MOLECULAR DETECTION OF *bla*$_{NDM-1}$ GENE IN BACTERIA CAUSING URINARY TRACT INFECTIONS

A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Philosophy

In

Microbiology

By

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Islamabad 2012


بسم الله الرحمن الرحيم

DEDICATED

TO

My dear ***Father***

My Sweet & loving ***Mother***

&

My beloved ***HUSBAND***

DECLARATION

The material contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

SANA REHMAN

CERTIFICATE

This thesis submitted by SANA REHMAN is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan; as fulfilling the thesis requirement for the degree of Master of Philosophy in Microbiology.

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Dated: 05-10-2012

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LIST OF ABBREVIATION

AMR	Antimicrobial Resistance
ARG	Antibiotic Resistance Genes
E.coli	Escherichia coli
CLSI	Clinical Laboratory Standard Institutes' criteria
EDTA	Ethylene Diamine Tetra Acetic acid (acetate)
OPD	Out Door Patients
ESBL	Extended-spectrum beta-lactamases
IPD	Indoor Patients
MBL	Metallo-beta-lactamases
MDR	Multidrug-Drug Resistant
MHA	Mueller Hinton Agar
NaCl	Sodium Chloride
NDM1	New Delhi Metallo-beta-lactamases
UTI	Urinary Tract Infection
Mg	Milligram
PCR	Polymerase Chain Reaction
PK	Proteinase k
µg	Microgram
SDS	Sodium Dodecyl Sulfate
USA	United States of America
WHO	World Health Organization
Ul	Microliter
GNR	Gram Negative Rods

Acknowledgement

Praise be to **Allah, Lord of the Worlds**. The Beneficent, the Merciful, Who is the entire source of knowledge and wisdom endowed to mankind; Who gave me courage and potential to pursue this goal Whom I believe that He never spoils any effort of good deeds. Blessings of Allah be upon **His Prophet Mohammad (P.B.U.H.)**, The City of knowledge and blessing for entire creature, who has guided his Ummah to seek knowledge from Cradle to Grave, and enabled me to win honor of life.

It is a matter of great pleasure to express my sincere regards to my honorable supervisor **Dr. Rabaab Zahra,** Assistant Professor, Department of Microbiology for affectionate supervision, inspiring attitude, masterly advice and encouragement. Without her useful intellectual suggestions, it would have been impossible for me to complete this tedious work. I would like to extend my thanks to **Dr. Safia Ahmad,** Chairperson of the Department Microbiology, for providing me all the privileges during my research work. I am also really thankful to **Dr. Fariha Hasan** for her appreciable cooperation, kind help and practical guidance.

Words are inadequate to express my thanks to my honorable seniors, , **Faisal Rasheed, Tawaf Ali Shah, Zeeshan Haider Naqvi,** and **Fouzia**, and my colleague **Mohammad Kashif Munir** for their valuable suggestions, extensive help, critical and constructive discussions, for their help and cooperation during my research work.

With deep sense of gratitude and benevolence, I offer words of appreciation to my friends **Mohammad Usman Qamar, Mehnaz Akram, Sana Mumtaz, Irum subtain, Rani kiran, Marayam adnan, Urooj kiran , Sidra Murtaza, Tayiba saba, Saniya Saleem, Tehreem Naz, Afshan and Waqas Ahmad.** I cannot forget to thank my junior lab fellows, **Asma khan, Neelam zaib, Ameer** and **Adam sher Khan** who provided me a pleasant environment and company during my research work.

Words fail to express the intensity of gratitude to my loving **Grandfather, Grandmother, loving Father** and my **husband** whose prayers and inspiration gave me the courage to dream high and opened up avenues for me to explore my abilities, my sweet and loving **Mother** whose self-sacrificing love will always be cherished but can never be repaid.

SANA REHMAN

In healthcare system, antibiotic resistance has been an increasing problem. New Delhi Metallo-β-lactamase 1 (NDM-1), a novel Metallo-β-lactamase reported for the first time in 2008 in *K. pneumonia* was isolated from a patient in India. This novel enzyme (NDM-1) enables the bacteria to resist wide range of beta-lactam antibiotics by hydrolyzing them and inactivating their activity. In Pakistan, there is no adequate data available to estimate the updated prevalence of NDM-1 positive pathogens causing Urinary Tract Infections (UTIs). This study was conducted to detect the prevalence of $bla_{\text{NDM-1}}$ gene in bacterial isolates causing UTI.

A total of 130 Gram negative isolates were collected from UTI samples submitted to two tertiary care hospitals; Pakistan Institute of Medical Sciences (PIMS), Islamabad and Mayo hospital Lahore. These isolates were further confirmed by conventional biochemical tests. Out of 130 isolates, the majority were collected form PIMS (n=75) among which dominant microorganisms were *E. coli* (41.5%), *K. pneumoniae* (22.2%) *P. aeruginosa* (20.2%), *Proteus spp.* (12.1%) and *Enterobacter spp* (4%) while 55 isolates were collected from Mayo Hospital, Lahore among which predominant were *E.coli* (41%), *P. aeruginosa* (25%) *K. pneumoniae* (23%) and *Proteus spp.* (11%). Most of the samples collected were from patients between the age range of > 41 years to <60 years. Metallo-beta-lactamase production in pathogens was screened by phenotypic combine disk method and Modified Hodge Test (MHT). Out of 130 isolates, 68 (52.3%) isolates were carbapenem resistant while 42 (32.3%) showed both carbapenamase and MBL production and 20 (15.4%) were Non-carbapenamase and MBL producers. Molecular detection of $bla_{\text{NDM-1}}$ gene was carried out through PCR amplification which demonstrated the presence of $bla_{\text{NDM-1}}$ gene in 7 (16.6%) out of 42 MBLs producing pathogens. Among these, three microorganisms were identified as *P. aeruginosa*, two as *E. coli* and two as *K. pneumoniae* (n=2). In conclusion, the study reveals a high rate of antimicrobial resistance and NDM-1 positive microorganisms in UTI patients. Therefore a surveillance study should be implemented in different hospitals of Pakistan, and up-to-date information is required to know the exact prevalence of this MBL in pathogenic strains causing UTI.

Keywords: Metallo-beta-lactamases, $bla_{\text{NDM-1}}$, meropenem, UTI.

Chapter 1

Chapter 1

INTRODUCTION

1.1. Urinary Tract Infection (UTI):

The continuous occurrence of vigorously multiplying microorganisms within the urinary tract can be defined as UTI. In general, urine is sterile but UTI can be the result of microbial colonization of the urine or it can be due to invasion of the lower or upper urinary tract by the microorganisms (Bano *et al.*, 2012).

1.2. Classification of UTIs:

Four major types of UTIs have been recognized such as uncomplicated, complicated, asymptomatic and symptomatic UTIs (Mittal *et al.*, 2004; Nicolle, 2008).

Complicated UTI is mainly caused by anatomic deficiency which includes vesicouretic reflux (VUR), obstruction, surgery and metabolic diseases like diabetes mellitus and generalized immunosuppression especially in patients with organ transplantation (Bonadio *et al.*, 1999; Munoz *et al.*, 2001; Leone *et al.*, 2003). Catheterization of urinary tract is the main cause of complicated UTIs which predisposes the host to infection (Saint and Chenoweth, 2003; Shaw *et al.*, 2005). As a result of catheter placed inside the bladder, mucosal layer may become injured which disrupts the natural obstacle and allows colonization of bacteria (Kalsi *et al.*, 2003) and microbes can access through extra luminal route by moving across the outer lumen of catheter or by intra luminal route by directly entering the interior of catheter (Logan, 2003).

The patients having a normal, unobstructed genitourinary tract, with no account of fresh instrumentation are typically affected by uncomplicated UTI and symptoms remain limited to the lower urinary tract. Young sexually active women are mainly susceptible to this kind of UTIs (Mehnert-Kay, 2005).

Asymptomatic bacteremia (ASB) can be defined as two consecutive clean-catch midstream urine specimens of women with $\geq 10^5$ colony forming units (CFU)/ml of the same urinary tract pathogen in a patient without symptoms of UTI with no use of any urinary catheter within seven days of the first urine culture (Garner *et al.*, 1988; Schmiemann *et al.*, 2010). On the other hand it may also be defined as a single clean-catch midstream sample growing $\geq 10^5$ CFU/ml without any symptoms in men (Schmiemann *et al.*, 2010).ASB has been reported in

10% of men with ≥ 65 years of age and 30% of female patients of same age living at home, and up to 55% of females and a range of 15% to 40% of men inhabiting within long term care facilities (Nicolle, 2001; Ariathianto, 2011).In the aged inhabitants, *Escherichia coli* is the most commonly identified urinary tract pathogen, however, this percentage is very low in patients with symptomatic UTIs (Hassanzadeh and Motamedifar, 2007).

According to Centre for Disease Control and Prevention (CDC), symptomatic UTI can be defined as a clean-catch urine culture which grows ≥ 10^5 CFU/ml of no more than two species of pathogens in a patient with symptoms of UTI (Garner *et al.*, 1988). Another explanation projected by the CDC includes two symptoms from the following; fever >38 °C, dysuria, frequency of micturition, urgency in micturition, or suprapubic pain, and at least one of the following symptoms; positive Gram stain, pyuria ≥10 white blood cells/ml^3, presence of leukocyte esterase enzyme and nitrite done by dipstick method or two positive urine cultures with the same pathogen (≥10^2) in a non voided specimen (Nicolle *et al.*, 2005).

1.3. Epidemiology of UTI:

UTIs have turned out to be the most frequent disease encountered in clinical practice and are one of the most commonly diagnosed cases. The annual burden of UTIs is expected to be 150 million globally (Gobernado *et al.*, 2007). Patients of all ages, infected with GNRs are the main source of UTI and is linked with an elevated rate of morbidity and mortality, predominantly in aged population (Ahmadzadeh and Askarpour, 2007; Hasan *et al.*, 2007). UTI is responsible for 8 million physician visits (84% by women) and around 100,000 hospitalized patients annually in United States only (Memon, 2007). Although UTIs occur in both genders of all ages, they are more prevalent in females. Two out of one female has suffered from UTIs at least once in a life time with 12% of initial infection and 48% with repeat episodes (Hummers-Pradier and Kochen, 2002). UTIs are considered as the most common nosocomial infections and accounts for around 35% of all hospital based nosocomial infections annually in the world. From uncomplicated urinary tract infection *Escherichia coli* is commonly isolated pathogen in UTI (Kariuki *et al.*, 2007; Taneja *et al.*, 2010).

1.4. Incidence of UTI:

UTIs are frequently seen in women and reported range is up to 12%. Mostly by 32 years of age, women are reported to have suffered from at least one UTI (Foxman and Brown, 2003). Infection of cystitis among young, healthy individuals recurs in 25% of women within six months after the primary UTI (Foxman *et al.*, 2000) and the repetition rate enhances with more than one previous UTI (Czaja *et al.*, 2009). Acute uncomplicated pyelonephritis is greatly less frequent (estimated ratio, 1 case of pyelonephritis to 28 cases of cystitis) with a peak annual frequency of 25 cases per 10,000 women 15 to 34 years of age (Czaja *et al.*, 2007).

1.5. Microorganisms causing UTIs:

UTIs occur due to both Gram positive cocci (GPC) and Gram negative rods (GNR). However most prominently it is caused by GNR (Memon, 2007). Microorganisms causing UTIs more or less come from the skin at or near the aperture of the urethra. Uropathogens differ in virulence factors and pathogenic mechanisms that allow them to colonize and communicate a disease in urinary tract. Gram negative bacteria are responsible for 80-85% of UTIs where as Gram positives are responsible for 15-20% of the cases. Among GNR, the primary contributory microbe is *E.coli* causing 75% to 95% of all the UTIs cases and other GNR include; *Klebsiella spp*, *Proteus spp*, *Enterobacter spp*, *Pseudomonas spp*, and *Serratia spp.* while, among GPC, most common microbes belong to group B *Streptococci*, *Enterococcus* spp, *Staphylococcus aureus* and *Staphylococcus saprophyticus*. (Ehinmidu, 2003; Bakhsh *et al.*, 2006; Kariuki *et al.*, 2007; Tanvir *et al.*, 2012).

