphylococci, thus increasing the level of sensitivity and specificity. BrillianceTM MRSA agar is a medium which is supplied as pre-poured culture plates. It is used for the identification of MRSA in human specimens.

All the 133 clinical isolates of *S. aureus* were tested on this medium. Before inoculation, the medium was allowed to warm up to room temperature and dried up. The agar screening plates were inoculated directly by picking isolated colonies of test isolate and incubated for 18-24 hrs at 37°C. The denim blue color colonies were reported positive for MRSA.

2.13 Biocides assay

Biocides including benzalkonium chloride, cetylpyridinium chloride, chlorhexidine, cetrimide, PCMX and silver nitrate (AgNO₃) were obtained from Sigma-Aldrich (UK). Copper sulphate (CuSO₄) was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Triclosan and triclorcarban were gifts from Pz Cussons (Stockport, Cheshire, UK). Ethidium bromide was purchased from Sigma-Aldrich (UK). *S. aureus*, ATCC 9144 was used as a control strain. Biocides used in this study, their diluents and concentrations are listed in Table 2.4.

2.14 Determination of Minimum inhibitory concentration (MICs) for Biocides

Iso-sensitest agar was used for the determination of MICs of biocides in the agar incorporation method, using BSAC guidelines [149]. A multipoint inoculator (Denley Industries, Sussex, UK) was used to dispense approximately 1µl of each bacterial suspension to the plate. Different concentrations were used for different biocides. The ranges of concentrations are given in the Table 2.4. The concentrations for AgNO₃ and CuSO₄ were used in 0.05-1.0mM and 0.1-10.0mM respectively, while µg/ml concentrations were used for the rest of biocides.

2.15 Ethidium Bromide (EtBr) Agar Screening Method

The isolates expressing MICs on the extreme of upper and lower sides for quaternary ammonium compounds (QACs) were tested using a method described by Martin et al., [150]. All the isolates were streaked out on the TSA plates containing the EtBr with a concentration range from 0.5-30.0µL. All the inoculated plates were incubated for 18-24 hrs at 37°C in air. After incubation all the plates were viewed under the UV light for the florescence.

Table 2.4: Biocides, diluents and ranges of concentrations used in the study.

Biocides	Diluents	Concentrations ($\mu\text{g/ml}$)
Benzalkonium Chloride	Water	0.1-10.0
Cetrimide	Water	0.05-100.0
Cetylpyridinium Chloride	Water	1.0 -100.
Triclosan	Ethanol	0.05-5.0
Chlorhexidine	Water	0.1-20.0
PCMX	Ethanol	0.05-100.0
Trichlorocarban	Acetone	0.005-5.0

2.16 DNA extraction from *Staphylococcus aureus*

DNA from *Staphylococci* was extracted by using achromopeptidase (Sigma, Germany) method. Achromopeptidase stock solution was prepared in Tris-EDTA (TE) buffer. The crude powder containing 100,000 units of the enzyme was suspended in 10 ml of 1x TE buffer, and was properly vortexed. The solution was then stored in the form small aliquots in sterile tubes and stored at -20°C for further use.

Two or three bacterial colonies were emulsified in a solution containing 50µl NET (10mMNaCl +1mMEDTA +10mMTris) buffer and vortexed for 1-2 mins and then 10µl of achromopeptidase solution was added. This mixture was then incubated at 50°C for 20mins and checked for lysis. The mixture was then centrifuged in centrifuge 1600 rpm (Sigma 1-4 Calserve Ltd, UK). After incubation, the lysate was diluted 10-15 times with NET buffer. It was then stored at 4°C and used within a month.

2.17 Detection of enterotoxin and toxic shock syndrome toxin genes

Detection of enterotoxin genes *sea*, *seb*, *sec*, *sed* and *tsst* in *S. aureus* were done by PCR using the sets of forward (F) and reverse (R) primers listed in table 2.5. *S. aureus* reference strains used for control were NCTC 10657 (*sea* and *seb*), NCTC 10655 (*sec*) NCTC 10656 (*sed*) and NCTC 11962 (*tsst*). The reaction mixture (20µl) for the PCR procedure for each gene contained 1µl of each primer (F+R), 10 µl of *Taq Polymerase* master mix and 7µl of nuclease free water (Fermentas, UK) and 1µl of DNA template were used. Amplification of DNA was carried out in a thermo cycler PX2 thermal cycler (Thermo Electron Corporation). The amplification cycles described by Johnson et. al., [151] were used as following: denaturation for 2 min at 94°C, annealing of primers for 2

min at 55°C, and extension for 1 min at 72°C. The PCR protocol was used for each gene by using only one primer pair in each tube.

2.18 Detection of Panton Valentine Leukocidin (*pvl*) gene

All *S. aureus* isolates were screened for *pvl* gene. DNA was extracted by achromopeptidase as described above. The PCR reaction was 20µl as used for the other toxin genes. The DNA amplification described by Lina et al., [152] was used as following 30 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C and 1 min of extension at 72°C. After amplification, PCR products were resolved by electrophoresis through 1.5% agarose gel. The strain *S. aureus* (MSSA 62 collected from Pakistan hospital in 2005-06) was used as a positive control.

Table 2.5: Primers and conditions used for the detection of *S. aureus* toxin genes.

Gene	Primer	Nucleotide Sequence (5'-3')	PCR product size (bp)	References	Annealing temperature
<i>sea</i>	SEA1	TTGGAAACGGTTAAAACGAA	120	[151].	1 min at 55°C
	SEA 2	GAACCTTCCCATCAAAAACA			
<i>seb</i>	SEB1	TCGCATCAAACGACAAACG	478	[151]	1 min at 55°C
	SEB 2	GCAGGTACTCTATAAGTGCC			
<i>sec</i>	SEC1	GACATAAAAGCTAGGAATT	275	[151]	1 min at 55°C
	SEC 2	AAATCGGATTAACATTATCC			
<i>sed</i>	SED 1	CTAGTTTGGTAATATCTCCT	317	[151]	1 min at 55°C
	SED 2	TAATGCTATATCTTATAGGG			
<i>tsst</i>	TSST1	ATGGCAGCATCAGETTGATA	350	[151]	1 min at 55°C
	TSST2	TTCCAATAACCA'CCCGTTT			
<i>pvl</i>	PVL1	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	[152]	30s at 55°C
	PVL2	GCATCAACTGTATTGGATAGCAAAAGC			

Initial denaturation for 4 min at 95°C followed by 34 cycles of denaturation (1 min at 94°C), annealing of primer (see details above) and primer extension (1min at 72°C)

Table 2.6 Chemicals used in different experiment

Chemical name	Brand	Typical composition g/L	
Muller Hinton Agar	CM 0337 (Oxoid)	Beef, dehydrated infusion	300.0
		Cesein hydrolysate	17.5
		Starch	1.5,
		Agar	17.0
		pH7.01 at 25°C (38g/L)	
Iso-sensitest Broth	CM 0473 (Oxoid)	pH 7.4± 0.2 at 25°C 23.4g in 12 litres	
Mannitol Salt Agar	CM 0085 (Oxoid)	Lab-lemco powder	1.0
		Peptone	10.0
		Mannitol	10.0
		Sodiumchloride	75.0
		Phenored	0.025
		Agar	15.0
		111 gram / L	
Blood Agar Base	Merk UM804586-722	40g/L	
Eosin methylene blue agar	Merk UM984550 823	Peptones	10.0
		Di-potassium hydrogen	
		Phosphase	2.0.
		Lactose	5.0
		Saccharose	5.0
		Eosin gelblich	0.4
		Methylen blue	0.07
		Agar	13.5
		36g/L, pH7.1 25 °C	
Nutrient Agar	Merk UM984550 823	Peptone from meat	5.0
		Meat extract	3.0
		Agar	12.0

		20g/L pH 7.0 at 25°C	
Triple Sugar Iron Agar	LAB53	Beef extract	3.0
		Yeast extract	3.0
		Blanced peptone	1.20.0
		NaCl	5.0
		Lactose	10.0
		Sucrose	10.0
		Glucose	1.0
		Fetric citrate	0.3
		Agar	2.12.
		Sodium thiosulphate	0.3
		Phenol Red	0.025.
		65g/L pH 7.1	
Simmons-citrate Agar	Merk Um667501/634	Ammonium	
		dihydrophosphaste	1.0
		Di-potassium hydrogen	
		phosphate	1.0
		Natrimucholond	5.0
		Natrimuctirat	2.0
		Magnesimsulfate	0.2
		Bromthymoblao	0.08
		Agar	13.0
		22.55g/L	
		pH 6.6 at 25°C	
MacConkay Agar	Oxoid	Peptone	20.0
		Lactose	10.0
		Bile salts	5.0

		NaCl	5.0
		Neutral red	0.075
		Agar	12.0
		pH 7.4+0.2 at 25°C	
Tryptone Water	Merk UM076659 922	Peptone from casein	10.0
		NaCl	5.0
		15g/L	
		pH 7.3+0.225°C	
DNAes Test Ager	Merk UM997949 827	Trypose	20.0
		Desoxy ribonucleisave	20.0
		Agar	15.0
		42g/L pH 7.3	
Urea Agar Base	CM053(Oxoid)	Peptone	1.0
		Glucose	1.0
		Sodium chloride	5.0
		Di-Sodium phosphate	1.2
		Potassium dihydrogen phosphate	0.8
		Phenol Red	0.012
		Agar	15.0
		2.4g/95mL	
MR-VP-Broth	Merk UM901712 744	Peptone from meat	7.0
		D (+) Glucose	5.0
		Phosphate buffer	5.0
		g/L pH 6.9±.2	
Kovacs Indole reagent	Merk UN2920		
100mL			
Pseudomonas certramide	CM 0579 (Oxide)	Gelatin peptene	20.0

Agar		Magnesium chloride	1.4
		Potassium sulphate	10.0
		Cetrimide	0.3
		Agar	13.6 5
		45.3g/L	
Isosensitest agar	CM047 (Oxide)	31.4g/L	
Trytone Soya Broth	CM01298 (Oxide)	Pancreatic digest of casein	17.0
		Paplic digest of soya bean meal	3.0
		sodium chloride	5.0
		Pibasicpotassium phosphate	2.5
		glucose	2.5
		30g/L	
Trisma Hcl	Sigma Batch 057k54041		
NaOH	Sigma CAS 1310-73-2	Trizma base Lot:	
Trizma Base	Sigma 090m54101v		
EDTA dehydrate disodium	Simga Batch 095K0204		
Norgen PCR 100 bp DNA	Norgen Cat NO. 11300		
ladder			

Chapter 3

Results

Bacterial isolates included in this study were collected from two main hospitals of Peshawar, Khyber Teaching Hospital (KTH) and Lady Reading Hospital (LRH), Peshawar, Pakistan. A total of 386 strains were collected. Among these 107 were *Escherichia coli*; 67 *Klebsiella pneumoniae*; 79 *Pseudomonas aeruginosa* and 133 *Staphylococcus aureus*. The biochemical tests for the identification of all these strains were done in Centre of Biotechnology and Microbiology, University of Peshawar, Khyber Pakhtunkhwa, Pakistan. Further experiments have been done in Bradford University, United Kingdom (UK).

3.1 *Staphylococcus aureus*

In this study n=133 isolates of *S. aureus* were obtained from pus. In these, 70 isolates were collected from male patients and 63 isolates were collected from female patients. The age range was from 8 months to 78 years with mean average age 26.1 years (Fig: 3.1). Fourteen antibiotics for susceptibility testing were used for all isolates. Vancomycin was found to be the most active among all used antibiotics. Next to vancomycin (VAN) was the linzolid (LZD). Only 2.25% and 98.0% isolates were found resistant and susceptible to LZD respectively. Among the fluoroquinolones ofloxacin (OFX), ciprofloxacin (CIP) and levofloxacin (LEV) showed 33.0%, 51.87% and 57.89% activities respectively. Amikacin (AK) was very effective among the aminoglycosides which showed susceptibility to 80.45% isolates while 55.63% isolates were susceptible to gentamicin (CN). The erythromycin (E) showed activity of 59.39%. Doxycycline (DO) showed 65.4% activity. The fusidic acid (FD) showed activity of 51.87%. Among the β -

lactams the susceptibility pattern was more than 50%. In *S. aureus* 52.6 %, 57.14% and 60.90% isolates were susceptible to FOX, CAZ and CEC, respectively. AMC showed activity of 63.90% (Fig 3.2). Among 133 *S. aureus*, 63(47%) isolates were MRSA and 70(53%) were MSSA (Fig 3.3).

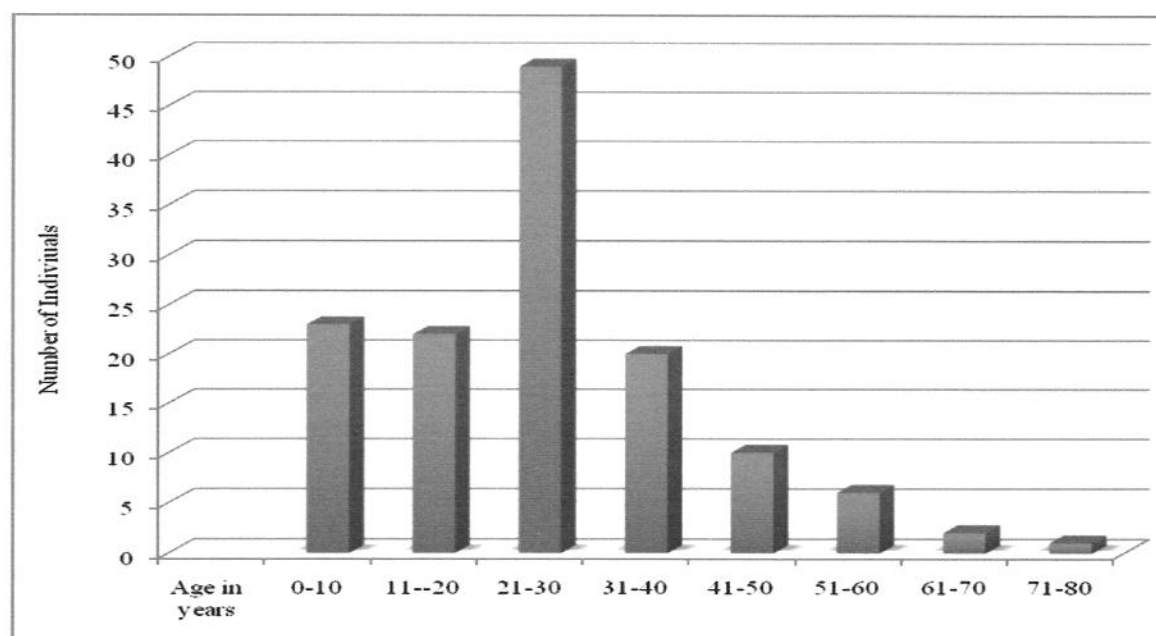


Fig 3.1: Age wise distribution of *S. aureus* infection.

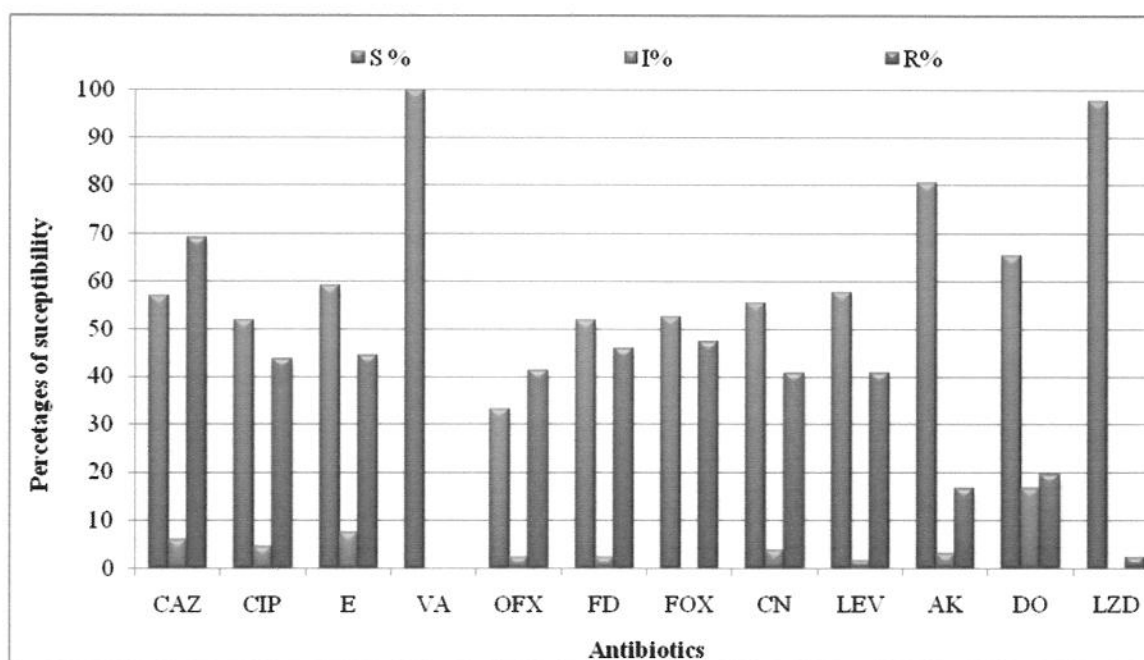


Fig 3.2: Sensitivity pattern of *S. aureus* (n=133) against different antibiotics.

Abbreviations: Amoxycillin+clavulanic acid (AMC), Cefaclor (CEC), Ceftazidime (CAZ), Ciprofloxacin (CIP), Erythromycin (E), Vancomycin (VA), Ofloxacin (OFX), Fusidic acid (FD), Cefoxitin (FOX), Gentamicin (CN), Levofloxacin (LEV), Amikacin (AK), Doxycycline (DO) and Linzolid (LZD).

