MOLECULAR DETECTION OF NEWDELHI METALLOBETALACTAMASES IN ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE IN CLINICAL ISOLATES OF A TERTIARY CARE HOSPITAL

Dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University

In partial fulfillment of the regulations

For the award of the degree of

M.D. MICROBIOLOGY

Branch - IV

DEPARTMENT OF MICROBIOLOGY,

PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH,

PEELAMEDU, COIMBATORE-641004,

TAMILNADU, INDIA.

PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH

COIMBATORE-4

CERTIFICATE

This is to certify that the dissertation work entitled **"Molecular detection of New Delhi metallobetalactamases in Escherichia coli and klebsiella pneumoniae in clinical isolates of a tertiary care Hospital** submitted by **Dr.M.Uma maheswari**, is the work done by her during the period of study in Department of Microbiology from April 2014 to August 2015. This work was done under direct guidance of **Dr. B.Appalaraju, Head of the Department, Department of Microbiology, PSGIMS & R.**

Dr. B. Appalaraju, M.D
Professor and Head
Department of Microbiology
HOSPITALS
PSG IMS &R

Dr. S. Ramalingam, M.D
Dean
PSG IMSR &PSG

Dr.M.Uma maheswari
Post graduate
Department of Microbiology
PSG IMS&R

Place: Coimbatore

Date:

ACKNOWLEDGEMENT

I am most great full to the God almighty for the blessings showered by him upon me to carry out this work.

With deep gratitude I express my sincere thanks to my guide **Dr.B.Appalaraju M.D** professor and Head of Department of Microbiology, P.S.G Institute of Medical sciences and Research for his vast knowledge in microbiology, constructive ideas, guidance, valuable advice, motivation and inspirations which has made the study possible and purposeful.

I am thankful to the Dean **Dr.S.Ramalingam MD** for permitting to carry out this study. I express my hearty thanks to the faculty, lab technicians and staffs of Microbiology who helped to carry out this study. I express my immense gratitude to my husband Dr. G.Thanaseharan MD, my kids Sharmila Nandhini ,Prem rajaseharan and my parents who sacrificed, encouraged and helped me to carry out my post graduation with ease. Finally I extend my sincere thanks to Dr. Saikeerthana, Dr. Rizwana, Dr.J.Lavanya, Mr.A.C.Baskkaran and Mrs. Manonmani for their immense help during the study period.

TABLE OF CONTENTS

	PAGE NO
INTRODUCTION	1
REVIEW OF LITERATURE	5
AIM AND OBJECTIVES	25
MATERIAL AND METHODS	27
RESULTS	37
DISCUSSION	64
SUMMARY	74
CONCLUSION	77
BIBLIOGRAPHY	
ANNEXURE	
ETHICAL CLEARANCE FORM	
TURNITIN DIGITAL RECEIPT	
CONSENT FORM	

ABSTRACT

INTRODUCTION

Enterobacteriaceae are inhabitants of human, animal gastrointestinal tract and various other environmental sites.Escherichia coli and klebsiella pneumoniae initially responded to betalactam antibiotics but there was an increase in resistance to betalactam antibiotics due to beta lactamase produced by them. Resistance to carbapenems has now become more prevalent due to production of carbapenemases. NDM-1 is a novel metallo betalactamase that is capable of hydrolysing all beta lactam antibiotics except Aztreonam.

AIM

Molecular detection of NDM-1 in Escherichia coli and klebsiella pneumoniae,Comparison of phenotypic methods of identifying carbapenemases and Screening of carbapenamases colonising in rectal samples of I.C.U Patients.

MATERIALS AND METHODS

After Ethical clearance 100 samples of carbapenem resistant Escherichia coli and klebsiella pneumoniae were randomly selected and phenotypically compared by Double disc synergy test,combined disc test,Modified Hodge test and Carba Np test.Molecular detection and sequencing of NDM-1was done.Screening for carbapenemases in Rectal swab of I.C.U patients were done using Carba chrome plate.

RESULTS

A total of 100 samples of Escherichia coli (42%) and klebsiella pneumoniae(58%) were selected. On comparison of phenotypic methods like Double -disc synergy, combined disc test, Modified Hodge test and Carba Np test , we observed Carba Np test was 100% sensitive and 100% specific. A total of 82% strains were found to be NDM-1 positive yielding 475bp amplicon by molecular methods. Nine samples were sequenced of which five were NDM-1, two were NDM-4, and remaining two were NDM-3, and NDM-5. About 8% carriers were identified on Screening of rectal swabs of 50 patients in I.C.U for carbapenemases

CONCLUSION

NDM-1 was detected in 82% 0f samples. Appropriate detection of emerging mechanism of resistance will prevent unnecessary mortality and morbidity. Carbapenamase carrier state was 8% and the less incidence is probably due to the infection control measures.