1.6. Antimicrobial Resistance:

Resistance to antimicrobials is an ever increasing problem in our hospitals. Antimicrobial resistance can be defined as the ability of microorganisms to grow and multiply in the presence of antibiotics (Macgowan, 2008). Multi Drug Resistance (MDR) can be reckoned as bacterial resistance to more than three antibiotics at the same time (Obritsch *et al.*, 2005).Resistance to β-lactam drugs is associated with production of β-lactamase enzymes that have ability to degrade beta-lactam ring by hydrolyzing it. Among these enzymes extended spectrum-β-lactamases (ESBLs) and Metallo-β-lactamases (MBLs) are widespread. The last remedy for these resistant

strains is the treatment with carbapenems (Bradford, 2001). The bacterial resistance against carbapenems drugs is due to the production of MBL. In clinical bacterial isolates, carbapenemases are also responsible for resistance in other β-lactam antimicrobial agents. In Enterobacteriaceae, these carbapenemases cause antibiotic resistance and contribute to risk of public health (Bush and Jacoby, 2010). There are two families of β-lactamases which include; (a) serine-β-lactamases and (b)Metallo-β-lactamases. In Serine-β-lactamases, serine is required at their active site while in MBLs zinc ions are essential co-factors and a chelating agent like EDTA is needed to inhibit these enzymes. The main types of MBLS are IMP-type, VIM-type, SPM-type (Osano *et al.*, 1994). The genes for MBLs are present on chromosomes and also on mobile DNA (Rolain *et al.*, 2010). The novel type of MBL, NDM-1 is very vital because its presence has been identified on chromosomes and plasmids, beside with movable gene cassettes, integrons, and insertion sequences. For distribution and spread to other strains in our surroundings, the insertion sequences are essential for mobility and motility (Walsh *et al.*, 2011).

1.7. New Delhi Metallo-β-lactamase-1 (NDM-1):

New Delhi metallo-β-lactamase 1 (NDM-1), was first identified in 2008 in single isolates of *K. pneumonia* and *E.coli* from a patient admitted in Sweden after taking treatment at a hospital in New Delhi, India (Yong *et al.*, 2009). Apart from Aztreonam, this enzyme has the ability to hydrolyze all β-lactam antibiotics by combined production of ESBL and/or AmpC β-lactamases. *Enterobacteriaceae* with $bla_{\text{NDM-1}}$ is extensively resistant to antimicrobials and is sensitive only to colistin and tigecycline. In various countries, this new NDM-1 has been found, mostly in patients with connection of Indian subcontinent. Patients suffering from various indications, including optional surgery and renal dialysis contained NDM-1 gene and had a travelling history to India or Pakistan (Kumarasamy *et al.*, 2010; Peirano *et al.*, 2011).

1.8. Epidemiology of $bla_{\text{NDM-1}}$:

From August 2010, the distribution and spread of NDM-1 positive strain occurred worldwide in countries including United States, Canada, Sweden, United Kingdom, Austria, Belgium, France, Netherlands, Germany, Africa, Oman, Australia, Japan and China (Kumarasamy *et al.*, 2010; Rolain *et al.*, 2010; Peirano *et al.*, 2011). It was anticipated that NDM-1 positive clinical isolates

were recognized in patients who took treatment or travelled in recent times to India, Pakistan or Bangladesh (Kumarasamy *et al.*, 2010; Poirel *et al.*, 2010). This has directed to the conviction that new bla_{NDM-1} gene originated from India. Additional research was carried out in India in order to estimate its prevalence in the country and it was found that the frequency of incidence of bla_{NDM-1} was high in *Enterobacteriaceae*. (Kumarasamy *et al.*, 2010). NDM-1 prevalence was mostly reported in *E.coli* and *K. pneumoniae* with few reports in other enterobacterial species. The bacteria containing NDM-1 cause a variety of infections, involving UTIs, pulmonary infections, septicemia, gastritis, peritonitis, infections involving soft tissues and contraceptive or other device-associated infections (Yong *et al.*, 2009; Poirel *et al.*, 2010).

In Pakistan, there is not sufficient information regarding the recent dominance of NDM-1 in Gram negative bacteria responsible for UTIs. Fecal specimens of 200 patients from combined military hospitals, Rawalpindi were examined to know the presence of NDM-1 containing isolates. The prevalence of bla_{NDM-1} gene was 18.5% in isolates from fecal specimens (Perry *et al.*, 2011). Due to lack of adequate knowledge regarding NDM-1 spread in Pakistan, our healthcare system is at high risk. There is a considerable requirement for advance information about the accurate prevalence of this MBL in pathogens causing UTI.

Aim and Objectives of the Study:

The aim of this study was to determine the prevalence of *bla*$_{NDM-1}$ gene in bacteria causing UTIs.

Objectives:

- To find out antimicrobial resistance pattern of organisms isolated from UTI patients.
- To determine the carbapenamase activity by Modified Hodge's test (MHT).
- To determine the Metallo-β-lactamase activity by Disc Potentiating method.
- To detect the presence of *bla*$_{NDM-1}$ gene in isolated strains by PCR technique.

Chapter 2

REVIEW OF LITERATURE

Antibiotic era started with the discovery of first antibiotic penicillin in 1940s. Within few years of the introduction of clinical use of first drug, penicillinase producing *S.aureus* was discovered which started the battle between antibiotics and microorganisms. Since then resistance against antibiotics has developed particularly by the production of β-lactamase enzymes such as; Extended-spectrum-β-lactamases (ESBLs), Carbapenamase and Metallo-β-lactamases (MBLs) (Gootz, 1990).

2.1. Classification of β-lactam drugs:

These are broad range of drugs which contain β-lactam ring that are hydrolyzed by the enzymes β-lactamases. As β-lactam is a large group of antibiotics, it is classified as follows:

1. Penicillin (penicillin G, penicillin M)
2. Cephalosporins (cefuroxime, ceftriaxone, ceftazidime, cefepime)
3. Carbapenems (imipenem, meropenem, ertapenem)
4. Monobactam (Aztreonam)
5. β-lactam inhibitors (clavulanic acid, sulbactam, tazobactam)

As more resistance developed, more drugs were introduced into market to overcome this problem (Gootz, 1990; Edwards and Betts, 2000). β-lactam drug structure is shown in Figure I.

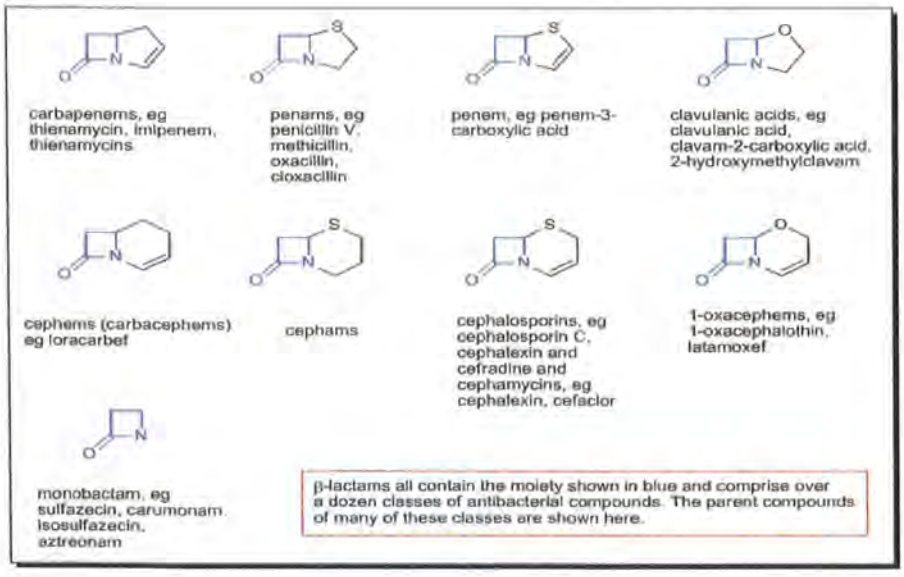

Figure1. Classes of β-lactam antibiotics

2.2. Mechanism of β-lactam resistance:

β-lactam drugs can produce resistance by five main mechanisms which are; β-lactamase enzymes, target alteration, porin proteins loss, efflux pump and mutation. There are many factors which can lead to Anti-Microbial Resistance (AMR). Firstly, cross contamination of pathogens due to unwashed hands of health care workers. Secondly, colonization of microbes that can leads to infections. Thirdly, irrational use of antimicrobial drugs leads to AMR (Fridkin and Gaynes, 1999).

2.3. Epidemiology of antimicrobial resistance in UTI patients:

Epidemiology of AMR in UTIs patients varies throughout the world. A study conducted in USA reported that urine culture was positive in 30% UTI patients. Predominant microbes were *E. coli* (n=44) followed by *Serratia spp* (n=11) and *E. fergusonii* (n=10). *E. coli* showed resistance against ampicillin (62%), cefalothin (46%), trimethoprim–sulfamethoxazole (39%) ciprofloxacin (32%) and ceftriaxone (21%). More effective drugs against these pathogens were amikacin and nitrofurantoin. Another study conducted in USA (2000-2010) documented a high resistance pattern of SXT (1%- 24%), ciprofloxacin (3%-17%) ceftriaxone (0.5%-2%) and nitrofurantoin(1%-2%) against *E.coli* isolated from urine. During this period an increased number of MDR *E. coli* isolate from UTI patients was observed (Sanchez *et al.*, 2012). A study form London (UK) found that more energetic mean of drug against UTI isolates was nitrofurantoin followed by cefpodoxime and gentamicin. They also reported ESBLs production in community (6%) and nosocomial infections (22%)(Bean *et al.*, 2008). A Canadian study revealed the resistance of UTI isolates for SXT (19%), ampicillin (41%), ciprofloxacin (5%) and nitrofurantoin (2%). The highest resistance of drugs was found against *E. coil* particularly for SXT, ampicillin and ciprofloxacin (Zhanel *et al.*, 2000). An Australian study documented that 97 pediatrics were positive for UTI and mostly infected by *E. coli* (n=90) and *P. mirabilis*(n=5). The resistance pattern of these organisms against ampicillin/amoxicillin, SXT, cephalothin was 52%, 14% and 24% respectively (Mehr *et al.*, 2004). A study from Germany reported *E.coli* (63.0%) followed by *Proteus spp.* (6.0%), *Klebsiella spp.*(3.0%), *Enterobacter spp* (1.5%) and *Pseudomonas spp.* (2.0%) in UTI patients (Beetz and Westenfelder, 2011). An African study on UTIs revealed that most commonly isolated microbes were *E.coli* (n=600), followed by, *K. pneumoniae* (n=87), *S. aureus* (n=35) and *P. mirabilis* (n=32). Seventy-seven per cent of GNR

showed resistant to amoxicillin (77%), SXT (66%), ciprofloxacin (15%), ceftriaxone (6%). Most pathogens were sensitive to nitroxolin. ESBLs producing *E. coli* were significantly found during the period of 2005-2006. Among GPC, *Staphylococcus spp.* and *Streptococcaceae* were resistant to penicillin (10%) and oxacillin (8%) (Randrianirina *et al.*, 2007). Another African study reported the presence of 84% *Enterobacteriaceae* in UTI patients among which 56% were *E.coli*, 17% were *K. pneumoniae*, 4% were *C. diversus* and 4% were *Salmonella spp.*Whereas 4% were non-*Enterobacteriaceae* isolated among which *P. aeruginosa* and *Acinetobacter spp.* accounted for 3.5% isolates. A total number of 10% isolates were GPC in which *S. aureus, S. agalactiae, E. faecalis* were 4.5%, 4.0% and 1.0% respectively (Hima-Lerible *et al.*, 2003). Surveillance reports from Netherland revealed that 10 – 20% of urinary pathogens were *E.coli* isolates from female outpatients which were mainly resistant to SXT (Karlowsky *et al.*, 2003). At Dalhatu Araf Specialist Hospital, Nigeria it was reported that 60% patients were positive for UTIs. Majority were *E. coli, P. aeruginosa, S.aureus,* and *P. mirabilis*. AMR revealed that GNR were sensitive to quinolones (ofloxacin, ciprofloxacin, pefloxacin) and erythromycin, while GPC were sensitive to lincomycin, erythromycin and quinolones (ofloxacin, ciprofloxacin, pefloxacin) (Kolawole *et al.*, 2009). An Asian study on UTIs reported majority of pathogens as *E.Coli* (30%), followed by *Pseudomonas spp.* (13%) *Klebsiella spp.* (10%) and *Enterococcus spp.* (10%). Resistance pattern of the organisms were below 45% for commonly used antibiotics. *Pseudomonas spp* and *Enterococcus spp* reported 70% resistant rates (Johansen *et al.*, 2006). A Malaysian study on UTIs revealed that UTIs cases were 57.1% positive. Majority of antibiotics prescribed were penicillin, macrolides and SXT which showed resistance (Teng *et al.*, 2011). An Indian study at Bangalore hospital reported 12 different species of uropathogens from 6350 cases. Among them 59% were *E.coli*, 12% were *Klebsiella spp*, 10% were *Enterococcus spp.* and 9.0% were *Pseudomonas spp.* Highest resistance was found against ampicillin (80%) followed by ciprofloxacin (73%), amoxicillin (70%), and norfloxacin(53%). Whereas most effective drugs against these uropathogens were penicillin, azithromycin, furomycin, linezolid, doxycycline and novobiocin (Manjunath *et al.*, 2011). Another Indian study also revealed that major uropathogens were *E.coli* (32%), followed by *S.aureus* (20%), *K. pneumonia* (16%) and *P. mirabilis* (8.0%). More than 80% of microbes showed resistance to SXT and Nalidixic acid, while 60% were resistant to amoxicillin and 45% were resistant to gentamycin, ciprofloxacin and cephalexin. Among GPC, *S.marcescens* and *Staphylococcus spp* showed susceptibility against ciprofloxacin,

gentamycin and cephalexin(Manikandan *et al.*, 2011). A similar study on UTI reported that 61% were *E.coli* and 22% were *Klebsiella spp.* In GNR, ampicillin and SXT showed high resistance rate. ESBL producing *E. coli* (35%) were also identified among these pathogens (Akram *et al.*, 2007). A study on uropathogens from Iran revealed the presence of *E. coli* (n=920) followed by *Klebsiella spp* (n=412), *Enterococcus spp* (n=202) and *Pseudomonas spp.* (n=158).About 30% resistance was shown by *Pseudomonas spp.*, *Klebsiella spp.* and *Enterobacter spp*, whereas *E. coli* showed 68% resistance against carbenicillin, 96% againt ampicillin , 70% against SXT 65% and 65% against kanamysin (Ranjbar *et al.*, 2009).