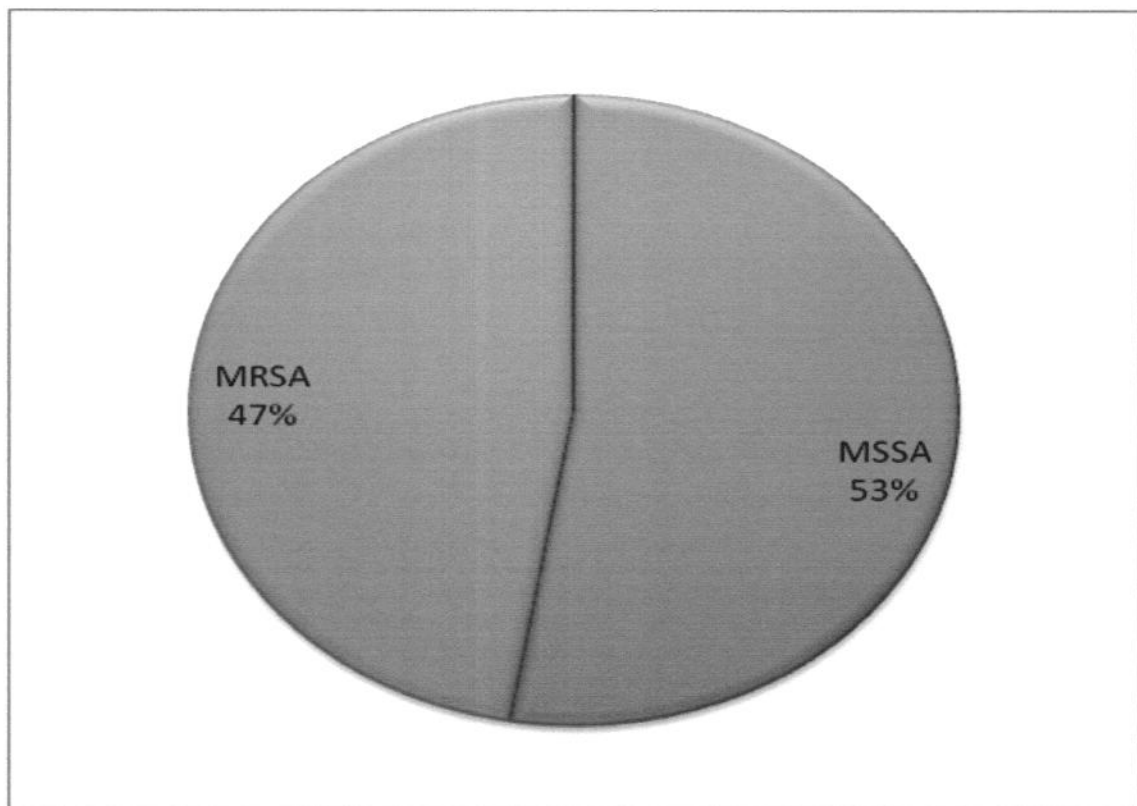


Fig. 3.3: Percent prevalence of MSSA and MRSA.

3.1.1 Methicillin Sensitive *S. aureus* (MSSA)

All the MSSA (n=70), were susceptible to FOX 30 and 10µg and VA (100%). Out of 70 MSSA 69(98.5%) isolates were susceptible to amikacin, 68(97.14%) to LZD and CEC, 66(94.14%), to AMC and DO, 65(92.8%) to LEV, 66(94.2 %) to CIP, E, 41(38.5%) to FD, 39(55.7%) to OFX and 25(57.1%) to CAZ. Out of 70 MSSA, 28 isolates were resistant to FD. Among these FD resistant isolates, 35.0% isolates were resistant to CAZ, LEV, CN, AMC and E. while 7.14% and 82.1% isolates were resistant to CIP and OFX, respectively (Table 3.1 and Fig 3.4).

Table 3.1: Susceptibility profile of MSSA (n=70) against different antibiotic.

Antibiotics	Sensitivity (%)	Intermediate (%)	Resistance (%)
AMC	66(94.14)	0(0)	4(5.7)
CEC	68(97.14)	2(2.85)	0(0)
CAZ	25(57.1)	41(35.7)	4(7.14)
CIP	64(91.4)	4(5.7)	2(2.8)
E	64(91.4)	3(4.2)	2(4.2)
LZD	68(97.14)	0(0)	2(2.8)
VA	100(100)	0(0)	0(0)
OFX	39(55.7)	0(0)	31(44.2)
FD	41(38.5)	1(1.4)	28(40)
LEV	66(94.2)	0(0)	4(5.7)
AK	69(98.5)	1(1.45)	0(0)
DO	66(94.2)	4(5.7)	0(0)

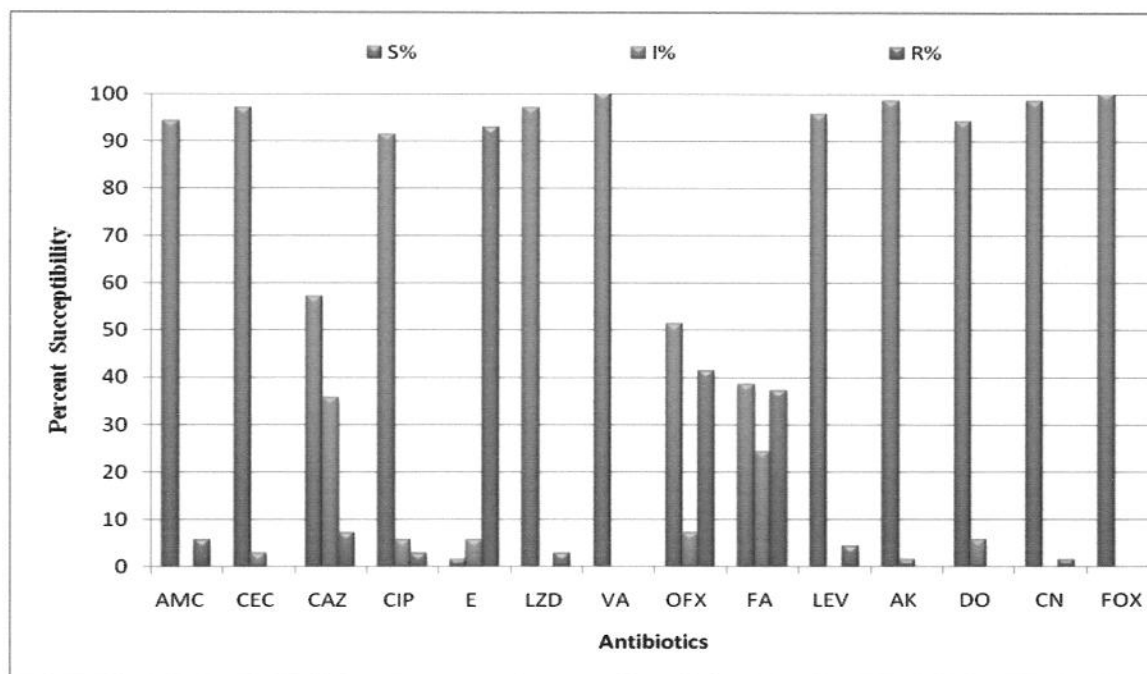


Fig 3.4: Antibiotic susceptibility profile of MSSA (n=70).

Abbreviations: Amoxicillin+clavulanic acid (AMC), Cefaclor (CEC), Ceftazidime (CAZ) Ciprofloxacin (CIP), Erythromycin (E), Linzolid (LZD), Vancomycin (VA), Ofloxacin (OFX), Fusidic acid (FD), Levofloxacin (LEV), Amikacin (AK), Doxycycline (DO), Gentamicin (CN), Cefoxitin (FOX).

3.1.2 Methicillin resistant *S. aureus* (MRSA)

There were 63 isolates which were resistant to ceftazidime according to CLSI guidelines of disc diffusion method. All the MRSA isolates showed 100% susceptibility towards VAN and 98.4% to LZD. Out of this 63 MRSA, 60.3% isolates were susceptible to amikacin 44.4%, to fusidic acid 33.3%, to DO 23%, to E 20.6%, to CEC 17.4 % to LEV, CAZ 12.6%, and 7.9% to OFX, CIP and CN. Table 2 shows the sensitivity pattern of different antibiotics for MRSA. Out of 63 MRSA isolates, 33 isolates showed resistance against FD. Among these FD resistant isolates, 72.7% isolates were resistant to AMC, while 69.6% and 62.0% isolates were resistant to CEC and CAZ, respectively. Among fluoroquinolones, 93.9%, 87.8% and 81.8% isolates were resistant to CIP, LEV and OFX, respectively. MRSA isolates of 65.0%, 42.4% and 81.8% were found resistant to E, DO and CN, respectively (Table 3.2 and Fig 3.5).

Table 3.2: Susceptibility pattern of MRSA (n=63) against various antibiotics.

Antibiotics	Sensitivity (%)	Intermediate (%)	Resistance (%)
AMC	19(30.1)	0(0)	44(69.8)
FOX	0(0)	0(0)	63(100)
CEC	13(20.6)	5(7.9)	45(71.42)
CAZ	8(12.6)	4(6.34)	51(80.9)
CIP	5(7.9)	2(3.17)	56(88.8)
E	15(23.8)	7(11.1)	41(65.07)
VA	63(100)	0(0)	0(0)
OFX	5(7.9)	3(4.7)	55(87.3)
DO	21(33.3)	18(28.57)	24(38.09)
FD	28(44.4)	2(3.17)	33(52.38)
CN	5(7.9)	5(7.9)	53(84.12)
LEV	11(17.4)	2(3.1)	50(79.3)
AK	38 (60.3)	3(4.7)	22(34.9)
LZD	62(98.4)	0(0)	1(1.5)

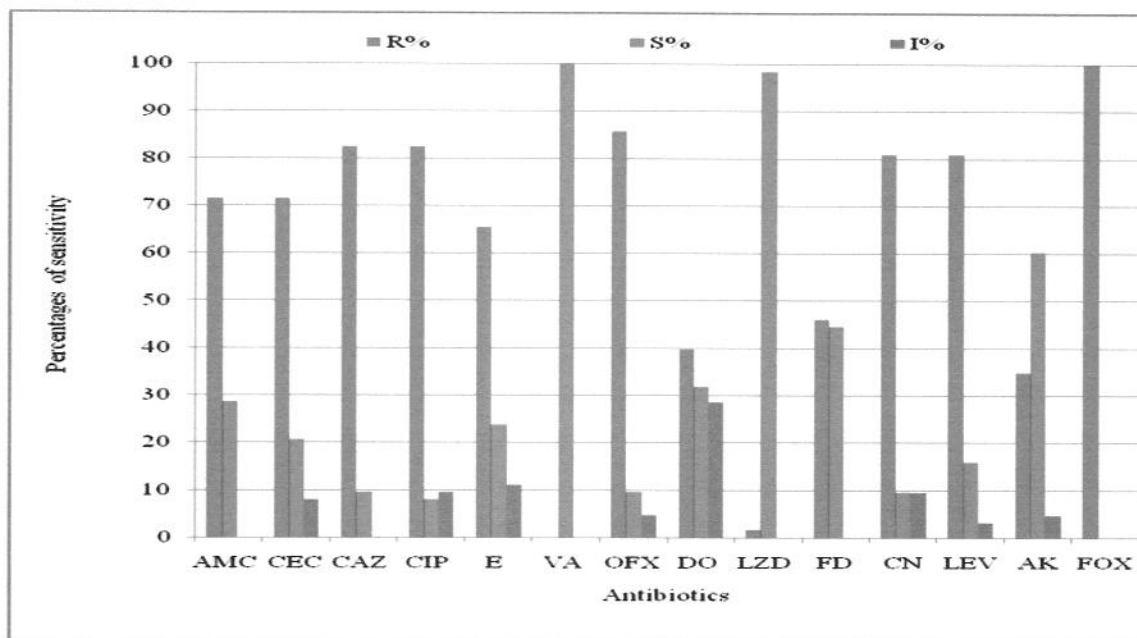


Figure 3.5: Susceptibility pattern of MRSA (n=63) against various antibiotics tested.

Abbreviations: Amoxycillin+clavulanic acid (AMC), Cefaclor (CEC), Ceftazidime (CAZ), Ciprofloxacin (CIP), Erythromycin (E), Vancomycin (VA), Ofloxacin (OFX), Doxycycline (DO), Linzolid (LZD), Fusidic acid (FD), Gentamicin (CN), Levefloxacin (LEV), Amikacin (AK) and Cefoxitin (FOX).

3.1.4 Comparison of three phenotypic methods for the Detection of MRSA

A total of 133 clinical isolates of *S. aureus* were screened by all three methods. All isolates were recovered from the wounds of both in and out door patients of two tertiary hospitals of Peshawar, Pakistan.

By using CLSI guidelines all isolates of *S. aureus* were tested for MRSA. Among 133 isolates, by using OX 1 μ g, 61(45%) were MRSA and 70(52.6%) were MSSA. Two (2.4%) isolates showed false negative results and gave intermediate zone diameters of 11mm. The specificity and sensitivity of OX 1 μ g was 100% and 96.8%, respectively. While FOX showed 100% sensitivity and 100% specificity. (Table 3.3) All the isolates showed the zone diameter <21mm.

Using BSAC recommendations, 63(47.36%) of the Staphylococci were MRSA and 70(52.6%) were MSSA. According to this guideline, FOX 10 μ g gave 100% specificity and 100% sensitivity. By using the OX 1 μ g method, two isolates gave false negative results, showing zone diameter greater than 14mm and the isolates were sensitive. Therefore, the OX 1 μ g showed 96.8 % sensitivity and 100 % specificity, (Table 3.3).

BrillianceTM MRSA agar (Oxoid) was used for the phenotypic identification of MRSA (Fig 3.6). Among 133, *S. aureus* 61(45%) were MRSA and 72(54%) were MSSA. It gave false negative results for one isolate which did not show any growth, while showed positive results for MRSA for both FOX (10 and 30 μ g) discs. The sensitivity and specificity of BrillianceTM MRSA agar was 98% and 100%, respectively, (Fig 3.6).

Table 3.3: Comparison of sensitivities and specificities of three methods used in this study.

Methods	Sensitivity (%)	Specificity (%)
1:CLSI		
a. (FOX 30µg)	100	100
b. (OX 1µg)	96.8	100
2.BSAC		
a. (FOX 10µg)	100	100
b. (OX 1µg)	96.8	100
3.Brilliance TM MRSA Agar	98.4	100

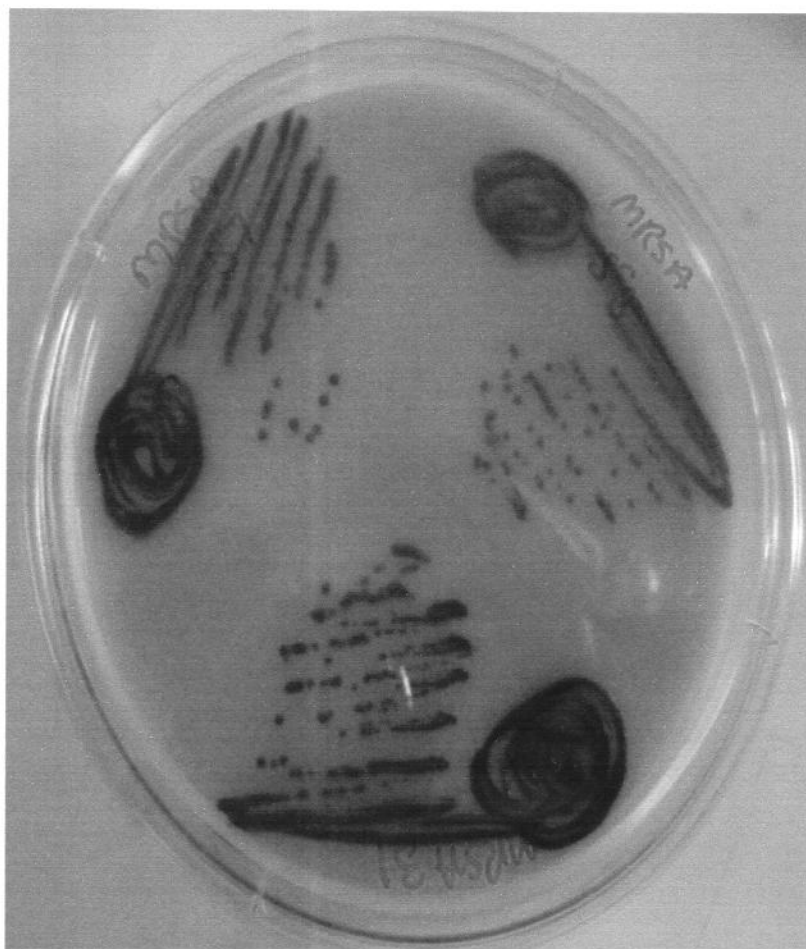


Fig 3.6: Morphological appearance of MRSA on Brilliance™ MRSA agar

3.1.5 Biocides

Among the biocides, the QACs showed the highest MIC value i.e. cetrimide 15.0µg/mL and cetylpyridinium chloride and benzalkonium chloride 3.0µg/mL. MRSA showed higher MIC₉₀ for cetrimide 15.0µg/mL, while the MIC₉₀ value for the MSSA was 6.0µg/mL. Overall, 22.9% *S. aureus* isolates showed resistance against cetrimide. Trichlorocarban showed MIC₅₀ value of 0.5µg/mL for MRSA which was higher than the MIC₅₀ value 0.25µg/mL recorded for MSSA. Triclosan showed MIC ≤0.05µg/mL and inhibited the growth of all the 70 *S. aureus* isolates on less than this concentration while PCMX showed the MIC ≥100µg/mL, to stop the growth of all isolates. In metals, silver nitrate had MIC₅₀ value of 0.4µg/mL while copper sulphate had MIC₅₀ value of 2.0µg/mL. Silver nitrate was found more effective than the copper sulphate. The isolates which showed resistance to cetrimide were selected for phenotypic detection of efflux pump by using EtBr agar screening method. Among the biocides no resistance was found except for cetrimide. Table 3.5 shows the MIC₅₀ and MIC₉₀ values for MRSA and MSSA for all biocides recorded in this study. The antibiotic resistance pattern of isolates used for biocides assay is shown in Table 3.4. The antibiotic disc diffusion method for these isolates was performed according to BSAC guidelines [149].