Key words :NDM-1-Newdelhi metallobetalactamases;I.C.U Intensive care unit

Introduction

ENTEROBACTERIACEAE

Enterobacteriaceae are inhabitants of wide variety of niches, including the human, animal gastrointestinal tract and various other environmental sites. The family Enterobacteriaceae includes a large number and diversity of genera .The clinically important members of this family are considered in two groups namely opportunistic pathogens and intestinal pathogens.

Typhi and shigella spp are intestinal pathogens. The opportunistic pathogens are *Citrobacter spp, Enterobacter spp, Klebsiella spp, Proteus spp, Serratia spp.* Escherichia coli is a normal bowel inhabitant and is classified between the overt pathogens and opportunistic pathogen. Yersinia pestis is not an intestinal pathogen ,it causes plague[1].

Escherichia coli causes urinary tract infections, cystitis, bacteremia, neonatal meningitis, diarrhea and dysentery. Shigella causes acute inflammatory colitis and bloody diarrhea. Salmonella is associated with enteric fever .Yesinia causes plague.

Other species like Klebsiella, Citrobacter, Enterobacter, Morganella, Providentia, proteus, serratia causes a wide variety of nosocomial infections of respiratory tract, urinary tract, and other normally sterile sites. They most frequently infect hospitalized and seriously debilitated patients. [1]

Treatment of *Escherichia coli* infections depend on the site and severity of the infection. Intraabdominal abscesses caused by *Escherichia coli* are treated with Ampicillin. Meningitis and pneumoniae are treated with third

generation cephalosporins like ceftriazone. Doxycycline, TMP/SMZ, fluroquinolones, Rifaximin are used in treatment of enteric infections .Uncomplicated cystitis requires TMP/SMZ and nitrofurantoin where as complicated cystitis responds to quinolones.

Klebsiella infections are intrinsically resistant to Ampicillin, Ticarcillin and nitrofurantoin is poorly active against them. In Severely ill patients with Klebsiella infections, the treatment options include third generation cephalosporins, quinolones and amino glycosides[2].

Escherichia coli and klebsiella initially responded to betalactam antibiotics there was a dramatic increase in resistance to betalactam antibiotics due to beta lactamases produced by them. These enzymes break the beta lactam ring, making them unable to bind to the penicillin binding proteins and terminate their activity[3].But this resistance was superseded by use of second and third generation cephalosporins and combination of betalactam antibiotics with betalactamases inhibitors.

With the emergence of ESBLs, carbapenems became the drug of choice. Resistance to carbapenems has now become more prevalent in Enterobacteriaceae due to production of carbapenamases. Dissemination of these pathogens is of great concern because there are only few antibiotics of choice for these resistant pathogens but none are orally available and are expensive[3]. Among the newly emerged beta lactamases, New Delhi Metallo betalactamases (NDM-1) represents a war between the bugs and drugs.NDM-1

producing organisms are called super bugs. This NDM-1 is a novel metallo betalactamases that is capable of hydrolysing all beta lactam antibiotics except Aztreonam[4].

In 2009, NDM-1 was first reported in Klebsiella pneumoniae, which was isolated in a Swedish patient, who was treated in an hospital in New Delhi previously. His faecal sample collected during his stay in hospital carried E.coli also. The gene encoding NDM-1 is the bla_{NDM-1} gene. Although this gene was identified in different members of the family Enterobacteriaceae, E.coli and Klebsiella remain the most frequent NDM-1 producers throughout the world. They are considered as significant pathogens causing infections in the hospital settings and the community[5].

In view of the above facts, the present study is undertaken to detect the presence of New Delhi metallo betalactamases in Escherichia coli and Klebsiella in clinical isolates of a tertiary care hospital.

Review of Literature

ENTEROBACTERIACEAE

Gram negative bacteria belonging to the family of Enterobacteriaceae are the most frequently isolated organisms from clinical specimens. They are widely dispersed in soil, water, plants. As the name indicates it is also present in intestinal tract of human and animals. The members of this family of Enterobacteriaceae exhibit general morphological and biochemical similarities. Within the family they exhibit wide biochemical and antigenic heterogeneity.