A study from Pakistan identified bacterial isolates as *E.coli* (46.98 %) and *E.cloacae,* methicillin resistant *S. aureus* (MRSA), *Staphylococcus saprophyticus* (1.20 %). *K. pneumoniae* showed highest sensitivity (80%) to cefepime and low susceptibility (13%) to ciprofloxacin, while the highest resistance (60%) to gentamicin and the lowest (6%) to meropenem, nitrofurantoin and ciprofloxacin. The susceptibility of *S. aureus* was highest (64%) to amikacin, augmentin and oxacillin and lower sensitivity for ampicillin and moderate for erythromycin, methicilline, and cefotaxime with 45% outcome (Bano *et al.*, 2012). Another Pakistani study on UTIs reported that major pathogens were *E. coli* followed by *P. aeruginosa* and *K. pneumoniae.* Antibiotic susceptibility pattern of the pathogens against co-amoxiclav, ciprofloxacin, cefotaxime, ceftriaxone, amikacin, Imipenem and pipracillin/tazobactam was 55%, 63%, 75%, 95% and 86% respectively (Naeem *et al.*, 2010). Another similar study from Pakistan on UTIs found that *E. coli* was most frequent causative agent (66%) followed by *Enterococci spp.* (8.3%), *Candida spp.*, *Pseudomonas spp.* (7.3%), *Klebsiella sp.p* (5.5%) and *Enterobacter sp.p* (2.7%). Another study on uropathogens identified *K. pneumoniae* (n=92) where 59% isolates were ESBL producers. Most of the pathogens were reported as MDR and most effective drugs were carbapenems (Ullah *et al.*, 2009). Another study from Pakistan found that *E. coli* were the predominant microorganism in urine samples. High prevalence of class 1 integron (43.56%), sulfamethoxazole resistance genes *sul1* (45.54%) and *sul2* (51.48%) along with occurrence of quinolones resistance genes was detected in multi drug resistance isolates (Muhammad *et al.*, 2011).

2.4. Classification of β-lactamases:

These enzymes have the ability to hydrolyze the β-lactam drugs. They are broadly classified into two main classes; Ambler class on the basis of homology in amino acid and Bush-Jacoby-Medieros class on the basis of biochemical properties (Queenan and Bush, 2007).

Ambler Molecular is further subdivided into four classes (A, B, C, D). A, C and D types require serine residue on the active site of the enzyme for their activity. Class A contains Pencillinases, TEM and SHV type β-lactamases and carbenicillin hydrolyzing β-lactamases, class C contain chromosomal and plasmid mediated Amp C enzymes, and class D contain OXA type β-lactamases. Whereas, class B requires zinc ion at their active site and includes Metallo-β-lactamases (MBLs) which are further divided into IMP, GIM, SPM and NDM.

Bush-Jacoby-Medieros class is divided into four main classes (1, 2, 3, 4). Classes 1 contain Amp C type enzymes. Class 2 is further sub-divided into 2a (penicillinase), 2b (TEM-1, SHV-1 type enzymes with inhibitors sensitive), 2be (ESBLs), 2br (TEM-1, SHV-1type enzymes but inhibitors resistant), 2c (carbenicillin hydrolyzing β-lactamases), 2e (cephalosporinase), 2r (Serine carbapenemases inhibited by calvulanic acid), and 2d (OXA type hydrolyzing β-lactamases). Class 3 is further divided into 3a, 3b, 3c (MBLs type enzymes) and class 4 include penicillinase which do not belong to other group (Queenan and Bush, 2007; Cornaglia *et al.*, 2011).

2.5. Metallo-β-lactamase enzymes (MBLs):

These are Ambler class B enzymes require zinc ion as a cofactor for their activity. Their activity will be lost by chelating these ions with ethylene-diamine-amino tetra acetic acid (EDTA). There are more than 900 sequences reported among which about 80 sequences are of MBLs (Bush and Jacoby, 2010). There are nine different types of acquired MBLs reported as shows in Table 1. The most clinically relevant and epidemiological disseminated enzymes are; IMP-type, VIM-type, SPM-type, and NDM-type enzymes (Cornaglia *et al.*, 2011).IMP-Type (IMP-1, IMP-2, IMP-3, IMP-4,IMP-5,IMP-6,IMP-7,IMP-8,IMP-9, IMP-10, IMP-11 IMP-12,IMP-13) which are detected in different countries including Japan, Italy,China,Taiwan and Portugal and these were reported in *P. aeruginosa, Enterobacteriaceae, A. baumannii, A. xylosoxidans.* While IMP-14,IMP-15 and IMP-16 were detected in Thialand, Maxico and Brazil and these enzymes were reported in *P. aeruginosa* (Cornaglia *et al.*, 2011).

2.5.2IMP-type enzyme:

First IMP-type enzymes were reported in late 1980s in Japan (Osano *et al.*, 1994). Then these types of enzymes speared globally in almost all the Gram negative isolates such as *E. coli, K. Pneumoniae, and P. aeruginosa, E. cloacae, and Acinetobacter spp.* The diversity of IMP enzymes (IMP-1, IMP-4, and IMP-7) has spread rapidly throughout the world (Miriagou *et al.*, 2010).

2.5.3VIM-type enzyme:

This enzyme was first detected in *P. aeruginosa* and *Enterobacteriaceae.* These enzymes show broader area for target substrate binding than IMP-types which enable them to hydrolyse 6-α-methoxy-penicillins. Moreover, these enzymes have also great affinity for carbapenems drugs (Docquier *et al.*, 2003). VIM-1 type of enzymes have been reported in different countries; VIM-1 in *E. coli, E. cloacae, K. pneumoniae* in Greece and France (Giakkoupi *et al.*, 2003; Miriagou *et al.*, 2003; Galani *et al.*, 2005; Kassis-Chikhani *et al.*, 2006), VIM-2,VIM-4 and VIM-5 were reported in *K. pneumoniae, P. aeruginosa, E .cloacae* in Portugal, France, Greece, Italy, Spain, Korea, and Turkey (Mavroidi *et al.*, 2000; Pallecchi *et al.*, 2001; Poirel *et al.*, 2001; Cardoso *et al.*, 2002; Lee *et al.*, 2002; Prats *et al.*, 2002; Luzzaro *et al.*, 2004; Gacar *et al.*, 2005).

2.5.4. SPM-1 type enzyme:

These were first time reported in Brazil from *P. aeruginosa* (Toleman *et al.*, 2002). Since then it is spread from Brazil to other parts of the worlds due to mobile genetic elements. These have wide range of substrate specificity for penicillins, cephalosporins and carbapenems (Murphy *et al.*, 2003).

2.5.5. NDM-1 type enzyme:

This enzyme is one of the novel types of MBLs which disseminated throughout the world with great transferable activity. NDM-1 was first detected in *K .pneumoniae* isolated in a Swedish patient returning from India in 2008 (Kumarasamy *et al.*, 2010).

2.5.6. SIM-1, GIM-1, AIM-1, and DIM-1 type enzyme:

These types of MBLs have less clinical impact and dissemination rate than NDM-1(Kumarasamy *et al.*, 2010).

There is less data available in Pakistani population so far on carbapenamases and MBLs. A case reported from Pakistan indicated the presence of carbapenamase and MBLs in *Acinetobacter spp.* and *P. aeruginosa* (Mirza, 2007; Kaleem et al., 2010)

2.6. New Delhi Metallo-β-lactamase (NDM-1):

Prevalence of NDM-1is different in different countries. This enzyme is encoded by gene $bla_{NDM-1}$and has the ability to hydrolyze broad class of β-lactam drugs except colistin. First NDM-1 was identified in *K .Pneumoniae* and *E. coli* isolates. A Swedish patient of 59 years old man was hospitalized twice in Indian hospitals in 2007 and 2008. There he acquired UTI and original carbapenem resistant *K. pneumoniae* and MBLs producing *E. coli* from urine and stool samples were isolated. NDM-1 producing *K. pneumoniae* showed resistance to all β-lactams and was sensitive to colistin whereas, *E.coli* was susceptible to ciprofloxacin, Aztreonam and cefepime (Yong *et al.*, 2009). NDM-1 is usually identified in clinical isolates of Gram negative pathogens that can lead to multidrug resistance. Mostly NDM-1 producer's pathogens showed sensitivity only against polymixin B and tigecycline (Walsh *et al.*, 2011). After this, NDM-1 was found in different countries particularly in those patients who had travelling history of Indo-Pak subcontinent. Therefore different studies were carried out in this region to find out the prevalence of NDM-1 producing organism. In Iraq, first bla_{NDM-1}producing *K. pneumoniae* was isolated in rectal swab of the patient in 2010. This report lead to the conclusion that NDM-1 producing microbes were also present in Middle East and that could be another reservoir for NDM-1 producers(Poirel *et al.*, 2011b).In Morocco, three bla_{NDM-1} producing *K. pneumoniae* were identified and these patients had no travelling history of Indian subcontinent (Poirel *et al.*, 2011a). Poirel*et al.*, (2010) reported bla_{NDM-1} harbouring *C. freundii* from a French woman in urine sample with history in Indian hospital admission and UTI from the urinary catheter (Poirel *et al.*, 2010a). First African report of NDM-1 was reported in Kenya (2010). They found 7 bla_{NDM-1} producing *K. pneumoniae* in patients hospitalized during 2007-2009 (Poirel *et al.*, 2011c). Later on another report of emergence of NDM-1 was found in South Africa (Brink *et al.*, 2012). Kumarasamy *et al* (2009) documented the prevalence of NDM-1 producing pathogens as 44% among the carbapenem-resistant isolates in UK. Among the 73 carbapenem-resistant

isolates, 37 were NDM-1 producer. Among these 37 NDM-1 producing microbes, predominant were *K. pneumoniae* (n=21), followed by *E. coli* (n=7), *Enterobacter spp.* (n=5), *Citrobacter freundii* (n=2), *Morganella morganii* (n=1) and *Providencia spp.* (n=1) (Kumarasamy *et al.*, 2010). After the first detection of NDM-1 positive strains now they have spread to different countries globally. It has been reported in Canada (Tijet *et al.*, 2011) Japan (Chihara *et al.*, 2011), Austria (Zarfel *et al.*, 2011), Belgium (Bogaerts *et al.*, 2011), Switzerland (Poirel *et al.*, 2011d), and Serbia (Jovcic *et al.*, 2011). In Israel, first bla_{NDM-2} producing *A. baumannii* was isolated with no travelling history or hospitalization in Indian subcontinent (Espinal *et al.*, 2011). Another novel NDM, bla_{NDM-1} producing *E. coli* was identified in Israel (Nordmann *et al.*, 2012).