Table 3.4: Resistance pattern recorded for MRSA and MSSA (n=70)

Antibiotics	Resistance (%)in MRSA	Resistance (%) in MSSA
CIP	94.3	5.7
OFX	94.3	82.9
N	88.6	54.3
CN	88.6	2.9
E	74.3	8.5
FD	71.4	77.1
TET	68.6	51.4
MH	65.7	40
SXT	54.3	14.28
RD	45.7	17.14
DA	42.9	11.4
LZD	2.9	2.9
MUP	8.6	11.4
VAN	0.0	0.0

Table 3.5: The concentration ranges of biocides and MIC₅₀ and MIC₉₀ for MRSA and MSSA

Biocides	Range (µg/ml)		MIC ₅₀		MIC ₉₀	
	MRSA	MSSA	MRSA	MSSA	MRSA	MSSA
Chlorhexidine	≥0.1-0.25	≤0.1-05.25	≤0.1	<0.25	0.25	0.25
Triclosan	≤0.05	≤0.05	≤0.05	≤0.05	≤0.05	≤0.05
Cetrimide	3.0-15.0	3.0-6.0	4	4	15	6
Benzalkonium Chloride	2.0-3.0	2.0-3.0	2	2	3	3
Trichlorocarban	0.25-0.5	0.25-0.5	0.5	0.25	0.5	0.5
PCMX	100	100->100	100	100	100	>100
Silver nitrate	0.4	0.4	0.4	0.4	0.4	0.4
Copper sulphate	2.0-3.0	2.0-3.0	2	2	3	3
Cetylpyridinium	<1.0-3.0	2.0-3.0	2	2	3	3

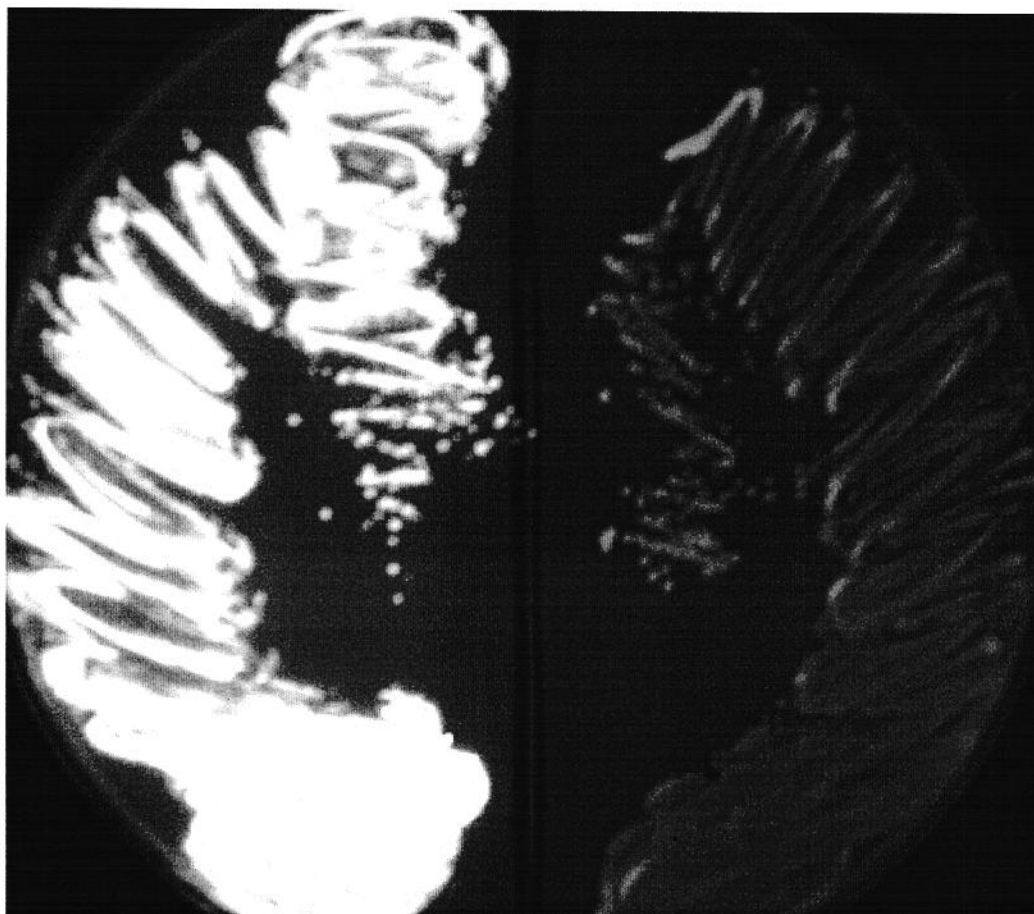


Fig 3.7: A positive efflux pump phenotype MRSA on right side and a negative efflux pump phenotype MRSA on left side.

3.1.6 PCR detection of enterotoxin genes in *S. aureus*

For molecular detection of enterotoxin genes, Polymerase Chain Reaction was used.

3.1.6.1 Distribution of Staphylococcus Enterotoxin (*sea–sed*) Toxic shock syndrome toxin (*tsst*) and Panton-valentine leukocidin (*pvl*) Genes

The most prevalent gene among the enterotoxin genes used in the present study in *S. aureus* isolates was *sea* which showed prevalence of 48.85% while *seb* showed prevalence of 28.57%. While *sec* was found only in 15.0% of Pakistani isolates, *sed* was not found in any of *S. aureus* isolate.

The *tsst* was found only in two MRSA isolates while no *tsst* was found in MSSA. The overall prevalence of *tsst* was 1.55% in this study.

pvl was detected in both MSSA and MRSA isolates. In MSSA the prevalence was higher than MRSA. In MSSA the prevalence of *pvl* was 85.70% (n=60), while in MRSA its prevalence was 19.0% (n=12).

In MRSA n=63 only 34 (53.9%) isolates harboured only one gene while 21(33.3%) isolates harboured 2 genes and only 3(4.7%) isolates harboured three genes. In 70 isolates of MSSA 51.4% (n=36) harboured one gene, 41.4% (n=26) harboured two genes while 10% (n=7) harboured only three genes (Table 3.6 and Fig 3.8, 3.9, 3.10, and 3.11).

Table 3.6: Distribution of Enterotoxin genes in *S. aureus* isolates.

Gene	Total n=133		MRSA=63		MSSA=70	
	n	%	n	%	n	%
<i>sea</i>	36+29	48.8	36	57.1	29	41.4
<i>seb</i>	14+24	28.57	14	22.2	24	34.2
<i>sec</i>	6+14	15.03	6	9.5	14	20
<i>sed</i>	0+0	0.0	0	0.0	0	0.0
<i>tsst</i>	2+0	1.5	2	1.5	0	0.0
<i>pvl</i>	12+60	54	12	19.0	60	85.7

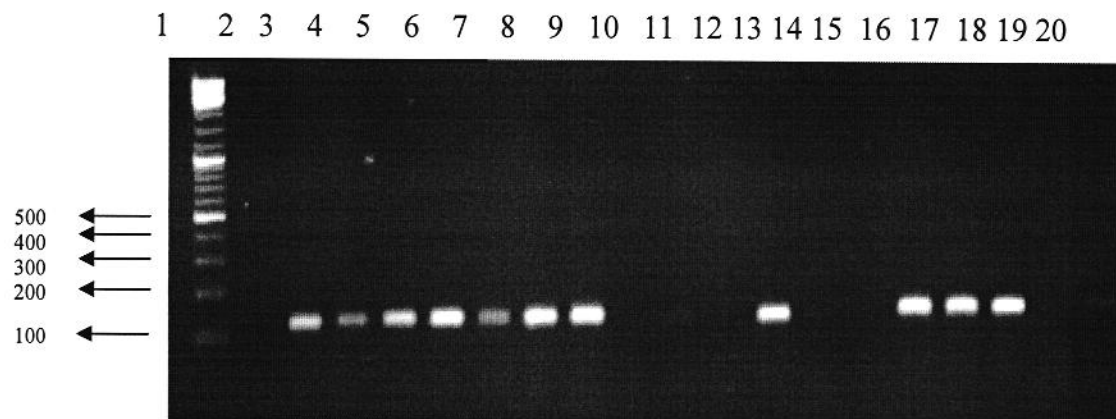


Fig. 3.8: Gel electrophoresis of *sea* PCR amplimers from *S. aureus*.

Lane 1, 1Kb DNA ladder. Lane 2, reagent negative control. Lane 3, *sea* positive control. Lane 4, MSSA 33 Lane 5, MSSA 34 Lane 6, MRSA 14 Lane 7, MRSA 21 Lane 8, MRSA 25 Lane 9, MRSA 15 Lane 10, MRSA 8 Lane 11, MRSA 11 Lane 12, MRSA 19 Lane 13, MRSA 20 Lane 14, MSSA 36 Lane 15, MSSA 39 Lane 16, MSSA 43 Lane 17, MSSA 44 Lane 18, MSSA 45 Lane 19, MSSA 47 Lane 20, MSSA 49

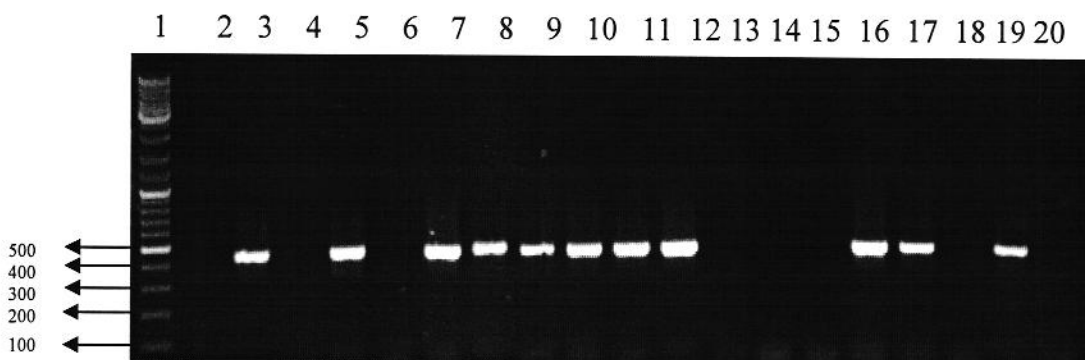


Fig. 3.9: Gel electrophoresis of *pvl* PCR amplimers from *S. aureus*.

Lane 1, 1 Kb DNA Ladder, Lane 2, reagent negative control, Lane 3, *pvl* positive control, Lane 4, MSSA 13 Lane 5, MSSA 8 Lane 6, MSSA 9 Lane 7, MSSA 14 Lane 8, MSSA 15 Lane 9, MSSA 16 Lane 10, MSSA 17 Lane 11, MSSA 18 Lane 12, MSSA 9 Lane 13, MSSA 30 Lane 14, MSSA 2 Lane 15, MSSA 33 Lane 16, MSSA 44 Lane 17, MSSA 45 Lane 18, MSSA 51 Lane 19, MSSA 52 Lane 20, MSSA 53

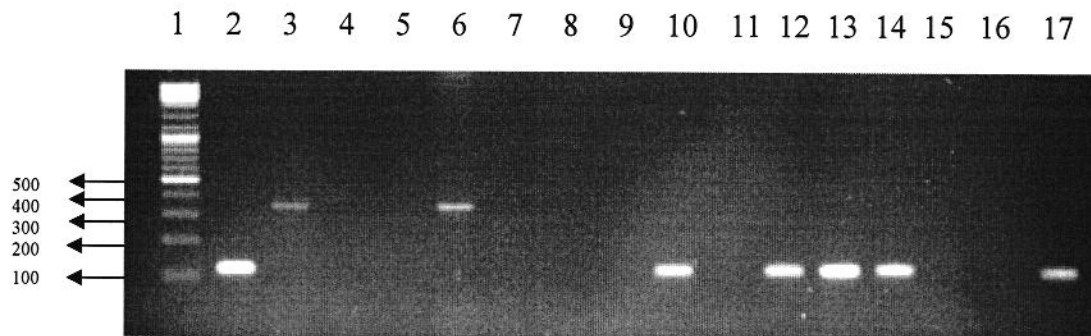


Fig. 3.10: *sea* and *tsst* Gel electrophoresis amplimers from *S.aureus*.

Lane 1, 1 Kb DNA ladder, Lane 2 *sea* positive Control, Lane 3 *tsst* positive Control, Lane 4, reagent negative control (*sea*), Lane 5 *tsst* reagent negative control, Lane 6, MRSA 11 Lane 7, MRSA 2 Lane 8 MRSA 4 Lane 9, MRSA 5 Lane 10, MSSA 15 Lane 11, MSSA 3 Lane 12 MSSA 12 Lane 13, MSSA 15 Lane 14, MSSA 17 Lane 15, MSSA 18 Lane 16 MSSA 30 Lane 17, MSSA 29

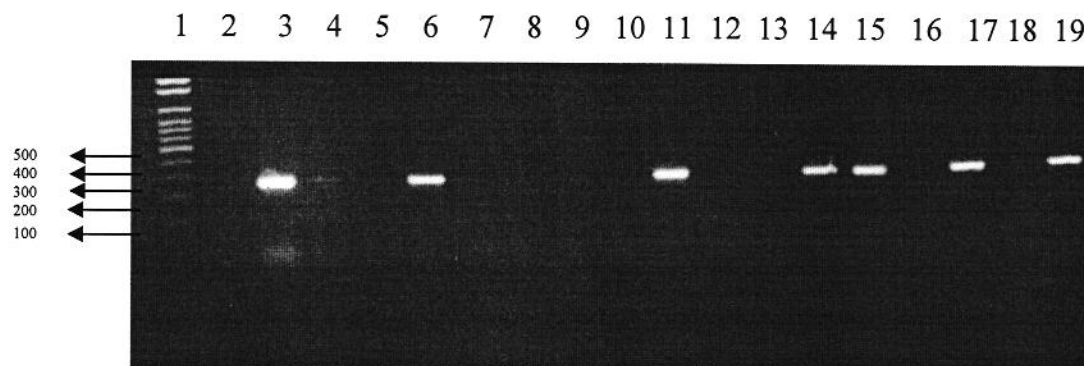


Fig. 3.11: Gel electrophoresis gel *sec* PCR amplimers from *S. aureus*.

Lane 1, 1Kb DNA ladder, Lane 2, reagent negative control, Lane 3 *sec* positive control, Lane 4, MRSA 53 Lane 5, MRSA 26 Lane 6, MRSA 38 Lane 7, MRSA 21 Lane 8, MRSA 45 Lane 9, MRSA 48 Lane 10, MRSA 24 Lane 11, MRSA 47 Lane 12, MRSA 6 Lane 13, MRSA 51 Lane 14, MRSA 23 Lane 16, MRSA 54 Lane 17, MRSA 43 Lane 18, MSSA 24 Lane 19, MRSA 62

3.2 *Klebsiella pneumoniae*

In total 67 *K. pneumoniae* isolates were collected from the urine samples of both in and out patients of both hospitals. More isolates were recovered from female 41 as compared to male which were 26. Mean average age was 29.4 years shown in (Fig 3.12).

All isolates were tested against 15 antibiotics. Among these antibiotics carbapenems had the highest activity, MEM and IMP had 100% and 98.5% activity respectively. Third generation of cephalosporins including CAZ, CTX and CFP had an activity of 39.6%, 43.2% and 37.35% respectively while the second generation including CEC had an activity of 46.2%. The susceptibility pattern of aminoglycosides including K showed excellent activity of 80.55% susceptibility while 64.1% isolates were found susceptible to CN. In fluoroquinolones including CIP, NOR and LEV showed the activity of 55.2%, 74.6% and 56.7%, respectively. The susceptibility results for AMC were 28.3%. While 14.9% and 17.1%, of *Klebsiella* isolates were susceptible to DO and SXT, respectively. Table 3.7 and Fig 3.13 show the susceptibility pattern of *K. pneumoniae* against different antibiotics. The MIC values of four antibiotics are shown in Table 3.8.

Among the 67 *Klebsiella* isolates, twenty one 31.3% isolates were found to be extended- β -lactamases (ESBL) positive and only 1.4% was carbapenemase producer. Morphological appearance of ESBL producer and carbapenemase producer on specific media are given in Fig 3.14 and 3.15. Among these ESBL producers, 19 isolates were recovered from female and only 3 were from male. Among ESBL producing isolates in *Klebsiella* spp. 19.4% isolates were resistant to NOR while 76.1% and 52.3% isolates were resistant to CIP and LEV, respectively. Among the aminoglycosides, 42.8% isolates

were resistant to both CN and K. While 90.4% and 100% isolates were resistant to SXT, AMC and DO, respectively in ESBL producers. (Table 3.9)

In 67 *Klebsiella* isolates six isolates showed resistance to only one antibiotic, five isolates were resistant to two antibiotics. Twenty four isolates were resistant two 3 and 4 antibiotics, 7 isolates were resistant to 5 antibiotics. Nine isolates were resistant to 6 antibiotics; five isolates were resistant to 7 antibiotics. Eight isolates showed resistance to 8 antibiotics while two isolates were resistant to 9 antibiotics. The isolate which was the producer of both ESBL and carbapenemase was resistant to 10 antibiotics. About 91.0% isolates were labelled MDR. The prevalent multi-drug resistance pattern was resistance to fluoroquinolones, cephalosporins SXT. High sensitivity was shown by carbapenems.