Prevalence of NDM-1 positive pathogens from India and Pakistan was investigated by Kumarasamy *et al.*, in 2010. In India, mostly NDM-1 producing organisms were found in Mumbai (n=32), Varanasi (n=13) and Guwahati (n=3) whereas, in Pakistan, from eight different cities 25 isolates were found to be NDM-1 positive (Kumarasamy *et al.*, 2010). Roy *et al* reported the presence of bla_{NDM-1} producing *K. pneumoniae* septicemia (Roy *et al.*, 2010). A SENTRY antimicrobial surveillance program (2006-2007) found 15 strains harboring bla_{NDM-1} out of 39 carbapenems-resistant microbes. This provides the evidence that NDM-1 producers pathogens were also present in India in 2006 (Castanheira *et al.*, 2011).

In Pakistan there is only little published data on NDM-1 positive pathogens. In a Military hospital, a study on stool samples from 200 patients was carried out. They reported a high prevalence of NDM-1 (18.5%) among these samples which required an important consideration (Perry *et al.*, 2011).

2.7. New Delhi Metallo-β-lactamase in UTIs isolates:

Prevalence of NDM-1 in UTIs patients is different in different parts of the world. Interestingly, first NDM-1 was isolated in *E. coli* from UTIs patients in 2008 in Sweden (Kumarasamy *et al.*, 2010). Recently, one *K. pneumoniae* and two *E. coli* strains were found to be NDM-1 producers from UTIs patients in Thailand (Rimrang *et al.*, 2012). CDC (2012) reported that a urine specimen from a Vietnamese patient was found to have a NDM-1 producing *K. pneumoniae* which was only sensitive to colistin, tigecycline, and polymxin B (CDC 2012). An outbreak of

NDM-1 producing *K. pneumoniae* (n=3) and *E. coli* (n=1) was found in urine sample in Italy (Gaibani *et al.*, 2011). A study from India reported that out of 19 *K. pneumoniae* urine isolates, 11 were *K. pneumoniae* which was NDM-1 producers (Bora and Ahmed, 2012). Another study from Mauritius reported a *K. pneumoniae* producing NDM-1 in urine sample (Poirel *et al.*, 2012). A NDM-1 producing *K. pneumoniae* was identified from UTIs patient in Sweden (Yong *et al.*, 2009). A Kenya study found that of 7 NDM-1 producing *K. pneumoniae*, 5 organisms from urine sample were NDM-1 producers (Poirel *et al.*, 2011c). In Australia, emergence of NDM-1 producing *E .coli* was isolated in urine sample (Poirel *et al.*, 2010b). In Canada, both NDM-1 producing *E. coli* and *K. pneumoniae* were isolated from UTIs patient (Peirano *et al.*, 2011a). Another Indian study revealed that out of 24 carbapenem-resistant GNR, 6 were NDM-1 positive *E.coli* followed by 5 NDM-1 positive *K. pneumoniae* and 1 NDM-1 positive *Morganellamorganii* in urine samples (Deshpande *et al.*, 2010).

In Pakistan there is low data available on NDM-1 producing organisms from UTIs patients or urine samples. Therefore, we design this study to know the prevalence of $bla_{\text{NDM-1}}$ gene in UTIs in patients in Pakistani population.

Chapter 3

MATERIALS AND METHODS

3.1. Setting

The major part of the study was carried out at the Department of Microbiology, Quaid-i-Azam University, Islamabad.

3.2. Study design

This study was carried out for molecular identification of a gene named bla_{NDM-1} in Gram negative clinical isolates from pathogens causing UTIs.

3.3. Collection of bacterial isolates

A total of 130 Gram negative isolates from patients with urinary tract infections were included in this study. Samples were collected from Microbiology Department of Pakistan Institute of Medical Sciences (PIMS), Islamabad (February 2012) and Microbiology Department of Mayo Hospital, Lahore (May 2012).

3.4. Phenotypic identification of bacterial isolates:

Sub-culturing of bacterial isolates was done on Blood agar (Sigma) and MacConkey (Sigma) agar medium. Primary identification of the bacterial isolates was carried on the basis of colony morphology, Gram staining and cultural characteristics. For final identification, conventional biochemical testing was done.

3.5. Gram staining

With the help of sterile wire loop, thin smear of isolate was prepared on a clean glass slide. Slide was air dried and heat fixation was done by passing the slide on the flame for 3-5 times. The procedure of Gram staining is given in appendix A.

3.6. Cytochrome Oxidase Test:

This test was used for differentiation of different Gram negative microbes on the basis of oxidase enzyme. This key enzyme has a role in aerobic respiration and is a part of electron transport system. Oxidase positive microorganisms are *Neisseria spp.* and *Pseudomonas spp.* oxidase negative microorganisms belong to *Enterobacteriaceae* [(Winn *et al.*, 2006)]. The detailed method is given in Appendix B.

3.6.1. Triple Sugar Iron (TSI) Test:

This test was used for differentiation of microbes on the basis of fermentation and H_2S production. With the help of 5 ml TSI agar, butt and slants were prepared in sterile test tubes. Bacterial isolates were inoculated in both butt and slant with the help of straight wire loop. Incubation was done at 37°C for 24 hrs. Color change in butt and slant was noted. Acid production was observed in the butt and yellow color appeared. Pink red color was observed in the slant due to alkaline reaction [(Winn *et al.*, 2006)] as shown in Appendix C.

3.6.2. Simmon Citrate Test:

This test was used for differentiation of the microbes by utilizing citrate as the sole source of carbon. Slants of the media were inoculated by using sterile wire loop and slants were incubated for 24 hrs at 37°C. Citrate utilizing microorganisms gave positive reaction and change in the color of media was observed from green to blue. No color change indicated negative result [(Winn *et al.*, 2006)] as shown in Appendix D.

3.6.3. Sulfide-Indole-Motility (SIM) Test:

SIM medium was used in this test which is semi-solid agar and was used for the identification of *Enterobacteriaceae* family on the basis of their ability for sulfide production, indole production and motility [(Winn *et al.*, 2006)] as shown in Appendix E.

3.6.4. Urease Test:

This test was used for the identification of microorganisms for their ability to produce urease enzyme. Bacterial inoculation of the media was done and tubes were incubated at 37°C for 24 hrs. Positive result was observed on appearance of light pink color whereas no change in color was reported as negative result [(Winn *et al.*, 2006)] as shown in Appendix F.

3.6.5. Methyl Red and Voges-Proskauer (MR-VP) Test:

These tests were used for the identification of gram negative microbes. These contained peptones and glucose in the broth as carbon and energy source. By using sterile wire loop, both the broths

were inoculated and were incubated at 37°C for over-night [(Winn *et al.*, 2006)]. Results were interpreted as shown in Appendix G.

3.7. Antimicrobial susceptibility testing:

Drug susceptibility testing was carried on Mueller-Hinton agar (Oxoid, UK) by using Kirby-Bauer disk diffusion method, according to Clinical Laboratory Standards Institute (CLSI) guidelines, 2011[(Cockerill *et al.*, 2011)]. Antibiotic discs used are given in Appendix H.

Inoculated plates were incubated at 35°C for 24 hours. Susceptibility results were noted according to CLSI guidelines [(Cockerill *et al.*, 2011)]. Micro-organisms resistant to antibiotics were further evaluated for cabapenamases and MBL production.

The inoculum was prepared by inoculating isolated colonies in 5ml of normal saline. Bacterial suspension was compared with 0.5 McFarland turbidity standards. The details of 0.5 McFarland turbidity preparation is given in Appendix I.

A sterile cotton swab was dipped in the suspension. The swab was rotated and then pressed resolutely against the tube walls. In order to ensure even spread of inoculums, the swabs were streaked on the MHA plate by rotating the plate at 60° angle. Inoculated plate was left for 3-5 min to absorb excess surface moisture before applying the drug impregnated discs [(Cockerill *et al.*, 2011)].

On inoculated plates, antibiotic discs (Oxoid) were placed and pressed gently in order to ensure contact with the medium. The discs were positioned at a distance of 15 mm from the edge of plate and distance from center to center was 24 mm. Plates were placed at 35°C for 18-20 hrs. After 18-24 hours of incubation, all plates were observed. The diameter of the zones of inhibition were measured by using digital Vernier calipers (Sylvac Fowler ultra-cal II) against black, illuminated with reflected light. The zones were interpreted according to the CLSI guideline [(Cockerill *et al.*, 2011)].

3.8. Carbapenamase Detection Method:

In *Enterobacteriaceae,* carbapenamase was detected by Modified Hodge Test (MHT) which is basically used as a screening and confirmatory test. Two types of carbapenemases are known, Serine and Metallo-carbapenemases.

3.8.1. Modified Hodge's Test

Principle:

The pathogens having ability for carbapenamase production will allow the carbapenem susceptible strain (*E. coli* ATCC 25922) to grow towards a carbapenem disc which will lead to a characteristic clover leaf-like indentation.

Procedure:

1. In 5 ml of normal saline. 0.5 McFarland dilution of the *E. coli* (ATCC 25922) was prepared.
2. By adding 0.5 ml (0.5 McFarland) to 4.5 ml of normal saline, suspension was diluted into 1:10.
3. By 1:10 dilution of *E. coli* (ATCC 25922), MHA plate was streaked and was permitted to air dry for 3-5 min.
4. A Meropenem (10μg) disc was placed in the centre of the test area.
5. The microorganism was streaked in straight line from periphery of disc to wall of plate.
6. Plates were incubated at 35°C for 18-20 hours.

Interpretation of Result:

MHT Positive: In disc diffusion zone a clover leaf-like indentation of *E. coli* (ATCC 25922) growth beside with pathogen growth streak (figure 5).

MHT Negative: In disc diffusion zone no clover leaf like indentation of *E. coli* (ATCC 25922) growth along with pathogen growth streak [(Cockerill *et al.*, 2011)].

Quality Control:

a) Positive Control *E. cloacae* (Known carbapenamase producer)

b) Negative Control *S. paucimobilis* (Known carbapenamase negative)

3.9. Metallo-β-lactamse Detection Method:

For MBL detection, combined disc diffusion method was used.

3.9.1. Combined disc diffusion method

Principle:

Cofactors like zinc ions are required by these enzymes for their activity. So in order to lose their activity, chelating agent like ethylene diamine tetra-acetic acid (EDTA) can be used to capture those [(Franklin *et al.*, 2006)].

Procedure

1. In order to prepare the solution of 0.5 M EDTA, 186.1 gm of disodium EDTA (Sigma) was dissolved in 1000 ml of distilled water.
2. PH was set to 8.0 by adding NaOH in the solution.
3. Through autoclaving the solution was sterilized.
4. Then two antibiotic discs of imipenem (10μg) and meropenem (10μg) were placed on MHA plate.
5. On each of imipenem and meropenem discs, 5μl of 0.5M EDTA (which is equal to 750 μg EDTA) was added.
6. Plate were incubated at 35°C for over-night.

Interpretation of results:

If zone size of carbapenems plus EDTA discs increases 8-15mm than carbapenems discs with no EDTA then test will be positive for MBLs (Figure 6). Increase in the zone size from 0-5 mm indicates negative for MBLs [(Franklin *et al.*, 2006)].

Quality Control:

a) Positive Control *E.cloacae* (Known MBL Positive)

b) Negative Control *E.cloacae* (Known MBL Negative)

ATCC strains:

Standard strains of *P.aeruginosa* ATCC 27853 and *E.coli* ATCC 25922 were used as quality control. A known carbapenamase producing strain of *E.cloacae* was included as positive control whereas; a known *S.paucimobilis* strain was used as a negative control. A known *E.cloacae* was also used as a positive control and a known *S.paucimobilis* strain as negative control for the detection of MBL.

3.10. Molecular Methods for Identification of Clinical Isolates:

3.10.1. DNA isolation:

Boil colony method:

The resistant bacterial isolates were first cultured on nutrient agar plates for 24 hrs and were incubated at 37°C. In an eppendorf tube, about 3-4 colonies were picked with sterile wire loop and were mixed in 40 µl of autoclaved distilled water. The tubes were vortexed for 20 sec and were incubated at 99°C for 5 min. in PCR machine. Then freezing was done at -20 for 20 min. All the tubes were again vortexed for 20 sec and were boiled for 15 min. at 99 °C [(Rohde, 1995)].

3.10.2. Ethanol precipitation of DNA:

For bacterial DNA extraction, this method was used and the procedure is as follows:

- From overnight culture of bacteria on nutrient agar, add 5-10 colonies in 250 µl of sterile normal saline in eppendorf tube.
- Then 20 µl of Sodium Dodecyle sulfate (10%) and 80 µl of Proteinase K were added.
- The tubes were shaken well and were incubated at 55°C for 1 hr in water bath.
- Subsequently add 100 µl of 6M NaCl to the solution and vortex the tubes for 1min.
- Tubes were centrifuged for 5-10 min at 14000 rpm. The supernatant was transferred to other tubes.
- Chilled absolute ethanol (1 ml) was added in order to precipitate the DNA and tubes were vortexed for 1 min.
- The tubes were centrifuged at 14000 rpm for 5min and supernatant was discarded.
- Wash the tubes containing the DNA pellet with 1 ml of 70% ethanol. Tubes were vortexed and centrifuged at 14000 rpm for 5min.
- In tubes, 100 µl of TE buffer was added and stored at 4 °C [(Rohde, 1995)].

3.11. Molecular characterization:

3.11.1 Primers for detection of bla_{NDM-1}:

For PCR amplification of bla_{NDM-1}, primers were selected from published sequences [(Peirano *et al.*, 2011)]. Composition of master mixture and re-constitution of primers are given in Appendix J,K.

Primers sequence:

Primers	Nucleotide sequence	Product size
NDM-1 F	5'-CAGCGCAGCTTGTCG-3'	784bp
NDM-1 R	5'-TCGCGAAGCTGAGCA-3'	

3.12. PCR of *bla*$_{NDM-1}$:

Molecular characterizationof isolates for the occurrence of *bla*$_{NDM-1}$ gene was carried out by using specific primers reported in GenBank (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA). PCR based amplification technique was used. Following PCR conditions were used in T1 Thermo cycler (Biomerta): initial denaturation temperature was 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing temperature was 52°C for 1 min, primary extension at 72°C for 1 min and final extension at 72°C for 5 minutes. The amplified product was stored at -4°C

3.13. Gel electrophoresis for identification of PCR product:

1% agarose gel (0.8g agarose in 80ml of 0.5x solution of TBE (Bio life, ItalinaSrl, Italy) was used to load PCR product. The gel was heated for 1 min. cooled at 45°C and then 0.3µg/ml of ethidium bromide (Roche, Germany) was further added for fluorescence. About 5µl of PCR products were loaded in gel wells with 1kb DNA marker (Fermentas) for 50 min at 100V in gel electrophoresis instrument. DNA bands were visualized by using UV Trans-illuminator (UVItec, EEC) and with the help of Gel Doc (Bio-Rad), digital photographs were taken. By comparing test bands with 1kb DNA ladder, interpretation of the results was done [(Peirano *et al.*, 2011)].

3.14. Preservation of bacterial isolates: Preservation of bacterial isolates was done by using 16% (v/v) glycerol in brain heart infusion (Oxoid Ltd, UK). Storing of bacterial isolates was done at - 70°C [(Duguid, 1996)].

Chapter 4

Chapter 4

RESULTS

4.1 Clinical Isolates

A total of 130 clinical Gram-negative isolates were collected from UTIs patients from two main hospitals of Pakistan; Pakistan Institute of Medical Sciences (PIMS), Islamabad and Mayo hospital Lahore (Table1). Out of these pathogen most were collected form PIMS (n=75) among which dominant organisms were *E. coli* (41.5%), *K. pneumoniae* (22.2%) *P. aeruginosa* (20.2%), *Enterobacter spp.* (4%) and *Proteus spp.* (12.1%) while 55 GNR were collected from Mayo Hospital, Lahore among which predominant were *E. coli* (41%), *P. aeruginosa* (25%) and *K. pneumoniae* (23%) *Proteus spp.* (11%).

Most of the clinical GNR (n=118) were isolated from urine samples while (n=12) were isolated from urine catheter tips (figure 1).

4.2 Antibiotic resistance pattern of all isolates

Antibiotic resistance pattern of GNR isolated from PIMS Hospital, Islamabad is shown in (Table 2). All isolates showed moderate to high resistance against cephalosporins including cefuroxime (65-95%), ceftriaxone (60-86%), ceftazidime (40-92%), carbapenems including imipenem (30-94%), meropenem (30-94%), amikacin (30-67%), norfloxacin (30-66%) and β- lactam inhibitors (30-73%). Most effective drug was polymixin B (11-30%).

Antimicrobial resistance pattern of clinical isolates from Mayo Hospital, Lahore also showed moderate to high drug resistance against commonly used antibiotics including cephalosporins (30-93%), carbapenems(40-82%), amikacin(60-89%), nalidixc acid(20-45%), and β-lactam inhibitors(20-92%). PolymixinB (14-23%) was the most effective drugs against these pathogens (Table 3).

Figure 2 shows overall susceptibility pattern of GNR isolated from both hospitals. More than 50% microbes showed resistance against cephalosporins, aztreonam, β-lactam inhibitors and carbapenems. Forty percent of microbes were resistant against amikacin, nalidixc acid and nitrofurantoin. Effective drugs against these pathogens were Polymixin B followed by nitrofurantoin and nalidixc acid.

4.3 Carbapenamase and Metallo-β-lactamase production

Out of 130 isolates, 68 (52.3%) showed carbapenems resistance. Phenotypic tests showed that 42 (32.3%) were both carbapenamase and MBL producers, while 20 (15.4%) were Non-carbapenamase and MBL producers (Figure 3).MBLs producing pathogens were *K. pneumoniae* (52.5%) followed by *P. aeruginosa* (33.2%) and *E. coli* (14.3%) as shown in Figure 4. Molecular detection by PCR demonstrated the presence of $bla_{\text{NDM-1}}$gene in 7(16.6%) out of 42 MBLs producing pathogens. NDM-1 positive isolates from both hospitals are shown in Table 4. Among these, 3 were *P. aeruginosa* followed by *E. coli* (n=2) and *K. pneumoniae* (n=2).

Table 5 shows the resistance pattern of $bla_{\text{NDM-1}}$ gene producing pathogens. All 7 NDM-1 positive pathogens showed more than 60% resistance against cephalosporins, aztreonam, carbapenems, and amikacin and β-lactam inhibitors. Most effective drug was Polymixin B and nalidixic acid.

Overall susceptibility pattern of $bla_{\text{NDM-1}}$producing microbes is shown in Figure 8. More than 70% of pathogens showed resistance against ceftriaxone, ceftazidime, carbapenems and pipracillin/tazobactam. About 50 to 60% organisms showed resistance against co-amoxiclav, cefuroxime, aztreonam and nitrofurantoin. Most effective drugs were polymixin B and nalidixc acid.

4.4 Association with age and gender

Table 6 shows the clinical isolates according to age and gender. Among these most of the patients were between the age range of >41 years to <60 years and $bla_{\text{NDM-1}}$ gene was also isolated in isolates from patients in this age group. Among gender male to female ratio was ~1:1.Out of 7 $bla_{\text{NDM-1}}$gene producing pathogens, 4 were isolated from females and 3 were isolated from males.

Table 1: Total number of Gram-negative clinical isolates causing UTIs (n=130)

Microorganism	Distribution (n)
Isolates from PIMS Hospital, Islamabad (n=75)	
E.coli	**31**
K.pneumoniae	**17**
P.aeruginosa	**15**
Proteus spp	**9**
Enterobacter spp	**3**
Isolates from The Mayo Hospital, Lahore (n=55)	
E.coli	**23**
P.aeruginosa	**14**
K.pneumoniae	**13**
Proteus spp	**5**

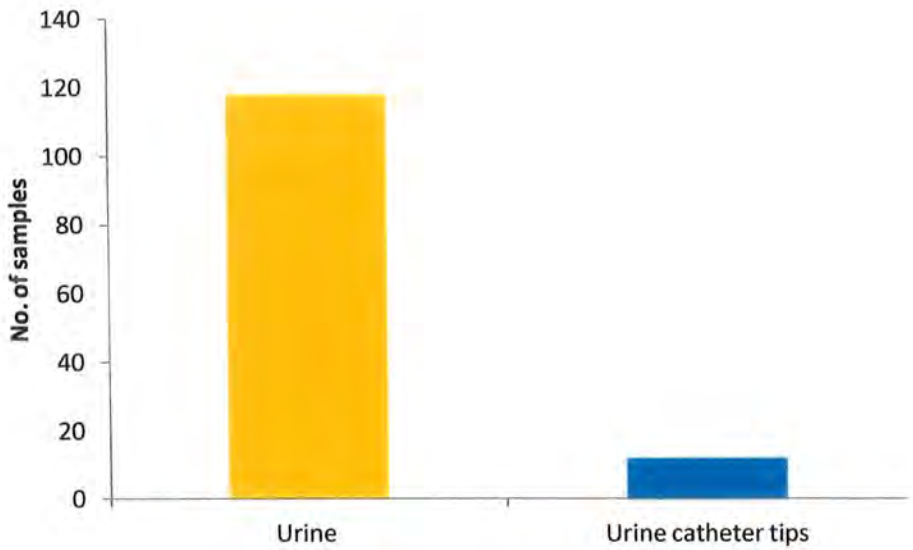

Figure 1: Total no of clinical samples

Table 2: Antimicrobial resistance pattern of isolates from PIMS Hospital, Islamabad.

Antimicrobial	*E. coli* (n=31)	*K. pneumoniae* (n=17)	*P. aeruginosa* (n=15)	*Proteus spp*(n=9)	*Enterobacte rspp*(n=3)
Co-amoxiclav	42%	53%	67%	44%	0%
Cefuroxime	68%	47%	73%	78%	0%
Ceftriaxone	48%	76%	67%	78%	33%
Ceftazidime	58%	76%	53%	78%	33%
Aztreonam	32%	71%	47%	44%	0%
Imipenem	64%	94%	60%	89%	33%
Meropenem	77%	94%	60%	67%	67%
Pipracillin/tazobactam	25%	18%	53%	45%	0%
Cefoperazone/sulbactam	42%	47%	73%	33%	66%
Gentamicin	25%	53%	33%	56%	0%
Amikacin	55%	35%	67%	44%	0%
Norfloxacin	54%	55%	40%	56%	66%
Nalidixic Acid	18%	35%	37%	26%	33%
Nitrofurantoin	32%	41%	73%	45%	33%
Polymixin B	19%	18%	27%	11%	33%

Table 3: Antimicrobial resistance pattern of isolates from Mayo Hospital, Lahore.

Antimicrobials	*E. coli* (n=23)	*P. aeruginosa* (n=14)	*K. pneumoniae* (n=13)	*Proteus spp*(n=5)
Co-amoxiclav	39%	36%	38%	40%
Cefuroxime	74%	71%	92%	100%
Ceftriaxone	43%	93%	46%	100%
Ceftazidime	43%	79%	54%	100%
Aztreonam	48%	50%	62%	100%
Imipenem	61%	43%	62%	40%
Meropenem	56%	46%	57%	20%
Pipracillin/tazobactam	35%	21%	38%	20%
Cefoperazone/sulbactam	48%	71%	54%	60%
Gentamicin	48%	29%	15%	60%
Amikacin	43%	21%	54%	60%
Norfloxacin	65%	21%	38%	20%
Nalidixic Acid	25%	34%	24%	20%
Nitrofurantoin	39%	27%	18%	30%
Polymixin B	22%	14%	23%	0%