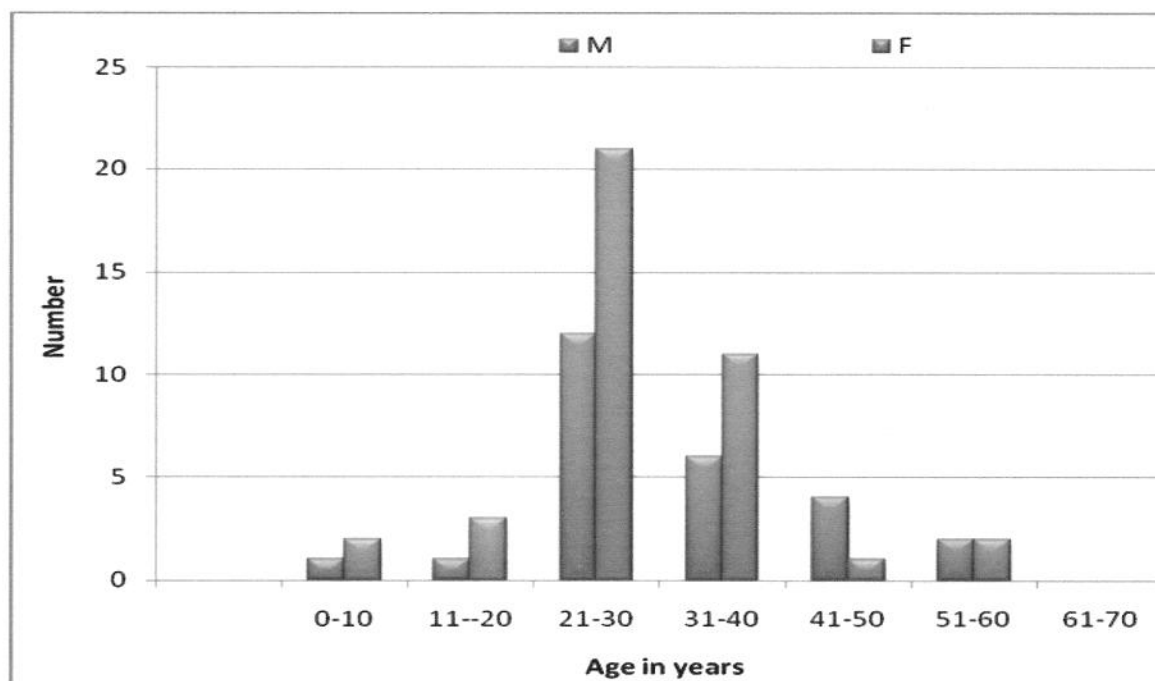


Fig 3.12: Age and gender wise distribution of *Klebsiella* infection.

Table 3.7: Antibiotic susceptibility pattern of *Klebsiella* spp. (n=67)

Antibiotics	Sensitive (%)	Intermediate (%)	Resistance (%)
CTX	29(43.2)	3(4.4)	35(52.2)
AMC	19(28.3)	2(7.4)	46(64.1)
CAZ	26(39.6)	9(13.4)	33(50.7)
CAR	20(29.86)	6(8.9)	41(61.1)
IMP	66(98.5)	1(1.4)	0(0)
STX	12(17.9)	2(0.9)	53(29.1)
CFP	25(37.3)	10 (12.9)	32(47.7)
CIP	37(55.2)	2(2.9)	28(41.7)
LEV	38(56.7)	7(14.9)	22(32.8)
CN	43 (64.1)	11(16.4)	13(19.4)
DO	10(14.9)	3(4.4)	54(80.5)
CEC	29(43.2)	0(0)	38(56.71.)
K	54(80.5)	0(0)	13(19.4)
NOR	50 (74.6)	4 (5.9)	13(19.4)
MEM	67(100)	0 (0)	0(0)

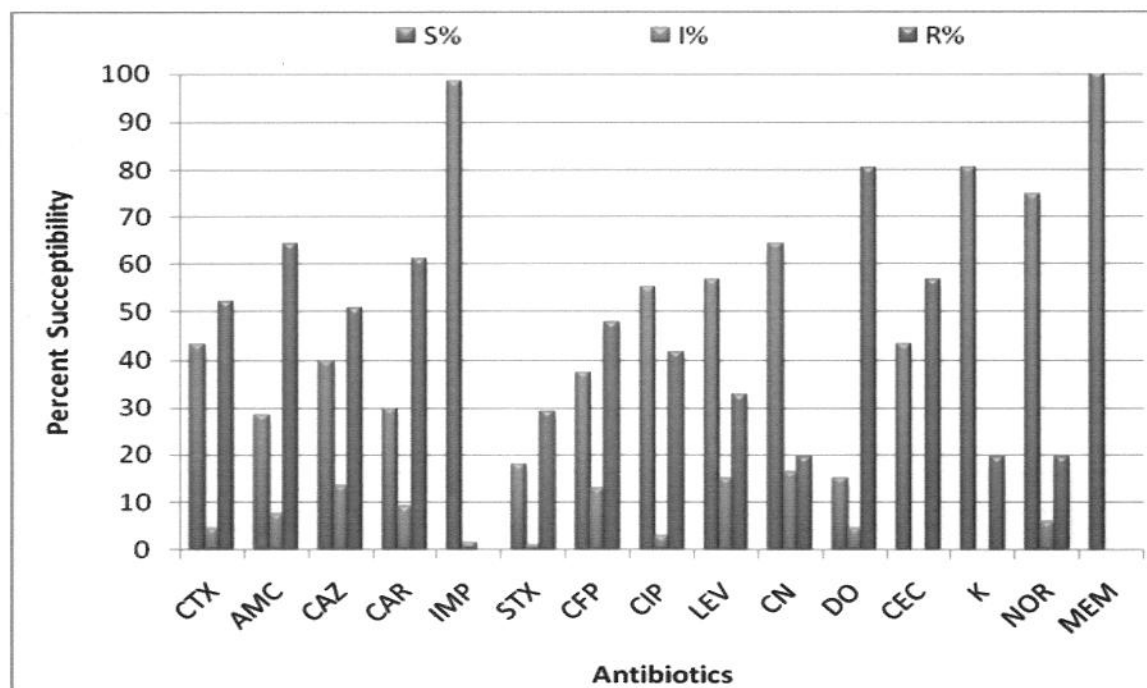


Fig. 3.13 Percentage of suceptibility of *Klebsiella* spp.(n=67) agaisnt different antibiotics.

Abbreviations: Cefotaxime (CTX), Amoxycillin+clavulanic acid (AMC), Ceftazidime (CAZ), Carbencillin (CAR), Imipenem (IMP), Trimethoprim+Sulphamethoxazole (STX), Cefoperazone (CFP), Ciprofloxacin (CIP), Levofloxacin (LEV), Gentamicin (CN), Doxycycline (DO), Cefaclor (CEC), Kanamycin (K), Norfloxacin (NOR) and Meropenem (MEM).

Table 3.8: Minimum inhibitory concentration of antibiotics for *Klebsiella* spp. (n=67).

Antibiotics	MIC ₅₀	MIC ₉₀
CIP	1µg/ml	≤64µg/ml
NOR	1µg/ml	≤256µg/ml
CN	≤4µg/ml	≤128µg/ml
K	2µg/ml	≤128µg/ml

Table 3.9: Comparison of antibiotic susceptibility of ESBL and non ESBL producers in *Klebsiella* spp.

Antibiotics	ESBL n=21		Non ESBL n=46		P. Value
	Sensitive (%)	Resistance (%)	Sensitive (%)	Resistance (%)	
SXT	2(9.5)	19(19.4)	10(21.7)	34(73.9)	.147
CIP	7(23.8)	16(76.1)	30(65.2)	14(30.4)	.001
LEV	7(33.3)	6(28.5)	31(67.3)	13(28.2)	.201
CN	7(3.3)	9(42.8)	33(71.7)	8(17.3)	.009
DO	0(0)	21(100)	10(21.7)	34(73.9)	.015
K	10(47.6)	11(52.3)	44(95.6)	2(4.3)	.000
NOR	20(95.2)	1(4.7)	34(73.9)	12(26.0)	.030

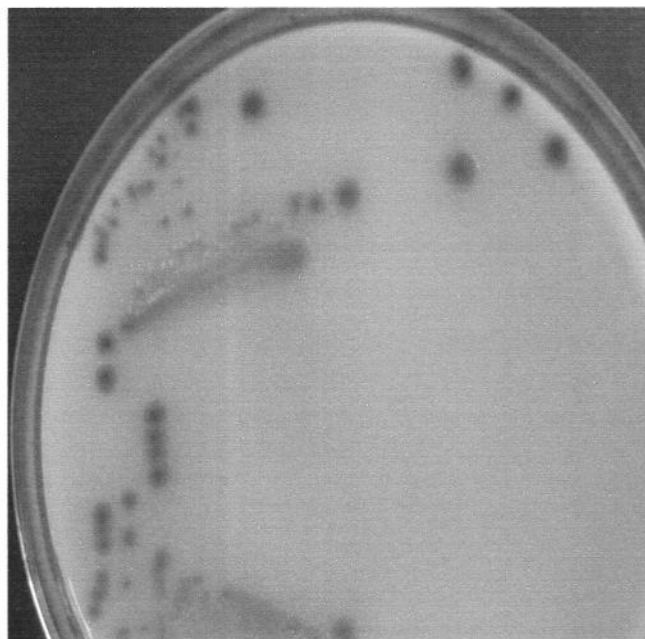


Figure 3.14: Morphological appearance of ESBL positive *Klebsiella* spp. on ESBL Brilliance Agar

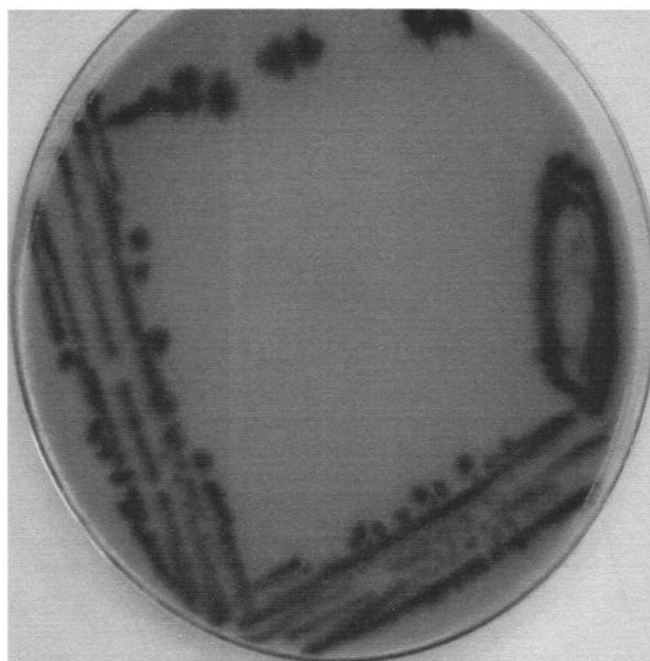


Figure 3.15: Morphological appearance of carbapenemase producer *Klebsiella* spp. on CRE Brilliance agar.

3.3 *Escherichia. coli*:

A total of 107 isolates of *E. coli* were recovered from urine samples. 81(75.7%) isolates were recovered from females while 26(24.2%) isolates were recovered from males. The average age of patients was 30.1 years (Fig 3.16).

In this study 89.7% *E. coli* isolates were found sensitive to IMP and 91.5% isolates were susceptible to MEM (Table 3.10 and Fig 3.17). Among the cephalosporins 39.7% isolates were susceptible to CTX, 48(44.8%) to CFP, 28(26.1%) to CAZ, 22(20.5%) to CE and 39(36.4%) to CEC. In other antibiotics 17(15.8 %) 15(14.0%), 11(10.2%) and 19(17.7%) were susceptible AML, DO, SXT and CAR, respectively. Among fluoroquinolones, 36.5%, 35.5% and 34.5% isolates were susceptible to NOR, CIP and ENX, respectively. In aminoglycosides 52(48.5%) isolates were sensitive to amikacin and 48(44.8%) isolates showed susceptibility to both gentamicin and kanamycin.

In 107 *E. coli* isolates 55(51.4 %) were ESBL producers which were detected on ESBL Brilliance agar shown in Fig 3.18. Among the 55 ESBL producer isolates of *E. coli*, 92.7% isolates were resistant to AML. In tested fluoroquinolones, 74.5% and 78.1 % isolates were resistant to CIP and NOR, respectively while 78.1% isolates were resistant to ENX. In aminoglycosides, 74.5%, 56.5% and 63.6% isolates showed resistant to AK, K and CN, respectively. While 81.8% and 83.6% isolates were resistant to SXT and CAR, respectively. Data of ESBL and non ESBL antibiotic susceptibility pattern (Table 3.11).

In 107 isolates of *E. coli*, 17(16.0%) showed positive results for carbapenemase on CRE Brilliance agar shown in Fig 3.19. In n=107, 6(5.6%) isolates were resistant to MEM while 3(2.8%) showed intermediate resistance to MEM. While 10(9.3%) isolates showed

intermediate resistance to IMP. Only one isolate showed full resistance to IMP. Only three isolates were susceptible to both IMP and MEM. But these three isolates gave positive result on CRE Brilliance agar. These isolates may be resistant to other carbapenems i.e. etrapenem or doropenem which were not used in this study.

Among these 17 carbapenemase producers 88.23% isolates were resistant to carbencillin while among the aminoglycosides 82.5%, 76.4% and 82.3% isolates were resistant to CN, K and AK, respectively. In fluroquinolones 94.11%, and 76.4% isolates were resistant to both CIP, NOR and ENX, respectively. To SXT 94.1% and 82.3% isolates were resistant to DO. Among these 17 carbapenemase producers 7(41.0%) were MBL producers (Fig 3.20). All MBL producer isolates were checked for NDM 1 using PCR but all the isolates gave negative result.

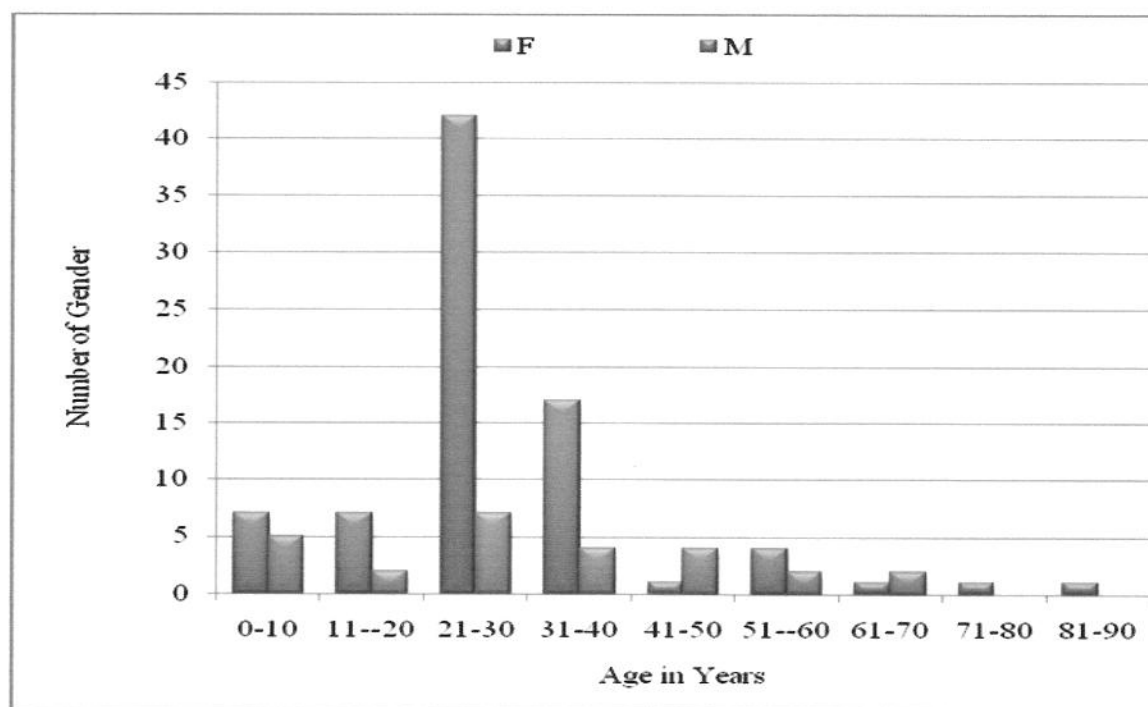


Fig 3.16: Age and gender wise distribution of *E. coli* recovered from urine specimens (n=107) of two tertiary care hospitals.

Table 3.10: Antibiotic susceptibility pattern of *E. coli* (n=107) recovered from urine samples.