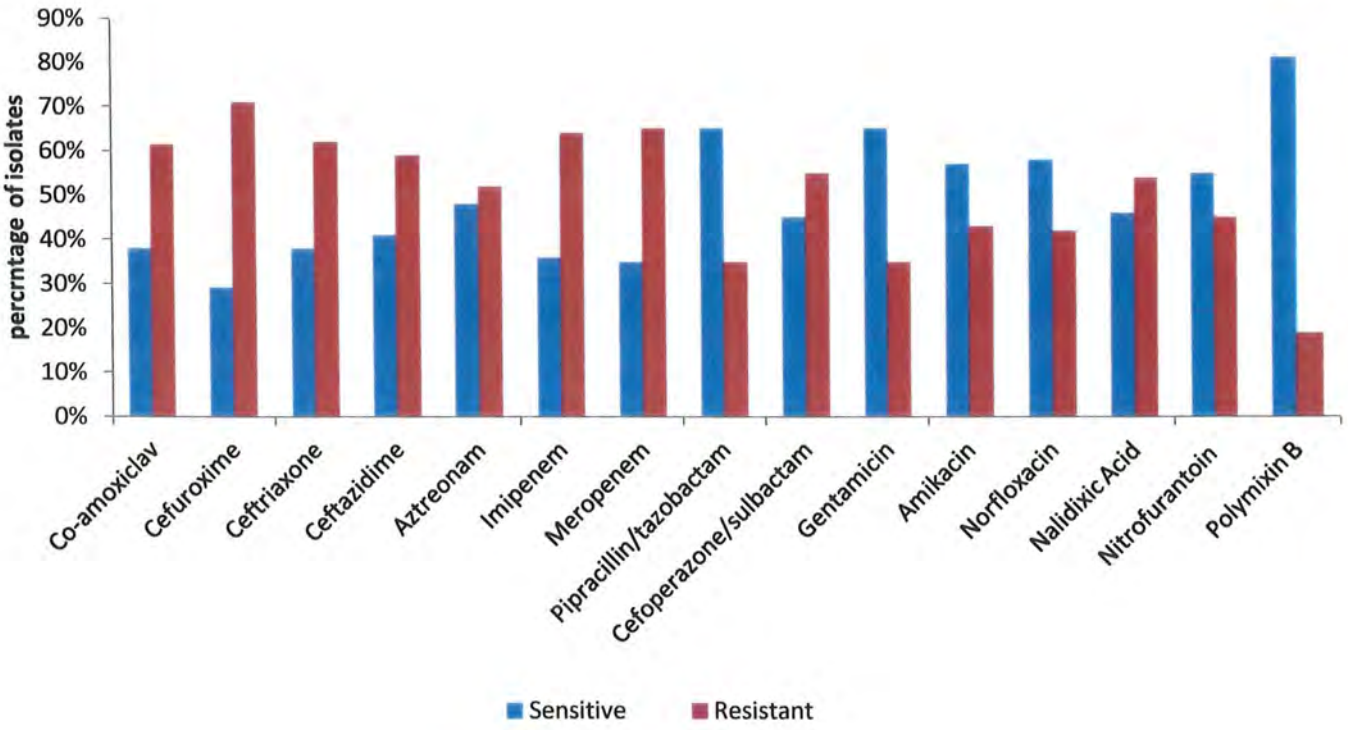

Figure 2: Overall susceptibility patterns of isolates collected from both hospitals

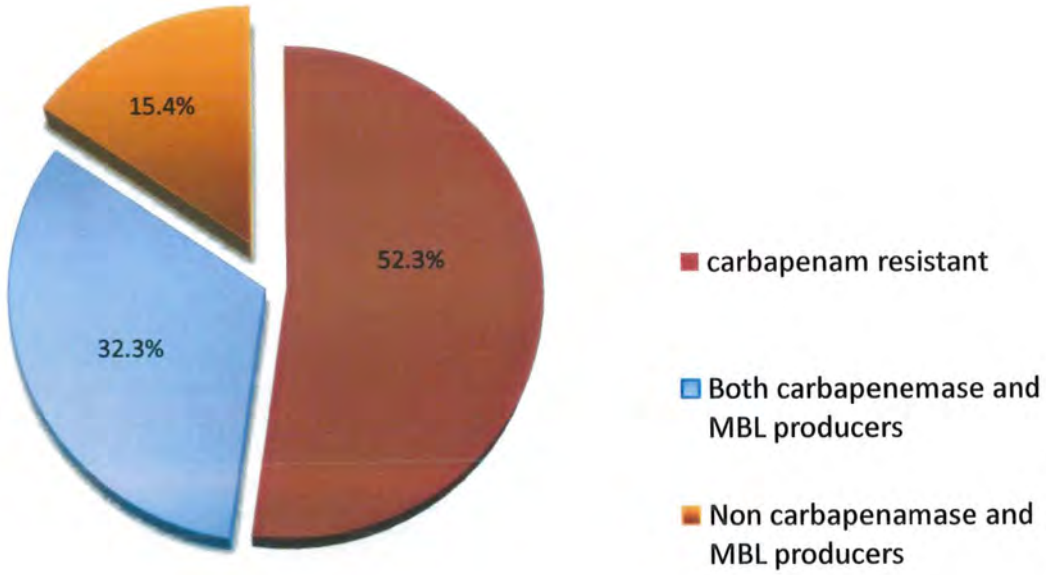

Figure 3: Resistance pattern of collected strains

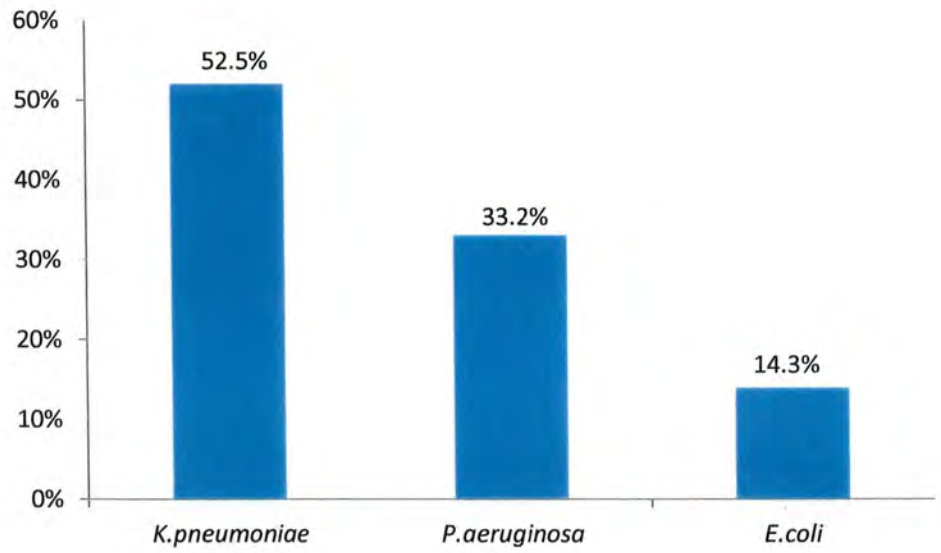

Figure 4: Total number of different carbapenamase and MBLs producing isolates

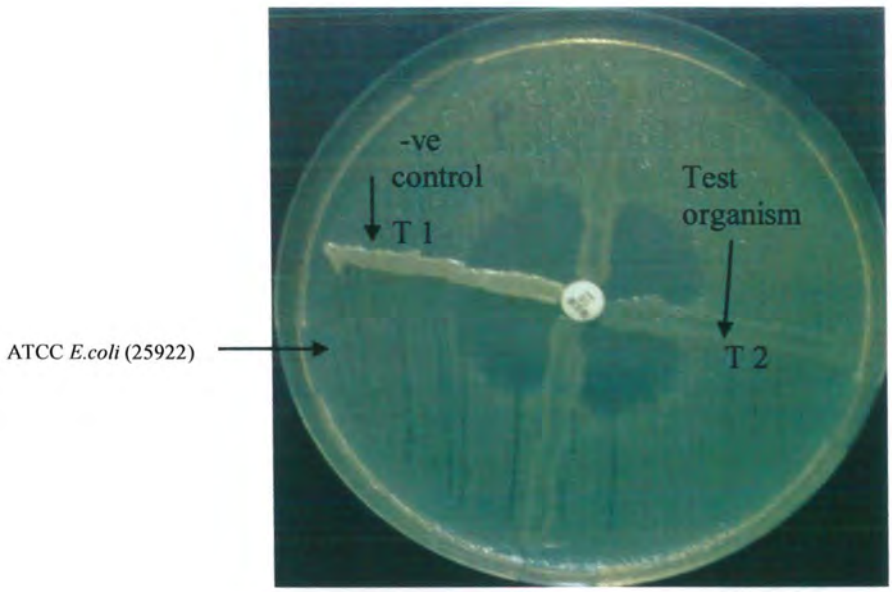

Figure 5: Demonstration of Modified Hodge's test. Cloverleaf like indentation of test strain *E.coli* (ATCC 25922) toward MEM disc along with negative (T1) and test organism (T2).

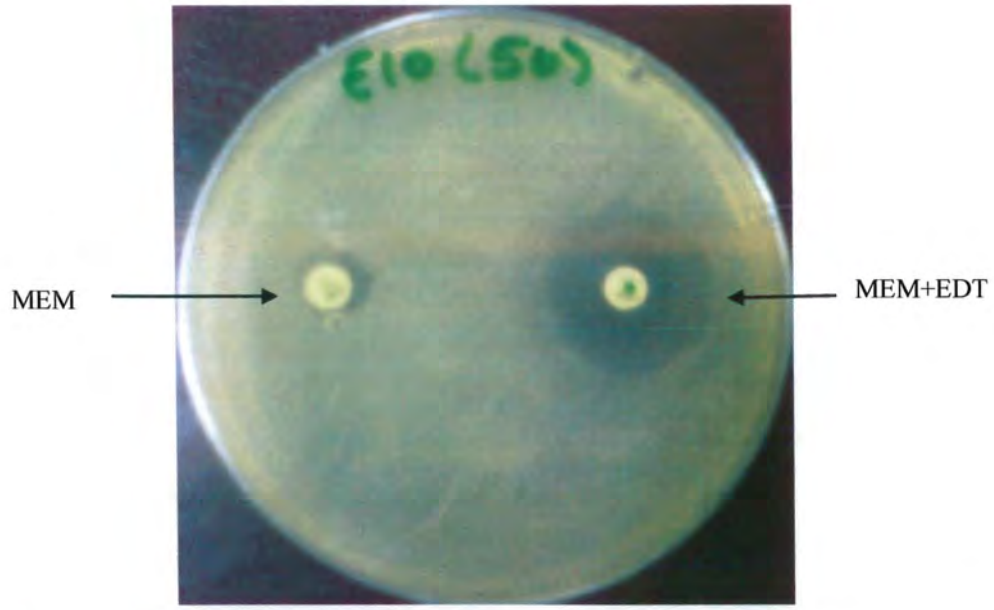

Figure 6: Demonstration of combined test method. MBL production by using meropenem (MEM) was containing EDTA on the right and no MBL production by non-EDTA containing MEM disc on the left.

Table: 4 NDM-1 positive isolates from PIMS and Mayo hospitals

Microorganisms	Frequency (n)
NDM-1 positive microbes from PIMS Hospital, Islamabad (n=4)	
E. coli	**2**
K. pneumoniae	**1**
P. aeruginosa	**1**
NDM-1 positive microbes from Mayo Hospital, Lahore (n=3)	
P. aeruginosa	**2**
K. pneumoniae	**1**

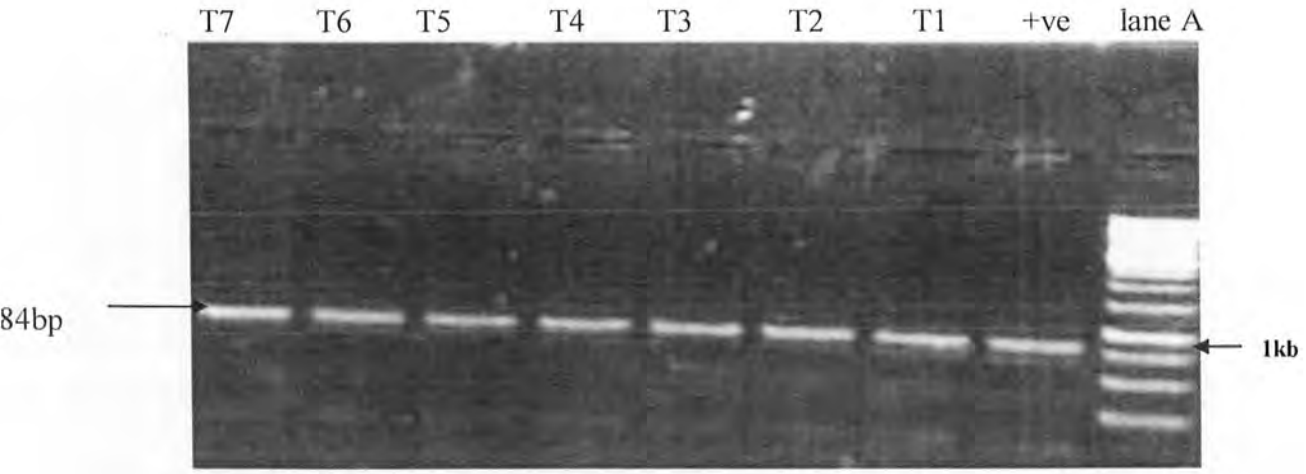

Figure 7: Gel electrophoresis analysis of PCR Amplicon of *bla*$_{NDM-1}$ gene on 1% agrose gel. Lane A shows 1kb DNA ledder.Positive control of known NDM-1 positive *K. pneumoniae*.Lane (T1, T2, T3) show positive bands for *Pseudomonas spp*.Lane (T4,T5) for *K. pneumonia* . Lane (T6, T7) for *E.coli*