Antibiotics	Resistance (%)	Sensitive (%)	Intermediate (%)
CFP	63(58.8)	43(40.1)	1(0.9)
CTX	65(60.7)	42(39.2)	0(0)
CAR	80(74.7)	19(17.7)	8(7.4)
IPM	1(0.9)	96(89.7)	10(9.3)
MEM	6(5.6)	98(91.5)	3(2.8)
AML	87(81.3)	17(15.8)	3(2.8)
CE	77(71.9)	22(20.5)	8(7.4)
CIP	69(64.3)	38(35.5)	0(0)
ENX	63(58.8)	37(34.5)	7(6.5)
CN	49(45.7)	49(45.7)	9(8.4)
DO	86(80.3)	15(14.0)	6(5.6)
SXT	96(89.7)	11(10.2)	0(0)
AK	49(45.7)	52(48.5)	6(5.6)
CAZ	77(71.9)	28(26.1)	2(1.8)
CEC	65(60.7)	39(36.4)	3(2.8)
NOR	68(64.4)	39(35.5)	0(0)
K	48(44.8)	53(49.5)	6(5.6)

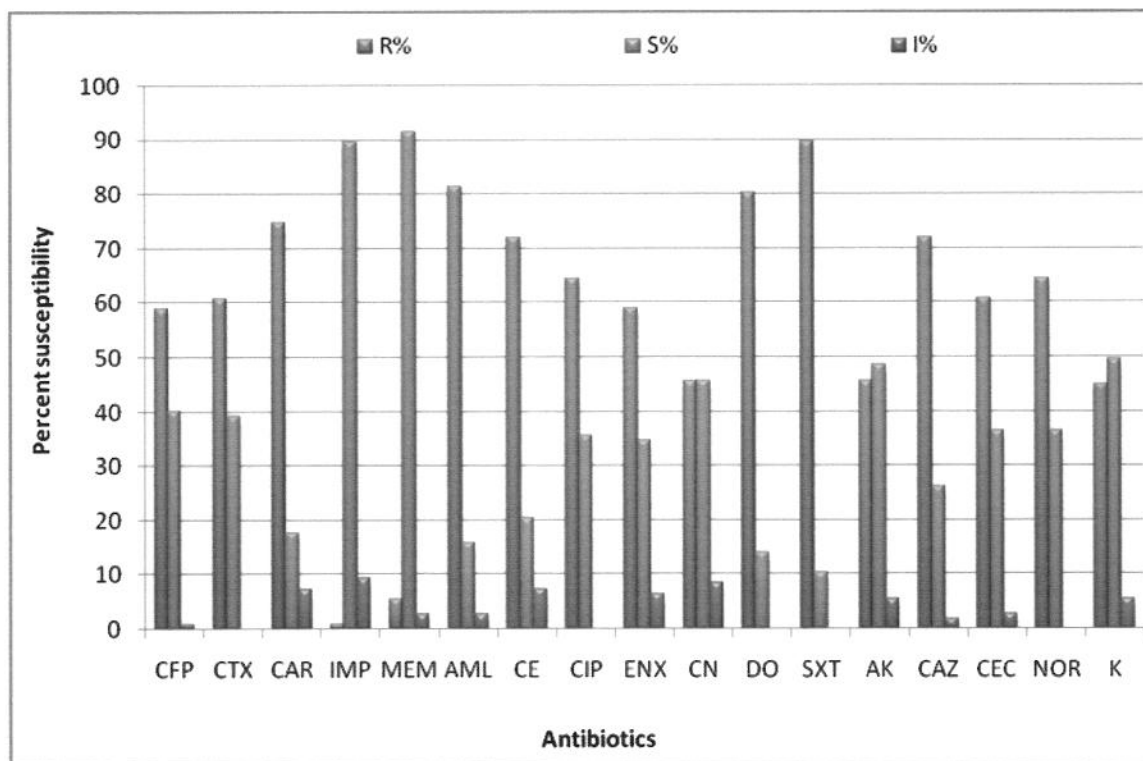


Fig. 3.17 Antibiotics sensitivity pattern of *E. coli* (n=107) recovered from urine samples.

Abbreviations: Cefoperazone (CFP), Cefotaxime (CTX), Carbencillin (CAR), Imipenem (IMP), Meropenem (MEM), Amoxicillin (AML), Cefradine (CE), Ciprofloxacin (CIP), Enoxobid (ENX), Gentamicin (CN), Doxycycline (DO), Trimethoprim+Sulphamethoxazole (STX), Amikacin (AK), Ceftazidime (CAZ), Cefaclor (CEC), Norfloxacin (NOR) and Kanamycin (K).

Table 3.11: Susceptibility of ESBL producing and Non ESBL *E. coli* against various antibiotics

Antibiotics	ESBL(n=55)		Non ESBL(n=52)		P. Value
	Sensitive (%)	Resistance (%)	Sensitive (%)	Resistance (%)	
CAR	0(0)	47(85)	15(14.0)	33(30.8)	.005
CIP	14(25.4)	41(74.5)	25(48.8)	28(53.8)	.021
NOR	12(21.8)	43(78.1)	26(50)	26(50)	.002
CN	17(5.8)	35(32.7)	32(29.9)	14(13.0)	.000
K	9(8.41)	41(38.31)	43(40.1)	8(7.4)	.000

Table 3.12: Minimum inhibitory concentration of antibiotics for *E. coli* (n=107).

Antibiotics	MIC ₅₀	MIC ₉₀
CIP	≤256µg/ml	≥512µg/ml
NOR	≤256µg/ml	≥512µg/ml
CN	32µg/ml	≤512µg/ml
K	32µg/ml	≤512µg/ml

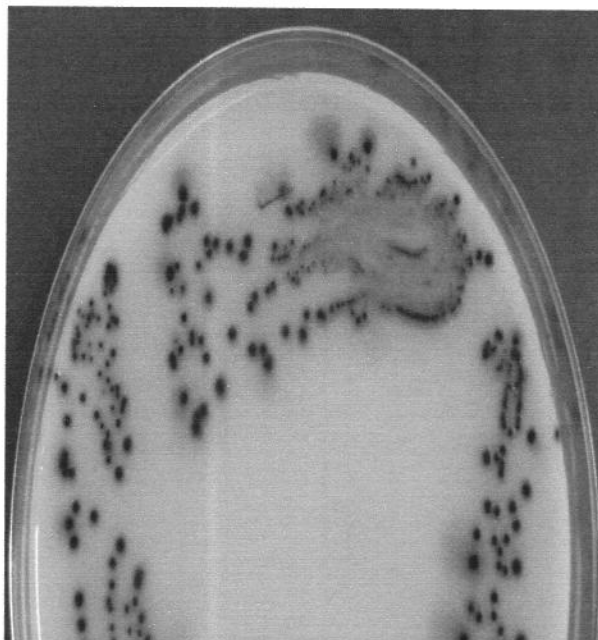


Fig.3.18: Morphological appearance of ESBL producer on Brilliance ESBL agar of *E. coli*.

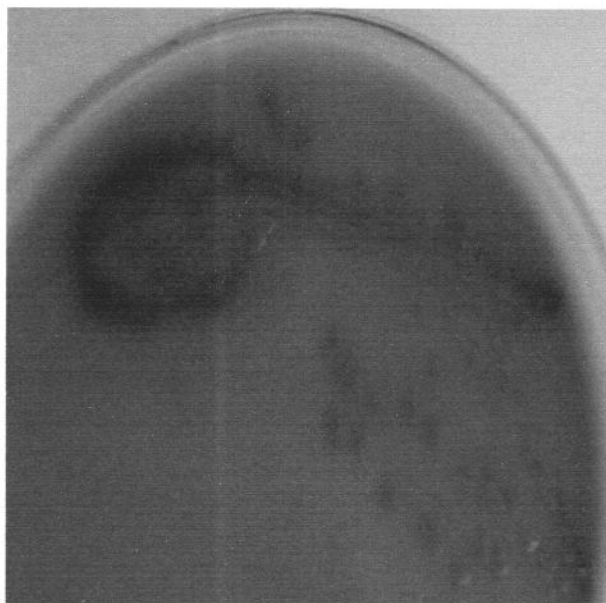


Fig.3.19: Morphological appearance of carbapenemase producer *E. coli* on CRE Brilliance agar.

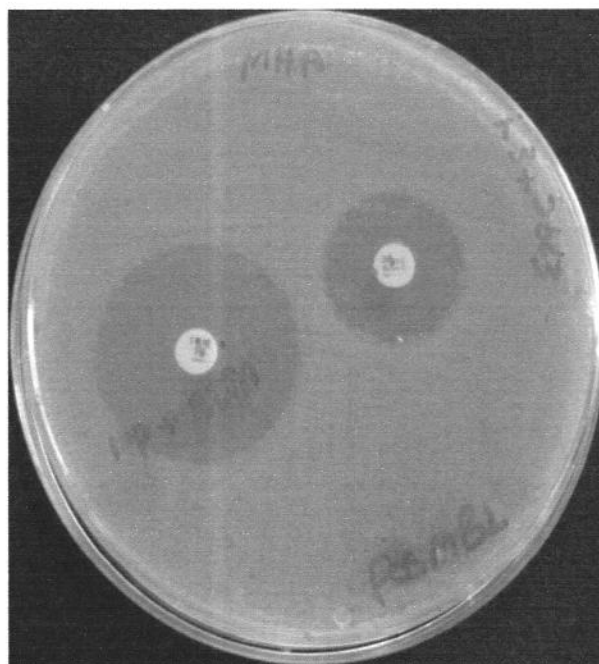


Fig.3.20: Phenotypic identification of MBL by Combined disc test (CDT) in *E. coli*.

3.4 *Pseudomonas aeruginosa*

A total of 79 isolates of *P. aeruginosa* were collected from clinical samples of wound in both in and out patients, in which 38% isolates were collected from female and 62% from male. The mean average age of patients from whom samples were collected was 30 years (Fig 3.21 and Fig 3.22).

Carbapenems were found the most effective among all the antibiotics showing 83.5% activity against *P. aeruginosa* (Table 3.12 and Fig 3.23).

Among other β -lactams a total of 56 isolates were sensitive to CE and showed activity of 28% while CRO and CTX had activity of 12.6% and 7.5%, respectively. CFM showed 34% activity. In aminoglycoside, 41.7% isolates were susceptible to CN. In fluoroquinolones CIP showed activity of 46.8%. All the isolates were resistant to AMC and AMP.

In 79 isolates of *P. aeruginosa* isolates 13(16.4%) were MBL producers (Fig 3.24). Among these 13 MBL positive isolates, 3(2.65%) showed intermediate zones towards IMP while the rest 10(76.9%) were found resistant to IMP. In β -lactams the resistance was also very high among the MBL producers i.e. 92.3% isolates were resistant to CRO, 76.9% to CAZ 92.3% to CFP, and 76.9% to CE. In these isolates 38.4% were resistant to ATM. All the MBL producers (100%) were resistant to AMC, AMP, and CN. These isolates showed 92.3% and 84.6% resistance against both CIP and CAR, respectively.

Table 3.12: Antibiotic sensitivity of *P. aeruginosa* (n=79).

Antibiotics	Sensitive (%)	Intermediate (%)	Resistance (%)
CAZ	23(30)	0(0)	56(70)
ATM	34(43)	16(20.2)	29(36.7)
CTX	63(79.0)	10(16.4)	6(7.5)
CRO	58(73.4)	11(13.9)	10(12.6)
AMC	0(0)	0(0)	79(100)
CE	51(64.5)	0(0)	28(35.4)
CN	44(55.6)	2(2.5)	33(41.7)
CAR	44(55.6)	0(0)	35(44.3)
CFM	52(68.8)	0(0)	27(34.1)
AMP	0(0)	0(0)	79(100)
IMP	10(12.6)	3(3.79)	66(83.5)
CIP	42(53.1)	0(0)	37(46.8)

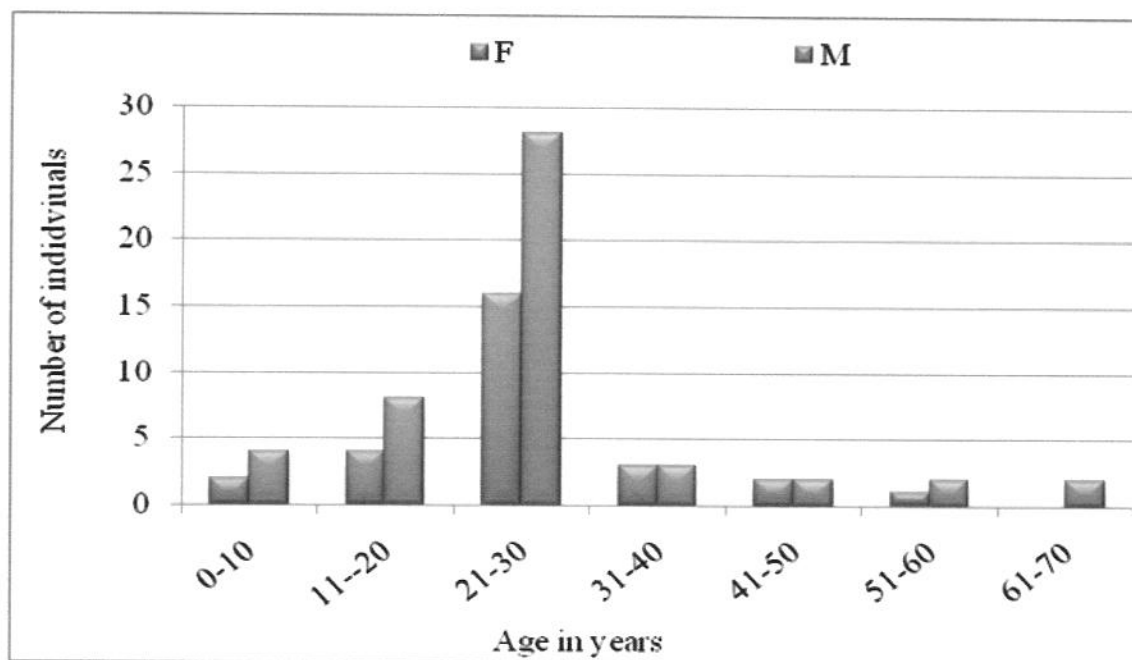


Fig.3.21: Age and gender wise distribution of infection caused by *P. aeruginosa*.

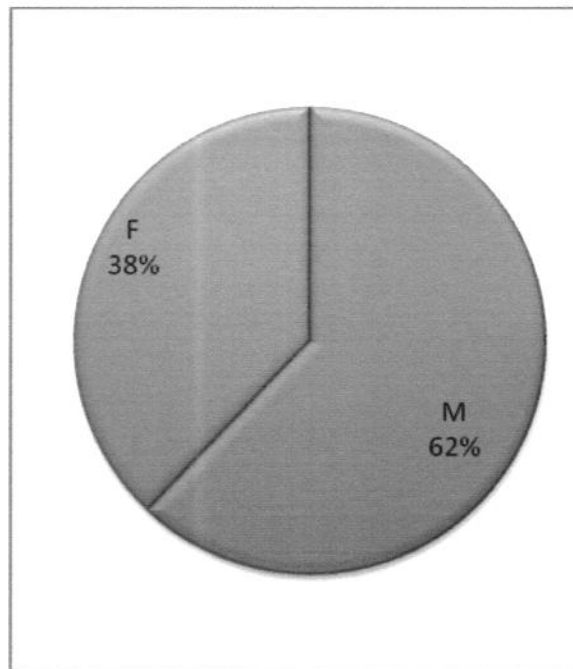


Fig. 3.22: Gender wise distribution of infection in male and female caused by *P. aeruginosa*.

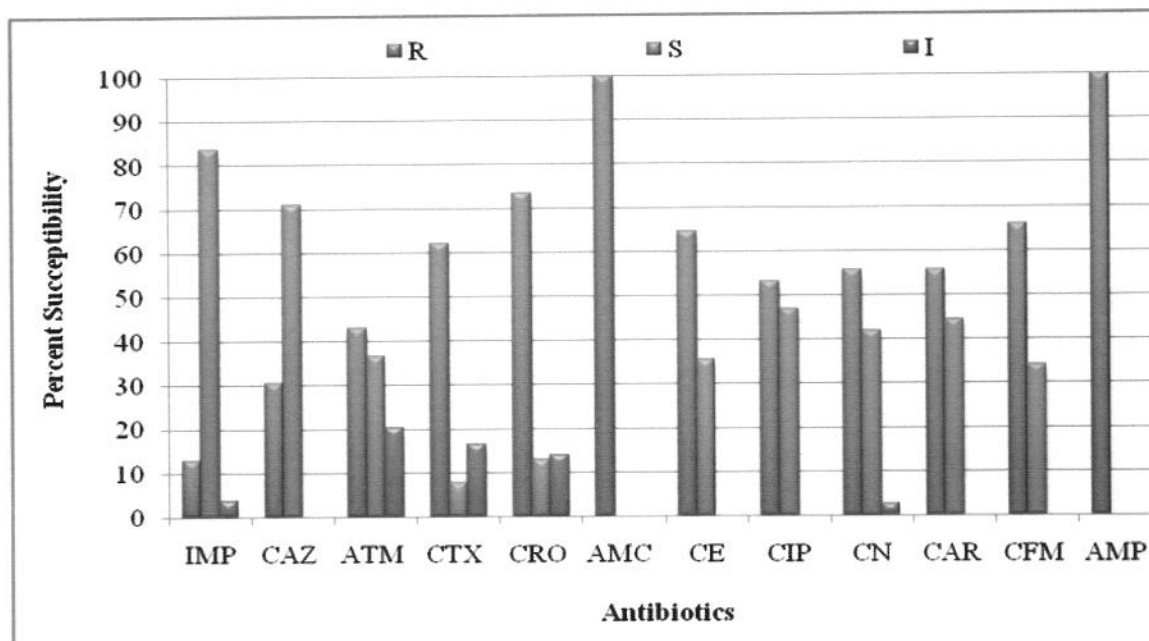


Fig.3.23: Antibiogram of *P. aeruginosa* (n=79) against different antibiotics.