Table 5: Percent resistance pattern of *bla*$_{NDM-1}$ producing organisms

Antimicrobials	*P. aeruginosa* (n=3)	*E. coli* (n=2)	*K. pneumoniae* (n=2)
Co-amoxiclav	67%	50%	50%
Cefuroxime	67%	50%	50%
Ceftriaxone	67%	100%	50%
Ceftazidime	67%	100%	100%
Aztreonam	67%	50%	50%
Imipenem	100%	50%	100%
Meropenem	100%	50%	100%
Pipracillin/tazobactam	100%	50%	100%
Cefoperazone/sulbactam	67%	0%	50%
Gentamicin	67%	0%	50%
Amikacin	33%	50%	50%
Norfloxacin	33%	21%	100%
Nalidixic Acid	33%	30%	12%
Nitrofurantoin	34%	20%	45%
Polymixin B	15%	30%	10%

Table 6: Total number of isolates from UTIs patients according to age groups and gender

Age groups	Organisms
0 - ≤20 years	20
>20 - ≤40 years	35
>41 - ≤60 years	47
>60 - ≤80 years	23
>80 years	5
Clinical isolates from gender	
Male	64
Female	66

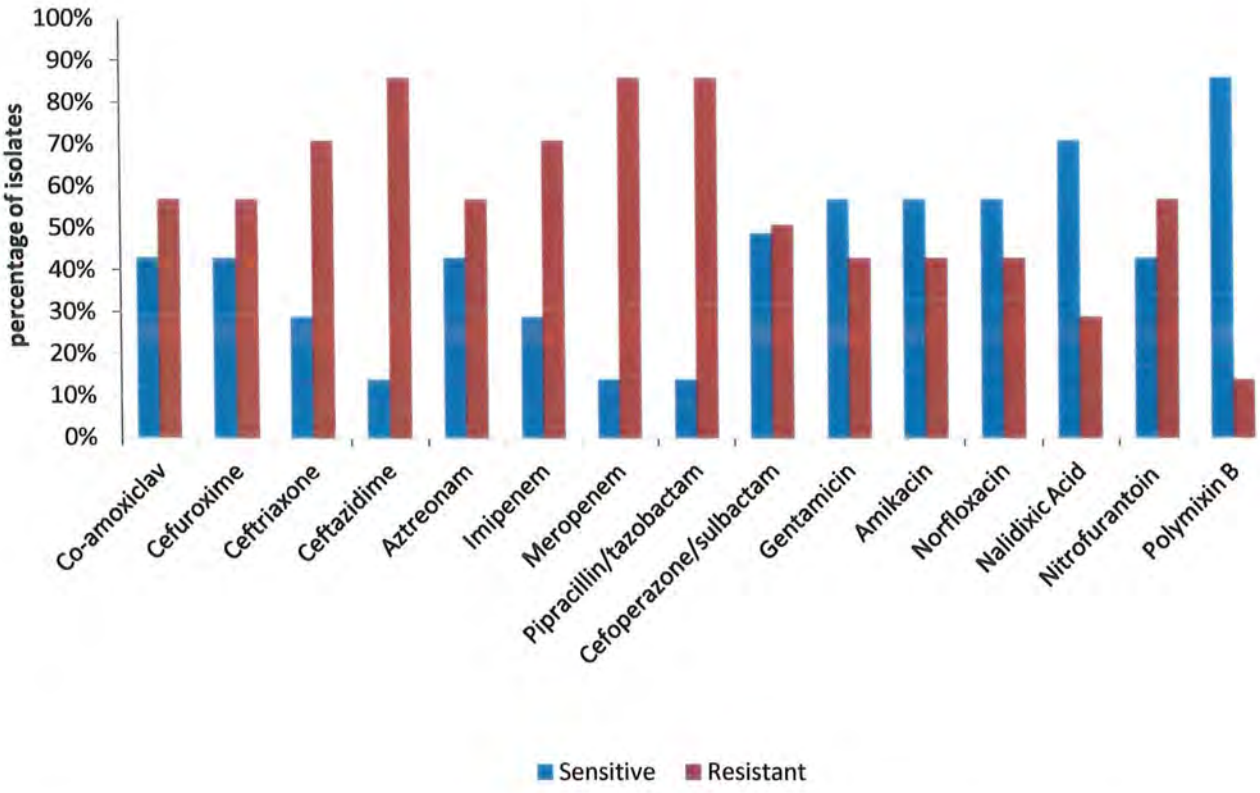

Figure 8: Overall susceptibility pattern of *bla*$_{NDM-1}$ gene producing microorganisms

Chapter 5

DISCUSSION

Escherichia coli are one of the main agents that cause UTIs in humans. A total of 130 clinical Gram-negative isolates were collected from UTIs patients from two main hospitals of Pakistan; Pakistan Institute of Medical Sciences (PIMS), Islamabad and Mayo Hospital Lahore (Table1). Among these pathogens the predominant were *E. coli* (41.5%) followed by *K. pneumoniae* (22.2%), *P. aeruginosa* (20.2) *Enterobacter* (4%) and *Proteus spp.* (12.1%). A similar study has reported that the most frequent organism were *E. coli* (55.6%) followed by *K. pneumoniae* (16.9%) and *C. diversus* (4.2%) (Karlowsky *et al.*, 2003). A Pakistani study also documented similar results where *E.coli* was main causative agent of UTIs followed by *K. pneumoniae* and *P. aeruginosa* (Naeem *et al.*, 2010). The prevalence of UTI causing causing pathogens in our study are in accordance with various previous studies conducted throughout the world (Mehr *et al.*, 2004; Johansen *et al.*, 2006; Bashir *et al.*, 2008; Kolawole *et al.*, 2009; Bours *et al.*, 2010; Beetz and Westenfelder, 2011; Manikandan *et al.*, 2011; Manjunath *et al.*, 2011; Muhammad *et al.*, 2011; Bano *et al.*, 2012).

Increased drug resistance particularly in uropathogens is becoming a global threat. In the present study we also found high level of resistance among these pathogens (Tables 2, 3, Figure 2). More than 50% organisms showed resistance against cephalosporins (cefuroxime, ceftriaxone, ceftazidime), aztreonam, nalidixic acid and carbapenems (imipenem, meropenem). Forty percent of microbes were resistant against amikacin, norfloxacin and nitrofurantoin. Effective drugs for these pathogens were Polymixin B followed by gentamicin and pipracillin/tazobactam. These findings are similar to a study conducted in India, where uropathogens showed highest resistance against ampicillin which was 80.4% followed by ciprofloxacin (73%), amoxicillin (70.4%), norfloxacin (53.3%) (Manjunath *et al.*, 2011). Another study found the percentages of resistance of all uropathogens as; 83.3% for SXT, 80.6% for nalidixic acid, 67.3% for amoxicillin, 61% for co-trimoxazole, 48.8% for gentamycin, 46% for ciprofloxacin and 43% for cephalexin (Manikandan *et al.*, 2011). A recent study from Pakistani has reported high drug resistance pattern of uropathogens that amoxicillin/clavulanate (55%), ciprofloxacin (63%), levofloxacin (73%), cefixime, cefotaxime (70% and 38%), ceftriaxone (75%), cefoperazone/sulbactam (93%), amikacin (95%), imipenem (86%) and pipracillin/tazobactam (86%) (Naeem *et al.*, 2010). In contrast, drug resistance pattern for uropathogens is low to moderate in developed countries as

compare to developing countries (Zhanel *et al.*, 2000; Mehr *et al.*, 2004; Bours *et al.*, 2010; Sanchez *et al.*, 2012). The increased drug resistance in our set up could be due to unhygienic conditions of our hospitals, irrational use of antibiotics, lack of diagnostic microbiological testing laboratory in hospitals and use of broad spectrum antibiotics.

In present study an alarming increase of carbapenems resistance and carbapenamase and MBLs producing were found in UTIs patients. Out of 130 uropathogens, 52.3% were carbapenems resistant, 32.3% were carbapenamase and MBLs while 15.3% showed non- carbapenamase and MBLs production respectively (Figure 3).MBLs producing pathogens (Figure 4) were *K. pneumoniae* (52.5%) *P. aeruginosa* (33.2%) and *E. coli* (n=14.3%). A study conducted in Indian Kashmir on MBLs producing pathogens in a tertiary care hospital. Found that out of 283 clinical isolates, 38 were carbapenem resistant and 33 were MBLs producing *P. aeruginosa*. MBLs producing *P. aeruginosa* were mostly isolated from urine source (Bashir *et al.*, 2011). An earlier study from Poland also reported that 33 *P. aeruginosa* isolated from urine samples were MBL producers (Fiett *et al.*, 2006). Whereas, two Canadian studies found the presence of MBLs producing *E. coli* in urine sample (Peirano *et al.*, 2011a; Peirano *et al.*, 2011b). A study conducted in Spain documented the presence of MBLs producing *E. coli* and *K. pneumoniae* in urology ward (Tato *et al.*, 2007). Recently a study conducted in South Africa also reported MBL producing *K. pneumoniae* from a UTI patient (Brink *et al.*, 2012). A study conducted in AFIP Rawalpindi also reported the presence of MBL producing *P. aeruginosa* in UTI patient (Butt *et al.*, 2005). The high rate of MBLs producing uropathogens is because of many reasons but one of the main causes is the empirical prescribed antibiotics on clinical grounds and no proper diagnosis made by the physicians. The present study shows that there is a need for surveillance study and an extensive screening for MBL production all over the country in both hospitals and community surroundings especially in UTI patients.

New Delhi Metallo-β-lactamase (NDM-1) is a novel type of MBL first detected in *E. coli* and *K. pneumonia* in UTI patient who had traveled to India. Since then $bla_{\text{NDM-1}}$ producing bacterial infections have spread and increase all over the world (Yong *et al.*, 2009; Kumarasamy *et al.*, 2010; Poirel*et al.*, 2011). The $bla_{\text{NDM-1}}$ gene has the ability to turn MDR pathogens into XDR. The $bla_{\text{NDM-1}}$ gene is chromosomal as well as plasmids mediated therefore these genes are

transferable easily to sensitive strains at high rate (Yong *et al.*, 2009). Plasmids detected most in NDM-1 positive organisms were easily moveable and capable of wide rearrangement which can lead to widespread transmission among bacterial population (Rolain *et al.*, 2010).

In the present study molecular detection demonstrated the presence of 7 (16.6%) bla_{NDM-1} genes containing organisms among 42 MBLs producing pathogens (Figure 7). Among these, 3 were identified as *P. aeruginosa* followed by *E. coli* (n=2) and *K. pneumoniae* (n=2). Interestingly, first ever NDM-1 was isolated in *E. coli* from UTI patient in 2008 in Sweden (Kumarasamy *et al.*, 2010). Presence of NDM-1 positive uropathogens is different in different region of the world. Recently, one *K. Pneumoniae* and two *E. coli* strains were isolated from UTIs patients in Thailand were found to contain NDM-1(Rimrang *et al.*, 2012). A CDC report showed that a patient's urine specimen which was recently hospitalized in Viet Nam, but who was getting care at a hospital in Rhode Island, was detected to have a *K. pneumoniae* isolate containing NDM CDC (2012). Whereas, an outbreak of NDM-1 producing one isolate of *E. coli* and three isolates of *K. pneumonia* and was found in urine sample in Italy (Gaibani *et al.*, 2011). Similarly an Indian study reported that out of 19 *K. pneumoniae* urine isolates, 11 were NDM-1 producers (Bora and Ahmed, 2012). Another study was conducted from Mauritius in which they reported a NDM-1 producing *K. pneumoniae* from urine sample (Poirel *et al.*, 2012). Another similar study was at Kenyain which they found that out of 7 NDM-1 producing *K. pneumoniae*, 5 organisms were NDM-1 producer collected from urine (Poirel *et al.*, 2011c). In Australia, NDM-1 producing *E. coli* was isolated in urine sample (Poirel *et al.*, 2010b). In Canada, both NDM-1 producing *E. coli* and *K. pneumoniae* were isolated from UTIs patient (Peirano *et al.*, 2011a). Another Indian study revealed that out of 24 carbapenem-resistant GNR, 6 were NDM-1 positive *E. coli* producers followed by 5 were NDM-1 *K. pneumoniae* and 1 was NDM-1*Morganella morganii* in urine samples (Deshpande *et al.*, 2010). In Pakistan there is little data available on NDM-1 producing organisms from UTIs patients or urine samples. A multicounty study conducted in India, Pakistan and UK in 2010. They found NDM-1-positive bacteria from Varanasi (13), Guwahati (three) and in Mumbai (32 isolates), and from eight cities in Pakistan 25 isolates (Charsadda, Faisalabad, Gujrat, Hafizabad, Karachi, Lahore, Rahim Yar Khan, and Sheikhupura). These isolates were from a range of infections including ventilator-associated

pneumonia, bacteraemia, and community-acquired urinary tract infections. But most of the samples (n=15) were from UTIs (Kumarasamy *et al.*, 2010).