Abbreviations: Imipenem (IMP), Cefazidime (CAZ), Azteronem (ATM), Cefotaxime (CTX), Ceftriaxone (CRO), Amoxycillin Clavulanic acid (AMC), Cefradine (CE), Ciprofloxacin (CIP), Gentamicin (CN), Carbencillin (CAR), Cefixime (CFM), Ampicillin (AMP).

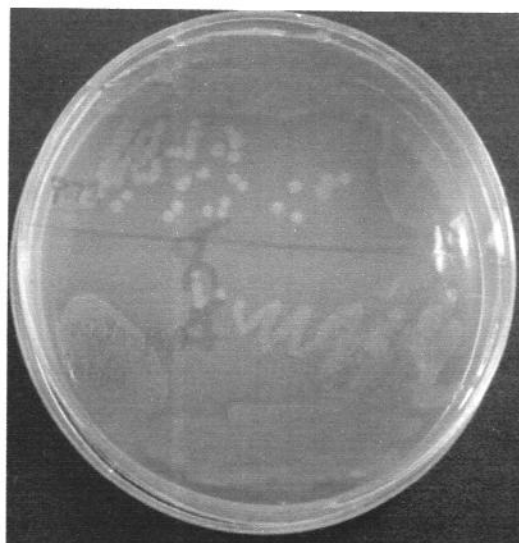


Fig.3.24. Morphological appearance of *P. aeruginosa* on Pseudomonas Cetrimide Agar

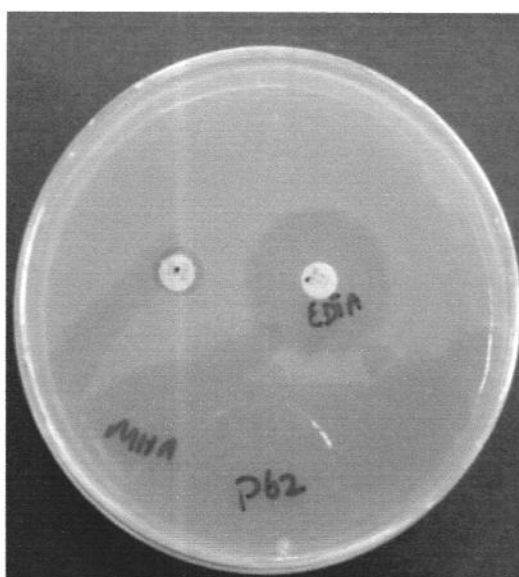


Fig.3.25: Phenotypic identification of Metallo- β -lactamases (MBL) by combined disc test (CDT) in *P. aeruginosa*.

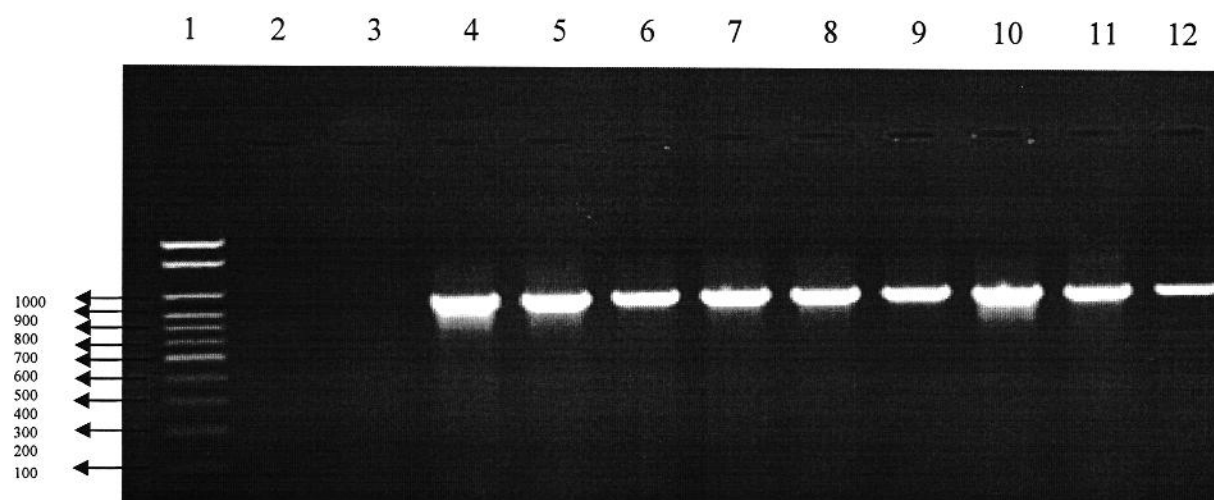


Fig.3.26: Gel electrophoresis of 16S rDNA amplimers from *P. aeruginosa*.

Lane 1 1Kb DNA ladder, Lane 2 *B-capecia* negative control, Lane 3 reagent negative control, Lane 4, positive control, Lane 5 P1, Lane 6 P2, Lane 7 P3, Lane 8 P4, Lane 9 P5, Lane 10 P6, Lane 11 P7, Lane 12 P8

Chapter 4

Discussion

Antimicrobial drugs are readily and extensively available from pharmacists over the counter in most parts of Asia, where frequent self-medication and ease of purchase are likely to be major cause in the emergence of antibiotic resistance [153]. In underdeveloped countries 98% mortality in children are mainly due to pathogenic microbial infection [154]. Antimicrobial resistance in bacteria to most of the currently used antibiotics is an important issue worldwide. A lot of factors are contributing in this problem like over use and miss use of antimicrobials, high frequencies of drug-resistant bacteria in the indigenous faecal, the flora, low quality of sanitation, and lack of education. All these problems are found in underdeveloped countries like Pakistan [155]. Nosocomial infections are also contributing to morbidity and mortality in this part of the world [156].

4.1 *Staphylococcus. aureus*

This study reveals the antibiogram of both MSSA and MRSA from two tertiary hospitals of Pakistan. The isolates were taken from the wounds and skin infections from both out and in patients. In this study we found the prevalence percentages of both MSSA and MRSA infections were 52.6% and 47.3% respectively.

S. aureus is responsible for many infections like folliculitis and food borne intoxications and severe infections like osteomyelitis, endocarditis or septicaemia. On many skin surfaces it lives harmlessly, mostly around the nose, mouth, genitals, and rectum [30].

The sensitivity of all *S. aureus* isolates were observed against various antibiotics by disc diffusion method. Vancomycin was the most effective drug for all *S. aureus*. It showed

100% sensitivity which is in good agreement with Zafar et al., [157]. It is the most effective drug in this study against *S. aureus*. Linzolid was the next effective drug followed by vancomycin in this study. Its susceptibility was 98% in *S. aureus* isolates. Mahmood et al., [158] reported 100% sensitivity to linzolid which is a fair agreement with our study.

Among the fluoroquinolones 57.89, 51.87% and 33.0% of *S. aureus* isolates were susceptible to levofloxacin (LEV), ciprofloxacin (CIP) and ofloxacin (OFX), respectively. In a study done by Goswami et al., [159] found 60.5% isolates were susceptible to LEV which can be compared to our study. Balandó et al., [160] reported 54.6% of *S. aureus* isolates susceptibility to CIP which supports results of our study.

4.1.1 Prevalence of MRSA

MRSA are seen in the local population with frequencies varying between 2-61% highest incidences seen in the major cities of the country [42]. We found 47.3 % MRSA prevalence in our study. Our findings are in agreement with the findings of Parwaiz et al., [55]. But the percentage reported by Hafeez et al., [161] are less than our findings.

The prevalence varies considerably from one region to another and among hospitals in the same city. MRSA emergence, particularly as a hospital pathogen has been attributed to a multifactorial etiology including host factors, infection control practices and antibiotic policies of the hospital [162].

4.1.2 Antibiotics Susceptibility pattern of *S. aureus*

The antibiogram of MRSA isolates, vancomycin showed excellent activity of 100%. Our findings are consistent with the findings of Hussain et al., [162] and Mahmood et al., [158]. Vancomycin is considered the antibiotic of last resort against multi-drug-resistant infections caused by Gram-positive bacteria. However, resistance to vancomycin has emerged, first in enterococci and, more recently, in *S. aureus* [163].

In MRSA, 98.4% isolates were sensitive to linezolid. Susceptibility of vancomycin was found best among all the antibiotics. This could be because of less use of its high cost in Pakistan. Linezolid was approved by the Food and Drug Administration in 2002. Linezolid is the first oxazolidinone in clinical use. It prevents the formation of 70S ribosomal initiation complex which cause the inhibition of protein synthesis. It is effective in the treatment of infections caused by various gram-positive pathogens, i.e. MRSA [164].

4.1.3 Aminoglycosides

In this study we found CN and AK resistance 84.12%, 34.9% respectively. Naqvi et al., [165] reported CN and AK resistance in *S. aureus* 80% and 90% in isolates of *S. aureus* from burn patients. The resistance frequency in case CN is in fair agreement to our study but with disagreement in case of AK resistance. Zafar et al., [157] reported 96% resistance to CN which is in fair agreement with this study. In another study 58% resistance to CN was recorded in *S. aureus* by Farzana and Hameed, [166]. Another study conducted in January 1999 to June 2002, reported 81.03% resistance to CN by Hafeez et al., [161] which can also be compared with our findings.

4.1.4 Fluoroquinolones

In this study 88.8% isolates were resistant to ciprofloxacin. There has been an increase in *S. aureus* resistance to fluoroquinolones in recent years. A study conducted in 2007-2008 by Hannan et al., [167] also reported a very high level resistance in MRSA to CIP (96.2%) which is much more than the frequency of resistance recorded in this study. Similarly in the present study other members of fluoroquinolones also showed high resistance i.e. 77.7% and 87.3% in case of LEV and OFX respectively. In a study conducted by Zafar et al., [157] 96% isolates were resistant to ofloxacin which is much more than this study finding has been reported. The resistance to fluoroquinolones develops as a result of spontaneous chromosomal mutations in the target of the antibiotic, DNA gyrase or topoisomerase IV or by the induction of a multidrug efflux pump. However the reasons for the difference in rates of quinolones resistance between MSSA and MRSA strains are uncertain. But antibiotic selective pressure could be a contributing factor, especially in the hospitals, resulting in the selection and spread of more antibiotic-resistant MRSA strains [168]. A study conducted on *S. aureus* isolated from burn patients in Rawalpindi in 2002-2003 by Naqvi et al., [165] reported frequency of resistance 100% to CIP which is much more than the findings of this study. Hafeez et al., [161] reported 86.6% of resistance in MRSA isolates taken from different clinical specimens including wound isolates which can be compared to our findings. There are several studies which alert on the customary dispensing of fluoroquinolones as over the counter drugs, could be the cause of increased resistance of the pathogenic bacteria [169].

4.1.5 Fusidic acid

Fusidic acid is a narrow spectrum antibiotic that had been used for more than 35 years in treatment of *S. aureus* infections. It is derived from *Fusidium coccineum* [170]. In the present study 52.38% resistance was observed. In studies conducted by Hafeez et al., [161] and Hussain et al., [162] reported 21.1% and 20% fusidic acid resistance in MRSA respectively, which is less than our findings. Resistance to fusidic acid in *S. aureus* has been reported throughout the world with prevalence ranging from 0.5% to 50% and is due to point mutation in bacterial *fusA* or *fuse* gene and acquired by FA resistance determinants *fusB*, *C* and *D* [171]. A study conducted in UK reported a sample of 64 consecutive patients all with fusidic acid resistant *S. aureus*. Another study conducted in Greece reported 88.9% resistance to fusidic acid [172] which is much more than the findings of our study. The resistance is known to increase with increasing use of an antibiotic, and particular concerns have been raised over the topical use of an antibiotic that is also used orally [173].

4.1.6 Doxycycline

In present study the *S. aureus* showed 38.09% resistance to doxycycline which is less than the findings of Hussain et al., [162].

4.1.7 Erythromycin

Bacterial resistance to erythromycin is becoming a serious clinical problem [174]. In our study we found 88.7% resistance to erythromycin in MRSA. A study conducted by Zafar et al., [157], a total of 126 consecutive, non-duplicated, clinically significant MRSA isolates were collected at the AKUH, Karachi, between June 2006 and July 2007. Their isolates showed 84% resistance which is comparable to this study. Hafiz et al., [175]

2002 reported 77% resistance to erythromycin in MRSA collected from different laboratories of Pakistan. Their findings are less than our findings. In another study Hafeez et al., [161] reported 85.5% resistance to erythromycin in MRSA from a cumulative study which is a good agreement to our findings.

4.1.8 Detection of MRSA

It is important to identify MRSA accurately because it will help in proper management of *S. aureus* born diseases. Many methods for the identification of MRSA have been developed but most of them are time consuming and not useful enough for the correct detection of MRSA Kumar et al., [176].

The most reliable method for detecting MRSA isolates is the detection of *mecA*, however in routine clinical practice; most of the laboratories do not use molecular techniques for the detection of MRSA. Therefore, those phenotypic techniques are very important, which are capable to identify MRSA strains quickly and accurately. This will also ensure the appropriate antimicrobial therapy at appropriate time and prevent the spreading of such nasty *S. aureus* strains in both health care and community settings. *S. aureus* with a *mecA* mediated resistance can be detected by cefoxitin [177]. In 2009, new breakpoints for the FOX 30µg disc test for identification of *mecA* in *S. aureus* have been validated by CLSI [178]. Their results showed that FOX disc diffusion test is the best technique and a better alternate for the OX disc diffusion test due to its easy interpretation and high accuracy. Findings of our study are similar to the findings of their study. In the present study, FOX 10µg and 30µg showed that they are the best for phenotypic detection of MRSA. In this study we have also found false negative results for detection of MRSA by using OX 1µg according to both CLSI (M100-S20) [146] and BSAC guidelines [149].

Skov et al., [179] also found that for identification of MRSA, FOX disc diffusion test was usually good, with sensitivity and specificity of 100% and 99%, respectively which is similar to the findings of this study. They used FOX 30µg on ISA [179]. In another study, Skov et al., [180] found that using semi-confluent growth on ISA and MHA along with standard incubations conditions, performance of FOX 5 and 10µg discs was excellent for the identification of MRSA. Their results showed that both of the FOX discs (5 and 10µg) performed well on both media [179]. In the present study the sensitivity and specificity was 100% for FOX 10µg which is similar to the findings of Skov et al., [180]. Several chromogenic media, and other differential MRSA selective agars have been used to identify MRSA within 18 to 24 hours [181,182]. In this study we have used Brilliance™ MRSA agar (Oxoid) for the detection of MRSA. The sensitivity and specificity was 98.4 and 100%, respectively. In a study by Kumar et al., [176] the sensitivity for Brilliance™ MRSA (Oxoid) was 97%, similar to this study and specificity was 86% which was different from this study. Another study modelled the effect of direct identification of MRSA carriage using a real-time PCR or chromogenic media on capture of patient isolation days (PIDs). The analysis showed that real time PCR captured 22.7% more PIDs and these more undesired PIDs were because of slightly low specificity as compared to chromogenic medium [183]. Similar performance of a chromogenic medium and RT-PCR has also been reported in another study [184]. However, yet another study has reported that molecular techniques give quick results with high sensitivity [185]. Brilliance™ MRSA agar (Oxoid) is also a good, cost effective medium for the detection of MRSA because cost of screening on conventional culture medium is similar to the cost of chromogenic medium. When downstream processing time and cost of the technician is

considered, variations range from 1.4-1.7 days, excluding time required for confirmatory test(s) [186]. On the basis of advantages of chromogenic media, it should be encouraged in the laboratories for rapid screening of MRSA [176]. Similarly, in a study conducted by Verkade et al., [186], Brilliance™ MRSA (Oxoid) has been found to be the best for the identification of MRSA. They used 788 isolates of staphylococci and after 20 hrs incubation, the sensitivity and specificity were 99.6% and 97.3% respectively. For the screening of MRSA in less time, this new medium is highly sensitive. Our results are similar to those reported by Verkade et al., [186]. By using Brilliance™ MRSA (Oxoid), results can be obtained within one day. Further studies should be performed to find out the usefulness of Brilliance™ MRSA (Oxoid) for the screening of MRSA directly from clinical specimens [186].

In conclusion, FOX 10µg and 30µg were the best for the phenotypic detection of MRSA because it is a superior test for the detection of MRSA than oxacillin. In most of the Pakistani labs oxacillin disc are still used for the detection of MRSA. It is therefore recommended that oxacillin discs should be replaced by the FOX discs test method because of its effectiveness. Brilliance™ MRSA agar is also effective and could be used as a second option for the direct inoculation of specimens in the laboratories.

4.1.9 Biocides

There is very limited data available in the literature regarding the use of biocides in hospitals of Pakistan; therefore, it is impossible to compare this data with findings of other researchers from Pakistan. As expected, most of the MRSA isolates were resistant to more than four antibiotics tested as compared to MSSA. In the present study three QACs showed higher MICs against the tested *S. aureus* isolates as compared to other

biocides used. These included cetrimide, benzalkonium chloride cetylpyridinium chloride.

Low level of resistance was found in isolates tested for cetrimide as compared to other QACs tested. Cetrimide showed MIC₅₀ values of 4.0µg/ml and 5.0µl/ml for MRSA and MSSA respectively. McDonnell and Russell [43] in their studies have reported similar results for cetrimide. In the present study 22.9% MSSA and MRSA had MIC₉₀ of 6.0µg/ml and 15.0µg/mL respectively which are similar to the findings of Al-Masaudi et al., (1991) who have reported resistance in MRSA to cetrimide with MIC₉₀ of 15µg/mL [187]. This resistance in Pakistani isolates towards QACs could be because of the fact that these compounds have been used for more than hundred years in the world including Pakistan as disinfectants [60].