In present study we also found an increased drug resistance in bla_{NDM-1} producing organisms (Table 5, Figure 8). More than 70% of bla_{NDM-1} gene producing pathogens showed resistance against ceftriaxone, ceftazidime, carbapenems and pipracillin/tazobactam. About 50% to 60% organisms showed resistance against co-amoxiclav, cefuroxime, aztreonam and nitrofurantoin. Most effective drugs were polymixin B and nalidixc acid. Similarly a Thailand study also reported high drug resistance in bla_{NDM-1} producing organisms and most effective drug against them was colistin and tigecycline (Rimrang *et al.*, 2012). A similar result was also found by Kumarasamy *et al* where high drug resistance to all antibiotics was found except colistin and tigecycline (Kumarasamy *et al.*, 2010). A CDC 2012 report published that NDM-1 producing pathogens were only susceptible to colistin, tigecycline and polymixin B (CDC, 2012). There are also various studies that reported the same kind of results worldwide (Deshpande *et al.*, 2010; Poirel *et al.*, 2010b; Gaibani *et al.*, 2011; Peirano *et al.*, 2011a; Poirel *et al.*, 2011c; Bora and Ahmed, 2012).

The study conducted in UK on isolates collected from pakistan found that all bacterial isolates were found to be resistant to most of antibiotics incuding (ampicillin, cephalosprins, carbapenems, β-inhibitors, amikacin, ciprofloxacin and aztreonam). Only drug which showed sensitivity were tygycyline and colistin (Kumarasamy *et al.*, 2010). This high resistance is maily linked with unhygenic practices in our hospitals, contaminated indewling use of urinary catheters, lack of helath care facilities, understaffed and overcroweded hospitals, prescription of antibiotics on clinical grounds, no infcetious control committee in hospitals, irretional broad spectram use of antibiotics, antibiotics policies not revised according to WHO policies. Therefore, there is a need of a surveillance study throughout various public and private sector hospital to overcome this emerging problem which can lead to more problems.

Chapter 6

Conclusions

- In present study molecular identification by PCR demonstrated the presence of 7(16.6%) bla_{NDM-1} gene containing organisms among 42 MBLs producing pathogens. Out of these, 7 bla_{NDM-1}genes identified as *P. aeruginosa* (n=3) followed by *E. coli* (n=2) and *K. pneumoniae* (n=2).

- We found a high degree of MDR positive microorganisms in UTI patients.

- All the 7 NDM-1 positive pathogens showed more than 60% resistance against cephalosporins, aztreonam, carbapenems, amikacin and β-lactam inhibitors. Most effective drug was Polymixin B and nalidixic acid.

- There was a high number of carbapenamase and MBL (32.3%) producing Gram negative microbes.

- The detection of bla_{NDM-1}gene in UTI indicates that there is a need of surveillance study conducted countrywide hospitals and healthcare systems in order to assess the actual prevalence of bla_{NDM-1} in Pakistan.

Chapter 7

Chapter 7

Future Prospects

- Further molecular studies are required for plasmid profiling which is essential to find different types and number of plasmids.

- There is a considerable need to detect other type of MBL producing resistant genes in pathogenic strains causing UTI.

- Molecular characterization of MDR and XDR and Co-existence of other types of MBLs genes including (IMP, VIM.SPM, DIM) along with NDM-1 in UTI causing pathogenic isolate.

- Advance research should be conducted for the sequencing of these genes in order to find out subtypes.

- Higher Studies are required to find different types of inhibitors to enhance the efficacy of antibiotics against $bla_{\text{NDM-1}}$positive strains.

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Appendix

Appendix A

Gram Stain Procedure:

Following steps were done in the staining:

1. The smear was covered with crystal violet stain for 60 seconds.
2. The crystal violet was poured off and the smear was covered with Lugol's iodine solution for 30 seconds.
3. The iodine solution was poured off and the smear was decolorized with acetone-iodine decolorizer until the colour ceased to come out of the smear.
4. The slide was thoroughly washed with water.
5. The slide was counterstained with diluted carbolfuchsin for 30 seconds, washed with water, blotted with absorbent paper and air dried.
6. Organisms that retained the crystal violet-iodine dye complexes, after decolorizing with acetone-iodine, stain purple and were termed as Gram-positive, those that lost this complex and became red due to counter stain (carbolfuchsin) were termed Gram-negative.

Following organisms were stained simultaneously for monitoring the quality control.

Controls:

Positive Control :	*S. aureus* (ATCC 25923)
Negative Control:	*E. coli* (ATCC 25922)

Appendix B

Cytochrome Oxidase Test

Procedure:

The cytochrome oxidase test uses tetramethyl-p-phenylenediamine di-hydrochloride, thatsubstitutes oxygen as artificial electron acceptors. In the reduced state, the dye is colorless; however, in the presence of cytochrome oxidase and atmospheric oxygen, p-phenylenediamine is oxidized, forming indophenol blue which gives deep purple color.

The test was performed by the indirect paper strip procedure, in which a few drops of 1% aqueous solution of the reagent were added to a filter paper strip. A bacterial colony to be tested was smeared into the reagent zone of the filter paper using a wooden stick.

Bacterial colonies having cytochrome oxidase activity developed a deep purple colour at the inoculation site within 10 seconds. As a precautionary measure, stainless steel or Nichrome inoculating loops or wires were not used for this test because surface oxidation products formed by metals when flamed for sterilizing may result in false positive reactions.

Bacterial species showing positive and negative reactions were run as controls at frequent intervals. The following organisms were used as controls:

Controls:

a) Positive Control: *P. aeruginosa* (ATCC 27853)

b) Negative Control: *E. coli* (ATCC 25922)

Appendix C

Triple Sugar Iron (TSI)

Procedure:

TSI medium contains Dextrose, Lactose and Sucrose, when these carbohydrates are fermented; phenol red ph indicator detects acid production. Sodium Thiosulfate reduces to hydrogen sulfide which reacts with iron salt resulting in black iron sulfide.

TSI slants were inoculated using a sterile needle. A single isolated colony was touched at the centre with a sterile needle and butt was stabbed while slant was slightly streaked. The tubes were incubated with caps loosened at 35±2°c in aerobic conditions. The tubes were examined after 18-24hrs for carbohydrate fermentation, gas production and hydrogen sulfide production. A yellow (acidic) color in slant and butt showed the organism ferments dextrose, lactose and sucrose. Both slant and butt were acidic for *E. coli* and *K. pneumoniae*, were positive for gas production while negative for H_2S production. A red (alkaline) color in slant and butt showed that organism is non-fermenter. For *P. aeruginosa*both slant and butt was alkaline, it was negative for both gas and H_2S production.

Controls:

a) Positive Control: *P. aeruginosa* (ATCC 27853)

b) Negative Control: *E. coli* (ATCC 25922)

Appendix D

Simmon Citrate Test

Procedure:

All isolates were tested for citrate utilization. The test is based upon the ability of microorganisms to convert citrate into oxaloacetate. The test is performed with Simmons citrate media (Oxoid) in which citrate is the only source of carbon available to bacteria. If bacteria can utilize citrate it will grow and the color of media will change to bright blue due to increase in pH of the media. This test is used for identification of *P. aeruginosa, K. pneumoniae and* for the differentiation between *E. coli* and *Citrobacterfreundii.*

Controls:

a) Positive Control: *P. aeruginosa* (ATCC 27853)

b) Negative Control: *E. coli* (ATCC 25922)

Appendix E

Sulfide-Indole-Motility (SIM) test

Procedure:

SIM medium (Oxoid) was inoculated with selected pure 18-24hr cultures by stabbing the centre of medium with an inoculating needle. Tubes were incubated at 35±2°C in aerobic conditions. After 18-24hrs tubes were analyzed for motility, H_2S production and for Indole production.

Turbidity or fuzzy growth away from the line of inoculation confirms that the isolate is motile. Growth only along the inoculation line gives negative result for motility. *K. pneumoniae*is immotile while *P. aeruginosa* and *E. coli* are motile.

Blackening of the medium gives a positive result for H_2S production. *E. coli*, *P. aeruginosa*and *K. pneumoniae*doesn't produce H_2S.

The Indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce Indole. After the addition of few drops of Kovac's reagent appearance of pink to red color gives positive result while yellow color indicates negative result. *E. coli* is Indole positive.

Controls:

a) Positive Control: *P. aeruginosa* (ATCC 27853)

b) Negative Control: *E. coli* (ATCC 25922)

Appendix F
Urease Test

Procedure:

Urease broth (Oxoid) was inoculated using heavy inoculums from an 18-24hr pure culture. The tubes were incubated at 35±2°c in aerobic conditions. The reaction was observed after 2, 4, 6, 24 and 48hrs. Intense pink-red color throughout the broth indicated positive result while no color change gave negative result.

Controls:

a) Positive Control: *P. aeruginosa* (ATCC 27853)

b) Negative Control: *E. coli* (ATCC 25922)

Appendix G

Methyl Red and Voges-Proskauer Test

Procedure:

For Methyl red test, added 5drops of the methyl red to 5mL of the broth. Red color at the surface of the medium indicated positive result while yellow color was considered negative. *E. coli* gives positive result.

For Voges-Proskauer test, added 0.6mL of α-naphthol and 0.2mL of KOH solution to 1mL of broth. Production of a red color at once or within 5min gave positive result. *K. pneumoniae*gives positive result.

Controls:

a) Positive Control: *P. aeruginosa* (ATCC 27853)

b) Negative Control: *E. coli* (ATCC 25922)

Appendix H

Antibiotic disks and their contents

Sr. No.	Antimicrobial Agent	Abbreviation	Disk Content
1	Ampicillin	AMP	10µg
2	Co-amoxiclav	AMC	20µg /10µg
3	Cefuroxime	CXM	30µg
4	Ceftriaxone	CRO	30µg
5	Ceftazidime	CAZ	30µg
6	Cefepime	FEP	30µg
7	Ciprofloxacin	CIP	5µg
8	Amikacin	AK	30µg
9	Imipenem	IMP	10µg
10	Meropenem	MEM	10µg
11	Pipracillin/tazobactam	TZP	10/100 µg
12	Polymixin B	PB	300U
13	Norfloxacin	NOR	30µg
14	Nalidixic Acid	NA	30µg
15	Nitrofurantoin	FD	30µg

Appendix I

0.5 McFarland standards

Turbidity Standard Preparation:

1. A 0.5 ml aliquot of 0.048 mol/L $BaCl_2$ was added to 99.5 ml of 0.18 mol/L H_2S0_4 (1 % v/v) with constant stirring to maintain a suspension.
2. The correct density of the turbidity standard was verified by using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0.08 to 0.10 for the 0.5 McFarland standards.
3. The barium sulfate suspension was transferred in 4 to 6 ml aliquots into screw-cap tubes.
4. These tubes were sealed and stored in the dark at room temperature.
5. The barium sulfate turbidity standard was vigorously agitated on a mechanical vortex mixer before use and inspected for a uniform turbidity.
6. The barium sulfate standard was replaced if their densities were not within acceptable limits.

Appendix J

Composition of PCR reaction mixture:

Components	Stock conc.	Final conc.	Volume/reaction (µL)
Forward primer	100 pmol/Ml	20 pmol/µL	0.6 µL
Reverse primer	100 pmol/Ml	20 pmol/µL	0.6 µL
Template DNA	-	-	3.0 µL
Master mix	5x	1x	4.0 µL
Nuclease free water	-	-	11.8 µL

Appendix K

Re-constitution of primers

Primer Reconstitution:

Stock concentration was 100pmol/µl while working concentration was 20pmol/µl. For 150821 pmoles of reverse primer, added 1508.21µl nuclease free water in lyophilized primer to make 100 pmol (stock conc.). To prepare working conc. of 20 pmol, took 20µl from this stock in 80 µl nuclease free water.

For 137450 pmoles of forward primer, added 1374.50µl nuclease free water in lyophilized primer to make 100 pmol (stock conc.). To prepare working conc. of 20 pmol, took 20µl from this stock in 80 µl nuclease free water.

TBE Buffer (5X /1000 ml):

Tris base: 53 g; Boric acid: 27.5 g; 20 ml 0.5 M EDTA, Up to 1000ml with dH_2O.

Agarose 1.0 %:

A 0.80g agarose was added in 80mL 0.5 × TBE buffer.

0.5 M EDTA (pH 8.0):

Added 9.3g of EDTA in 50ml with NaOH pellets to adjust pH.

Proteinase K:

1mg/ml (stock conc.) 100µg/mL.