Cetylpyridinium chloride and benzalkonium chloride showed MIC of 2.0µl/mL for 50% isolates both of MSSA and MRSA. The MIC values observed for BKC in this study lies in the susceptible range as reported by Zmantar et al., [188]. They found 4µg/mL and 8µg/mL for 54% staphylococci isolate which were considered resistant while those with MIC value $\leq 2.0\mu\text{g/mL}$ was reported in 28% of isolates were considered as sensitive [200]. Using the criterion of Zmantar et al., [188] all the isolates tested in the present study are susceptible to BKC. However, another study reported by Sekiguchi et al., (2004) classified *S. aureus* from a hospital in Tokyo resistant to BKC with MIC value 6.25µg/ml [189]. Al-Masuadi et al., (1988) reported that both MRSA and MSSA strains tested were susceptible to chlorhexidine [190]. Results of the present study are in agreement with their findings in the case of chlorhexidine susceptibility. However,

another study has pointed out that chlorhexidine MICs vary from strain to strain and are independent of the antibiotic resistance pattern [191].

Triclosan was found highly effective against all the *S. aureus* isolates tested in this study. MIC₅₀ and MIC₉₀ for triclosan were $\leq 0.05\mu\text{g/mL}$ for all *S. aureus* isolates used in the study. Doori et al., (2003) reported in their study MIC₅₀ 0.03mg/L and MIC₉₀ 0.06mg/L for the clinical isolates of MRSA. Although their MIC₉₀ value is slightly greater than our findings, they have labelled their isolates as sensitive to triclosan [192]. The MIC values observed in this study were different from the MIC values for *S. aureus* isolates tested by Schimd and Kaplan [193]. Another study which is also in disagreement to the findings of this study is reported by Aiello (2004) [194]. Both of those studies were conducted in the US where 76% commercial soaps contain triclosan and more than 140 home care and personal care products contain this compound [195]. Another reason for high MIC values of triclosan for those *S. aureus* in the US could be that a survey has reported that 74.5% of US population contain triclosan in their urine [196]. These studies support our results that why isolates from Pakistan have exhibited low MIC values for triclosan. Another study by Cole et al., (2011) found that the *S. aureus* isolates which were sensitive to the tested antibiotics had higher MIC for triclosan which reject the claim of cross resistance of biocides and antibiotics [197]. In the present study it was recorded that the antibiotic resistant isolates were sensitive to triclosan which supports the hypothesis of Cole et al., [197]. In Pakistan, very limited and infrequent use of antiseptic hand washes and gels could be a reason that most of the isolates from Pakistan are sensitive to the tested biocides [198].

Bamber and Neal (1999) reported that out of 16 MRSA isolates having low-level resistance to mupirocin, no one expressed high level MICs to triclosan also rejects the hypothesis of cross resistances between antibiotic and biocides [199]. In the present study no resistance was found to triclosan even not in antibiotic resistant isolates. Similarly, Cole et al., (2003) found no relationship between the use of triclosan and other biocides and antibiotic resistance in homes isolates where biocidal products were or were not being used [200]. In another study, Cole et al., (2011) found that *S. aureus*, had triclorcarban (TCC) MIC ranges from 0.0029-0.1875mg/L among TCC user group and 0.0469-0.1875mg/L among non users [197], which is less than our findings. In the present study TCC MIC range was found both for MRSA and MSSA 0.25-0.5 mg/L.

In the current study, among 70 *S. aureus* isolates efflux pump was detected in three MRSA isolates and in one MSSA isolate by using a phenotypic method as described by Marteins et al., [150]. In the current study it was found that MRSA isolates had the MIC values for EtBr >30.0µg/mL, 30.0µg/mL and 25.0µg/mL which is similar to a study conducted by Petal et al., [201]. They reported that the MIC value $\geq 25.0\mu\text{g/mL}$ for EtBr indicates the presence of efflux pump in such isolates. MSSA isolate which was phenotypically positive for efflux pump had MIC of 15µg/mL for EtBR which was in contrast to the findings reported by Petal et al., [201]. Some of the other MSSA isolates also had 15µg/ml MIC but were phenotypically negative for efflux pump. Therefore, the possibility of some other type(s) of efflux pump could not be excluded in such isolates. Further molecular studies are required to confirm this possibility. Three MRSA isolates were also resistant to EtBr, cetrимide, gentamicin, and neomycin. Cookson and Philips (1988) have presented a hypothesis that resistance to all these antimicrobials and biocides

transfer collectively [202]. Therefore, findings of the present study support the idea presented by Cookson and Philips (1988) because these isolates were also resistant to SXT. However, Chang et al., (2007) reported that four different types of *qac* (*qacA*, *qacB*, *qacC* and *qacD*) genes carried on plasmids are related to the presence of resistance to this particular antibiotic and biocides [203]. So the possibility of *qac* genes in these three isolates could not be excluded.

In the present study, 22.9% of *S. aureus* isolates were found resistant to cetrimide showing high level of resistance among all the biocides tested in this study. Mansouri et al., (2006) reported that cetrimide is useful and can be used as disinfectants against gram negatives and gram positives organisms [204]. Their findings are in contrast with the finding of this study. In this study, PCMX showed high MIC value of $\geq 100\mu\text{g/mL}$ for *S. aureus* isolates. Johanson et al., [205] found in their studies MIC value of $75\mu\text{g/mL}$ for their *S. aureus* isolates, which can be compared to our findings.

In the present study two metal salts were tested in which silver nitrate was found more effective having MIC_{50} of $0.4\mu\text{g/mL}$ while copper sulphate had MIC_{50} of $2.0\mu\text{g/mL}$. In the present study it was found that silver nitrate was more effective against clinical isolates of *S. aureus*. Copper sulphate was found less effective as compared to silver nitrate.

All the above mentioned studies were reported from developed countries where the use of biocides in the hospitals and community settings is very common. In contrast to developed countries, many hospitals in Pakistan lack proper use of biocides and antibiotics. Many studies in this regard have been conducted in the US and Europe, while limited data is available from developing countries like Pakistan. Results of a survey

conducted in 2007 in two tertiary hospitals of Karachi, Pakistan supports this fact that why isolates from Pakistan are more sensitive to most of the biocides used in this study [206] They have reported lack of facilities and resources such as sinks, water, soap, antiseptic lotions and gels in these hospitals. The use of antiseptics, disinfectants and sanitizing chemicals should be encouraged in our hospitals in order to minimize the rate of infection, morbidity, and mortality. All these practices are considered the first line defence against the infections and will also reduce the prescription of antibiotic [207].

This study suggests that there is no consistent pattern in the relationship between biocide and antibiotic resistance pattern. This study discounts the claim of cross resistance of biocides and antibiotics in bacteria. However resistance to aminoglycoside, cetrimide and EtBr supports the idea of cross resistance pattern.

4.1.10 Enterotoxin

There are many studies which have been done on the antibiotic resistance, infection control pathogenicity and diagnostic aspects of *S. aureus*. But, studies on enterotoxins in *S. aureus* need a lot of attention of researchers and require more research in this regard. However, the role of staphylococcal enterotoxins have not yet received the adequate attention of investigators and still require more research [208].

In the present study enterotoxin gene *sea-sed*, *tsst* and *pvl* were studied in the clinical wound isolates of *S. aureus*. This study revealed, the overall presence of enterotoxin genes in clinical *S. aureus* isolates were 72.9%. In another study by Ferry et al., [207] reported 83.7% isolates contained of one or more enterotoxin gene in *S. aureus* isolates but some other genes were also investigated in their studies compared to the four (*sea*, *seb*, *sec*, *sed*) in this study.

In the present study, the *sea* gene dominated over *sec* which were 48.85% and 15.1% respectively. Similar results have been reported from Germany among isolates of human origin [76]. Another study also reported that high prevalence of *sea* (57.1%) was found among the isolates associated with lower respiratory infections [210]. Similarly, Al-Bustan et al., [211] reported *sec* 16.4% in nasal samples of Kuwait population which can be compared with our results. In another study conducted by Pourmond et al., [212] 60 isolates (46.9%) of the total 128 clinical *S. aureus* isolates contained *sea* gene which showed a close agreement to this study.

Therefore it can be concluded that this difference in the frequency may be due the different origin of isolated strains and geographical differences. In the present study, we also found that the prevalence of *sea* 57.1% MRSA isolates which was higher than that of MSSA 41.4% in isolates which is similar to the study reported by Wang et al., [210]. Similar to our findings, another study reported by Garbacz et al., [213] reported that MRSA harbour more *sea* gene than MSSA from clinical isolates of *S. aureus*. This study shows that *sea* gene is the most prevalent among the enterotoxin genes from isolates of human origin. In clinical samples the quick detection of *sea* can help in minimizing severity of complications. Pourmond et al., [212] reported a significant relationship between the type of infection and *S. aureus* isolates carrying *sea*. It shows the relationship between the characteristic of pathogenic *S. aureus*, the host and also the pathogenic role of *S. aureus* [212].

In present study *seb* was found in 28.7% of Pakistani isolates. Similar findings were reported in another study on *S. aureus* in which the frequencies of *seb*, *sec* the isolates associated with SSTIs were 22.5%, 12.6%, respectively [214]. *S. aureus* strains contain

seb, the gene encoding SEB~10% (and perhaps up to 25%) [215]. So it is not surprising that 28.7% of the isolates recovered of *S. aureus* in this study contained *seb*.

In the present study none of *S. aureus* isolates had fourth classical enterotoxin *sed* gene. While in contrast to this study, Elhazhari et al., [216] reported 1.4% of *sed* genes in human origin *S. aureus* isolates. Another study conducted by Naffa et al., [217] from 100 clinical isolates of *S. aureus* no *sed* had been found which is similar to this study but in the same study they reported none of the isolates contained *seb* gene which is also in contrast of the present study.

In the present study, the overall presence of enterotoxin genes one or more in both MRSA and MSSA were (73%). Similarly, higher prevalence rate was reported for the classical *sea-see* genes in German multicentre clinical (43%) and nasal (39.5%) isolates [89] and in isolates collected from Japanese patients with food poisoning 76% [218]. Probably, the difference in the prevalence may be also affected by different ecological isolates origin i.e. food, animals and humans and as well as geographical differences [75, 86].

The prevalence of enterotoxigenic *S. aureus* from different human clinical samples has been reported previously and has ranged from 17.8% to 86.6%. So the frequency of staphylococcal enterotoxin producing strains from human clinical samples differs among studies in different countries or in different areas of the same country. In these studies the differences might be due to other factors like the sensitivity of detection methods, detected genes and number of samples and the type of clinical samples [219].

In this study the overall prevalence of *tsst* was 1.55% in Pakistani isolates. In this study only two isolates of MRSA had this gene while this gene was not detected in MSSA from

clinical isolates of Pakistan. The prevalence of *tsst* is reportedly low 7.5% among *S. aureus* clinical isolates and 4.5% among stable *S. aureus* nasal carriers. [220]. Prevalence of *tsst* in this study was higher in MRSA than in MSSA which is much similar to the study reported by, Shimaoka et al., [221]. In our study we found that in these two MRSA isolates one has only *tsst* gene and the other isolate had combination of *tsst* and *sec*. El-Ghaodhan et al., (2006) also found in their studies from Libya [220], that all the three *S. aureus* isolates harboured *tsst* and *sec* in combination which was similar to our one MRSA isolate which also harboured *tsst* and *sec* in combination. These super antigenic toxin genes are associated with mobile genetic elements such as phages, pathogenicity and plasmids. There is a horizontal gene transfer among strains of *S. aureus* [222]. In another study from Japan reported that HA-MRSA from 100 different hospitals were 80.3% positive for *tsst* gene [223] which is much more than our findings.

In present study *pvl* gene was detected in both MSSA and MRSA. Its prevalence in MSSA was found higher than MRSA. In MSSA the prevalence of *pvl* was 85.70% (n=60), while in MRSA 19.0% (n=12). Among these MRSA, *pvl* positive strains had two isolates from the hospital settings had this gene 3.17% while CA-MRSA had 15.8%. A study from China in January 2005 and January 2006, reported as 25 (12.8%) of 195 *S. aureus* isolates were positive for *pvl* in a teaching hospital in Wenzhou, China. Nineteen (11.9%) of 160 hospital-acquired isolates, and six (17.1%) of 35 community-acquired isolates, harboured *lukS/F-pvl* [224]. Our study also shows the high percentages of *pvl* in CA-MRSA and low prevalence in HA-MRSA. Another study described a low prevalence of *pvl* genes in *S. aureus* (<5%), and a particularly low prevalence in HA-MRSA [152].

A significant increase in *pvl* positive isolates of *S. aureus* was observed in Greece during 2001–2003, most of which were MRSA (77%). Of the CA-MRSA isolates, 72% carried the *pvl* genes, while 23% of HA-MRSA isolates were also *pvl* positive. The percentage of *pvl* found in MRSA in our study was lower than the previous study done by Chini et al., [225].

In contrast to our findings, Skiest et al., (2007) found 97% of *pvl* positive MRSA isolates in urban hospital [226]. This prevalence of *pvl* in MRSA is greater than the percentage of MRSA isolates having *pvl* found in our study.

In this study, the *S. aureus* which had only one gene in 52.6% while in 35.3% isolates, 2 genes were found and similarly in 7.5% isolates, three genes were observed. Adele et al., (2011) found in their studies 70.6% *S. aureus* isolates harboured at least one virulence gene [227]. Our findings can be comparable to their studies.

In this study we observed that a large proportion of *S. aureus* isolates had the property of producing toxin. However, molecular detection of enterotoxin genes by PCR does not provide the proof of their expression. Further investigation should be done by immunologic methods and bioassays for the confirmation of enterotoxin proteins production in *S. aureus* [228]. We should consider that detection of enterotoxin genes is not always concurrent with the toxin production [222].

In conclusion, the present investigation demonstrated that the *sea* gene was the predominant enterotoxin gene in clinical isolates from Pakistan. *pvl* was also found in MRSA but its prevalence was much lower than MSSA. *tsst* was only prevalent in MRSA isolates.

4.2 *E. coli*

4.2.1 Antibiotic susceptibility pattern

E. coli is the main pathogen among the enterobacteriaceae and is responsible for 75-90% UTIs [229]. In the present study we used 107 *E. coli* isolates which were isolated from the urine sample in which 75 % isolates were recovered from female. In our study we also found that the UTIs are more common in female than the male which is similar observation to Hassan et al., [230]. In another study which is a good agreement to this study was reported from Turkey [231].

In this study 89.7% of *E. coli* strains were resistant to SXT which is very high than the percentage reported by Randrianirina et al., [232] which was 70%. This higher resistance could be because of that for more than 30 years co-trimazole was considered as first choice drugs for the empirical therapy of UTI. In *E. coli* the resistance to co-trimazole has increased during the post 10 years and now exceeding 65% in some region of the world [233]. The increasing resistance for co-trimazole is worrisome since this antibiotic is frequently used for uncomplicated UTIs in many developed and under develop countries [231]. In our study the *E. coli* isolates were found highly resistant to co-trimazole. This situation is worrisome. The percentage of resistance found in the present study is much higher than the developed countries like Europe 4.9%, Canada 17.3% and 22.6% in US. More than 60% resistance was reported in less developed countries like India 76%, Madagascar 69.5% [231]. So it is concluded that SXT is not the appropriate drug for UTIs in Pakistan. In another study by Iqbal et al., [234] that most of

the strains of *E. coli* were found resistant to SXT which is in fair agreement to this findings.

4.2.2 Fluoroquinolones

E. coli is the most common pathogen of humans, and infections caused by it are mostly treated with the fluoroquinolones [235]. Among fluoroquinolones CIP has been given as first line drug for UTI's [236]. The increasing bacterial resistance to fluoroquinolones is evidently connected with their overuse [236].

In this study among the fluoroquinolones 62.6% and 64.4% isolates were resistant to CIP and NOR respectively. In another report by Iqbal et al., [234], 49% and 50% isolates were resistant to ciprofloxacin and norfloxacin respectively. The resistance against ciprofloxacin 85% reported by Ejaz et al., [130] is much more than the current study. The resistance to fluoroquinolones in our isolates is also much higher than studied in Turkey by Yalmaz et al., [231] which was 41.1%. The sensitivity reports of *E. coli* to the most common antibiotics, which are used for the treatment of UTI's, vary in different countries. But overall the fluoroquinolones resistance is increasing day by day which is alarming for the physicians. High quinolones resistance in our region may occur due to increased fluoroquinolones consumption over the years, necessitated by high TMP-SXT resistance [231]. Due to this resistance pattern, fluoroquinolones should be used with care, particularly if no antimicrobial susceptibility test result is available. Non-skilled practitioners also play important role in increasing high resistance rates in our country.

This resistance to the fluoroquinolones may be due to plasmid-mediated quinolones resistance (PMQR) which produces the production of pentapeptide repeat proteins has

been studied in different regions of the world in clinical isolates of enterobacteriaceae [237].

4.2.3 Aminoglycosides

Among the broad-spectrum antibiotics, aminoglycosides are mostly used as highly potent antibiotics in severe gram negative infections [238]. Among aminoglycosides in our study we found 45.7% and 44.8% resistance towards CN and K which is less than 61% resistance reported for CN in the *E. coli* isolates from Lahore by Raiz et al., [239]. In a study conducted by Ruppe et al., [240] from a developing country Cambodia, where high prevalence of resistance in gentamicin was 42.5% which is much more similar to this study. In another study which was conducted in Islamabad Alshifa hospital by Iqbal et al., [234] reported 40% resistance in the *E. coli* isolates from UTI's patients. Although we found low resistance against aminoglycosides but our findings are higher than the findings of Iqbal et al., [234]. In another study conducted in Pakistan institute of medical sciences reported the CN resistance in *E. coli* as 50% [241] which is more than our findings.

4.2.4 β -lactams

Among the cephalosporins, we tested CFP, CAZ, CEC and CTX against *E. coli* isolates. In all cephalosporins the resistance was >50%. In the present study 60.7% of isolates showed resistance to CTX which is less as compared to the Ejaz et al., [130] and Iqbal et al., [234]. In our study 71.9% of isolates were resistant to ceftazidime (3rd generation) which is more than reported by Ejaz et al., [130]. Nazir et al., [241] reported 75% of isolates resistant to CAZ which is near to our findings.

4.2.5 Ampicillin

In our study we found more than 80% of isolates resistance to ampicillin. In the beginning ampicillin was used as standard drug for the UTIs but *E. coli* developed a high frequency of resistance to ampicillin in most parts of the world [233]. Yalmaz et al., (2009) conducted a study in Turkey, using a total of 4,534 *E. coli* isolates in 5 universities and tertiary hospital in Izmir Turkey. They reported 61.8%, 78.7% resistance to ampicillin in male and female respectively [231]. These results can be compared to our results. One reason for such a high resistance may be the extensive use of this antibiotic in Pakistan because of its easy availability. In another study by Nazir et al., [241] reported 87% resistance in UTI *E. coli* isolates in three tertiary hospitals of Pakistan. In another study conducted by Noor et al., [242] reported 100% resistance to ampicillin in *E. coli* isolates from Pakistan which is in fair agreement to our findings. All the previous reports and this study showed that *E. coli* is highly resistant to ampicillin.

4.2.6 ESBLs

In the present study 51% *E. coli* isolates were ESBL producers collected from two tertiary hospitals of Peshawar. Urinary tract infections (UTIs) caused by ESBL-producing *E. coli* has been reported countries like Singapore (33%), Hong Kong 43% India 60% [243]. ESBL-producing organisms show significant difference in their frequency with geographic location. *E. coli* strains producing ESBLs are increasing. They are considered to be resistant to all β -lactams and are also resistant to other antibiotics [244]. In a study conducted in Karachi reported 41% ESBL producers in *E. coli* isolates which is less than our results [245]. But our findings have good agreement with [246]. In our study most of the ESBL producers showed cross resistance with other antibiotics like SXT, fluoroquinolones and aminoglycosides which is worrisome. Resistance to third

generation cephalosporins and quinolones, are very common in multidrug resistant isolates. This high level of resistance to beta-lactams is due to the production of ESBL [234].

4.2.7 Carbapenemase

Carbapenemases, hydrolyze carbapenems and makes them inactive. These are the most versatile family of β -lactamases [247]. In this study we also found 89.7% isolates which were susceptible to imipenem and 91.5% to meropenem. Hassan et al., [130], found 96.3% imipenem susceptibility in isolates of *E. coli* collected from UTI patients in a tertiary hospital in Karachi which is in good agreement to our findings.

We found only 17(15.8%) isolates as carbapenemase producers among the 107 *E. coli* collected from urine sample by using CRE Brilliance agar. Our results are in disagreement with a study conducted in Rawalpindi by Amjad et al., [248]. The previous study was conducted in a military hospital of Rawalpindi in isolates taken from different human specimens including urine, pus swabs, tissue cultures and bronchoalveolar lavage. They reported 38% *E. coli* isolates were carbapenemase producers which are more than our findings. Among these 17 carbapenemase producers 7(41.1%) isolates were found MBL producers. Enwuru et al., (2011), reported 25% MBL producers in *E. coli* isolates in Lagos Nigeria [249]. Their findings were less than our findings. All these isolates were then screened for NDM 1 by using PCR but none of the isolates were the NDM 1 producer. It could be possible that these MBL positive isolates have MBL other than NDM 1.

In order to minimize the spread of these pathogens will require effective laboratory screening methods to rapidly identify these organisms are required. Although the present

methods of screening these enzymes and methods for confirmation are useful, but the laboratories will still need new tools, probably molecular techniques, to make the process more reliable [250].

4.3 *Klebsiella pneumoniae*

4.3.1 β -lactams

All the isolates showed >30% isolates tested were sensitive to β -lactams used in this study. Our 50.7% and 53.7% of isolates were resistance to third generation of cephalosporin including CAZ, and 2nd generation CEC respectively. Bahrooz et al., [251] in his study found 100% resistance in *Klebsiella* isolates against CAZ which is more than our findings. While Ullah et al., [252] found in their study 54.8 % of *Klebsiella* isolates resistant to CAZ which can be compared to our findings. Less number of isolates showed resistance to CEC as compared to the study the reported Ullah et al., [252].

4.3.2 Aminoglycosides

In our study 19.4% of *K. pneumoniae* tested showed resistance to kanamycin and gentamicin. In another study conducted by Ahmed et al., [253] in Karachi, reported 14.28% and 7.14% of *Klebsiella* isolates were resistant to kanamycin and gentamicin respectively which are in good agreement with our findings. Aminoglycosides showed a very good activity against *Klebsiella* spp. in our isolates.

4.3.3 Fluoroquinolones

We found 41.7% of *Klebsiella* isolates resistance to ciprofloxacin. While Ullah et al., [252] found 52.1% resistance in *Klebsiella* against ciprofloxacin which can be compared to our findings. We found only 19.4% resistance in *Klebsiella* against norfloxacin. In a study conducted by Shahida et al., [254] reported only 10.4% resistance to norfloxacin in

Klebsiella which is in fair agreement to our findings. So norfloxacin was found most effective antibiotic in our study against *Klebsiella* spp.

4.3.4 Carbapenems

The carbapenems are the most effective class of antibiotic against many gram negative bacteria. We found 100% sensitivity of *Klebsiella* isolates against meropenem and 98.5% activity of imipenem which can be compared with the findings of Ullah et al., [252].

4.3.5 Trimethoprim+ Sulphamethoxazole (SXT)

In the present study we found 79.1% of *Klebsiella* isolates were resistant to SXT. Ullah et al., [252] found 93.48% resistance in *Klebsiella* which is more than our findings. Behroozi et al., [251] found only 45% resistance in *Klebsiella* which is less than our study. This may be due to over use of SXT in most of the UTIs infections in Pakistan.

4.3.6 ESBLs

In our study the frequency of ESBLs was 31.3% in *Klebsiella* isolates. Prevalence of ESBL producing strains is changing globally [255]. Padmini et al., [255] reported 40% ESBL in *Klebsiella* which can be compared to our studies. In contrast Ullah et al., [252] found 58% ESBLs in *Klebsiella* isolates. Another study conducted in Iran in tertiary hospital of 1000 bed reported only 12 % of ESBL in *Klebsiella* isolates. Behroozi et al., [252]. Their findings are different than our findings. Riaz et al., [256] also reported 26.1% ESBL in *Klebsiella* spp. which have fair agreement to our findings. Shukla et al., [257] reported 30.1% ESBLs producers in *K. pneumoniae* which is consistent to our findings. Jabeen et al., [245] recorded 36% ESBL in *Klebsiella* clinical isolates in Karachi which can be compared to our studies.

In this study we found more than 90% as multidrug resistant (MDR) which is in fair agreement with the MDR frequency reported as 100% by Subha and Ananthan [258]. But our findings are not in fair agreement with Ullah et al., [252]. They reported the MDR percentage i.e. 71.3% which is less than our findings.

4.4 *Pseudomonas aeruginosa*

In hospital settings *P. aeruginosa* acquires resistance to many of antibiotics classes which is due to extensive use of various antibiotics in the infections caused by it [259]. This high frequency of resistance in *Pseudomonas* spp. are the main cause of high deaths rates in many countries all over the world [260].

4.4.1 Percent prevalence of *P. aeruginosa* infection

The infection rate due to *P. aeruginosa* was high in males than in female. Our findings are opposite to the results of Anjum and Mir (2010) [261]. The reason in increase in the percentage of female in their findings was because they recovered isolates from both urine and pus. So females are more prone to the UTIs because of short urethra [261].

4.4.2 Antibiotic Susceptibility pattern of *P. aeruginosa*

Imipenem showed excellent activity against *Pseudomonas* spp. with susceptibility percentage of 83.5%.which are consistent to the findings of Ullah et al., [262], Khan et al., [263] and Anjum and Mir, [261]. The isolates' in our study showed very high level resistance to ampicillin which was 100%. Our results are in fair agreement with Khan, et al., [261] and Anjum and Mir [261].They found more than 98% and 100% isolates resistant to ampicillin respectively.

Similarly all the isolates were resistant to AMC. While Ullah et al., [262] reported only 61% resistances to AMC against *P. aeruginosa* in their study.

Among cephalosporins CAZ showed a good sensitivity against *P. aeruginosa* with percentage of 70.8%. This can be compared with the findings of Anjum and Mir [261] and Meenakumari et al., [264]. In our study CTX showed 7.5% activity against *P. aeruginosa* while the study conducted by Anjum and Mir [261] found 12% activity of CTX. In our study 43% isolates of *P. aeruginosa* were resistant to azetronem which is smaller to one recorded in burn patients in Karachi by Naqvi et al., [265]. They recorded 91% isolates were resistant. But in our study we found 20% isolates showed intermediate activity. Carbincillin showed an activity of 44%.

4.4.1 MBL

In our study we found only 16.0% MBL in *P. aeruginosa*. In this study 10 isolates were fully resistant to Imipenem but 3 of the isolates showed intermediate level sensitivity to Imipenem while all these isolates on testing with IMP and IMP/EDTA method gave a positive result for MBL.

MBL is the member of β -lactamases. This group of β -lactamases requires for its enzymatic activity divalent zinc as cofactor. It has very strong activity of hydrolysing both carbapenems and B-lactams. Antibiotic like tazobactam, sulbactam and clavulanic acid have no activity to hydrolyse these enzymes [266]. In study conducted in Kurdistan hospital in Iran reported 22% of MBL in *P. aeruginosa* [267] which can be compared to our studies. In another study conducted by Anuradha et al., (2010) reported 28.5% MBL in *P. aeruginosa* in ICU patients in a tertiary hospital in India [268]. Their findings were more than our findings.

In Pakistan the emergence of MBLs is very important issue for the physicians as well as for the patients. Carbapenems are the most effective drug for the infections caused by

gram negative bacteria. This class of β -lactamases is increasing in number by spreading among other gram negative bacteria and it could limit the choice of drugs for multidrug resistant gram negatives [269]. In another study 15.9% MBL in 478 *P. aeruginosa* clinical isolates was reported by Asghar, [270] in Makkah hospital. This is a good agreement with our findings. Bashir et al., [271] reported 11.6% MBL positive *P. aeruginosa* in a tertiary centre in Kashmir which is very similar to our findings. Similarly in India 17% and 20% MBL was reported in different studies respectively by Varaiya et al., [272] and Kumar et al., [273]. Their findings can be compared with our findings. The disseminations of such strains are worrisome, if immediate steps were not taken, i.e. proper prescription of antibiotics after investigating the antibiotic susceptibility pattern and right identification of MBL producers in laboratories, very soon the carbapenems will be useless drugs for dangerous infections caused by gram negatives bacteria.

CONCLUSION

Most of the pathogens investigated in this study were gram negative bacteria obtained from urine including *E. coli* and *Klebsiella* spp. while *P. aeruginosa* isolated from pus. In gram positive bacteria *S. aureus* was collected from skin infections i.e. pus samples and wound samples were investigated in this study. 45.0% of urine samples yielded growth while and 54.9% of pus yielded growth of both types of bacteria. Most of the isolates were collected from the females.

In *S. aureus* 53% were MSSA and 47% were MRSA. MRSA were identified by three methods i.e. CLSI, BSAC and Brilliance MRSA agar. Cefoxitin (10µg, 30µg) was found the most effective phenotypic method for the identification of MRSA. While Brilliance MRSA agar also was also found good for the phenotypic identification of MRSA.

Vancomycin and Linzolid were found the most effective drug among all the antibiotics used in this study. Both antibiotics were also effective against the multidrug resistant MRSA. All the MSSA were sensitive to most of the antibiotics.

Enterotoxin was also studied in both MRSA and MSSA. We found the *sea* was the dominant enterotoxin gene in clinical isolates of Pakistan. *Sed* genes were absent in both MRSA and MSSA. *pvl* was also found in MRSA but its prevalence was much lower than MSSA. *tsst* gene was only found in two MRSA isolates.

Biocides were also studied in this study against some *S. aureus* isolates. All the biocides showed good activity against the clinical isolates from Pakistan. Only cetrimide showed resistance in some *S. aureus* isolates. Efflux pump was also identified by phenotypic method. Only three isolates were efflux pump positive i.e. one MSSA and three MRSA.

Gram negative bacteria including *E. coli* were all collected (61.49%) from urine samples. Majority of isolates were recovered from female.

Carbapenems were the most effective drug among all the antibiotics used in this study against the *E. coli*. While most of the *E. coli* isolates showed resistance to other antibiotics. In *E. coli* 51% isolates were ESBL producers. All these ESBL positive isolates were identified by using Brilliance ESBL agar. Most of these isolates were multi drug resistant.

In *E. coli* 16% isolates were found carbapenemase producers and 6.5% isolates were MBL producers. No NDM -1 was found in these isolates.

All *Klebsiella* spp. were isolated from urine samples. The percentage of *Klebsiella* isolates were 38.5%. Carbapenems also showed good activity against *Klebsiella* spp. as well. Most of the *Klebsiella* isolates were multi drug resistant. In *Klebsiella* spp. 31.3% isolates were ESBL producers. Only one isolate was found carbapenemase producer.

P. aeruginosa were isolated from pus samples. Most of the isolates were resistant to more than three antibiotics. *P. aeruginosa* were also confirmed 16S rDNA by using PCR. All the isolates showed positive results.

MBL was also identified in 16.4% *P. aeruginosa* isolates. Ten isolates were resistant to imipenem.

RECOMMENDATIONS

Active role should be taken to protect our patients and healthcare workers from nosocomial infections. The following suggestions are made on the basis of current study results.

Correct and quick identification and antibiogram data of pathogenic organism

The correct and rapid identification of the pathogen will minimize the mobility and mortality rate. It also helps in correct therapy of the infection and also reduces the costs and hospital stay. This will also minimize the empirical therapy which is main cause of increasing antibiotic resistance in developing countries like Pakistan.

Culture and sensitivity test

The physician should encourage the patients for culture and sensitivity test this will helps the physician in appropriate prescription of the drugs for the infection.

Molecular diagnostic technique

In the laboratories, the diagnostic techniques at molecular level should be established. This will help the exact identification of the pathogenic agents.

Implementation of strict antibiotic policy

The antibiogram data of every organism should be recorded routinely. This will provide the information of sensitive and resistant organisms towards various antibiotics. This exercise must be done regularly to monitor antibiotic resistance in pathogenic bacteria.

Suggestions for patients

The following instructions should be followed by patients.

A: Patients should not use antibiotics without physician's prescription.

B: Avoid to pressurise your healthcare worker for the treatment of antibiotics common cold, flue or their viral infections.

C: Patients should take the full course of antibiotics.

Establishment of reference laboratory

Reference laboratory is necessary to cross check the identification and sensitivity report of the pathogenic organisms having unusual antibiogram pattern which should be sent to reference laboratory and which will provide further important information about the infections.

Establishment of Awareness Programs

Such programs will help in the awareness of surveillance of hospital and community acquired infections the hazards of misuse and overuse of antibiotics and also techniques of infection control.

Measures for minimizing the instrument related nosocomial infections

The instruments used in the hospital should be sterilized. Catheters should be used with great care to avoid the catheter associated bacteremia. The use of antibiotic added catheters should be encouraged.

Screening of MRSA

For the proper phenotypic detection of MRSA cefoxitin disc diffusion and MIC method should be preferred. The MRSA patients should be screened properly for colonization i.e. by doing cultures of nasal as well as the infected site. All the MRSA isolates must be screened by the reference laboratory as well. The screening of patients and health workers for MRSA colonization will also help in minimizing the spreading of MRSA in

both hospital and community settings. Such colonized patients and health workers should be treated fully till the MRSA is fully eradicated.

MRSA Flagging system will help in identifying those patients who were admitted because of MRSA infection previously. So such patients on readmitting can be easily identified and properly isolated.

Placement of patients according to the infection

MRSA patients should be kept separate from other patients in order to avoid spreading of MRSA. The health care worker should take proper care who come in direct contact with patients in order to avoid the spread of MRSA, similarly MRSA patients from community sitting should also be treated with great care and keep them in separate place from other patients.

Precisions for health workers

Health workers should take proper precautions while examining patients i.e. proper mask, gown must be put on. All these protective accessories should be changed after examining the patients. Those health workers, who are MRSA colonized must be treated, not allowed to control patients until become clear.

Sue of hand washes and disinfectants in both hospital and community setting

Hand washing is the most important step in reducing the risk of infections in both hospital and community settings. In hospitals before and after attending patients, the health worker must wash their hands with appropriate antimicrobial soap sanitizer. Even if gloves are used for the patients' examination hand must be washed after the removal of gloves. In hospital liquid soaps should be used. For the cleaning the surfaces in hospitals

disinfectants and detergents should be used on daily bases in order to minimize the rate of infection.

Health Care Education

All the staff members of hospital must be guided by the infection control team. The infection control guidelines must be given periodically in order to support the healthy habits i.e. hand washing.

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