The members of the Enterobacteriaceae are considered into two groups the opportunistic pathogens and intestinal pathogens. *Typhi and Shigella spp* are in the latter group. The opportunistic pathogens are *Citrobacter spp, Enterobacter spp, Klebsiella spp, Proteus spp, Serratia spp.* Escherichia coli is a normal bowel inhabitant and its position is somewhere between the overt pathogen and the opportunistic pathogen.

The members of Enterobacteriaceae family are incriminated in virtually any type of infectious diseases and recovered from any specimen received in the laboratory. Immunocopromised patients are highly susceptible to hospital acquired infections following invasive procedures like catheterization, bronchoscopy, colposcopy, surgical biopsies, in which mucous membrane are traumatized[1].

ESCHERICHIA COLI

Escherichia coli was first named after Escherich , who first described the colon bacillus under the name Bacterium coli immune. E.coli is a gram

negative, straight rod measuring 1-3 micrometer, arranged singly or in pairs. It is motile by means of peritrichate flagella. Some strains are non motile[1]. E.coli is the one of the most common gram negative bacteria causing sepsis and endotoxic shock, urinary tract infections, meningitis in neonates and pneumonia in immunocompromised.The members of Escherichia have the following key reactions. They are Gram negative rods , motile, catalase positive, Oxidase negative, lactose fermenters, indole positive; methyl red positive; Vogesproskauer negative; citrate and urease negative[6].

KLEBSIELLA PNEUMONIAE

The genus klebsiella was named after Edwin kleb, a German microbiologist of nineteenth century[1].They are non motile non capsulated rods that grow in ordinary media, forming dome shaped mucoid colonies of different degree of stickiness. They are short plump rods .The capsule is prominent and can be made out in gram stains as clear halo. Klebsiella exists as commensal in intestine and as saprophytes in soil and water.It is the well known cause of fatal pneumonia[6].They are Gram negative rods, catalase positive and Oxidase negative. They produce enormous amount of carbon dioxide, so that the deep portions of KIA and TSI slants are pushed half way up the tube.They are lactose fermenters forming mucoid colonies ;Indole positive ; citrate and urease positive ;Methyl red negative ;voges proskauer positive.[6]

TREATMENT PROTOCOL

The 20th century saw a series of discoveries that has created remarkable changes in medical field. Among them, the most important is the discovery of anti microbial agents which began with synthesis of Arsphenamine by Paul Ehrlich for the treatment of syphilis. Shortly after that optochin (ethyl cuprine) was tried for the treatment of pneumococcal pneumonia. This was then followed by sulphonamides, penicillin's, antituberculous drugs and antifungal drugs in the middle of 20thcentury[7].

Due to the growing array of antibiotic resistance, the treatment protocol for Escherichia coli and Klebsiella pneumoniae must be based on Antimicrobial susceptibility testing, site of infection and the co morbid conditions. Intraabdominal abscesses caused by *Escherichia coli* are treated with Ampicillin. Meningitis and pneumoniae are treated with third generation cephalosporins like ceftriazone. Doxycycline, TMP, TMP/SMZ, fluroquinolones, Rifaximin are used in treatment of enteric infections .Uncomplicated cystitis requires TMP/SMZ and nitrofurantoin where as complicated cystitis responds to ceftriazones and quinolones. Klebsiella infections are intrinsically resistant to Ampicillin, Ticarcillin .Nitrofurantoin is poorly active against them. In Severely ill patients with Klebsiella infections, the treatment options include third generation cephalosporins, quinolones and amino glycosides.[2]

MECHANISM OF RESISTANCE

Antibiotic resistance is increasing both in community and hospital. Resistance to antibiotic among the bacteria is widely seen in day to day practice .Among them Gram negative bacteria has created a greater threat to human health[8].Synthesis of betalactamases which are capable of hydrolysing betalactam, target alteration, efflux mechanism , alteration in metabolic activity are the main mechanisms of drug resistance[9].

Gram negative bacterial infections resistant to various antibiotics were successfully treated in the past with penicillin group of antibiotics like piperacillin and other penicillin groups.. Carbapenems posses maximum antimicrobial activity. This feature is due to high affinity towards penicillin binding protein 2, being stable against major serine based betalactamases and good outer membrane permeability. Carbapenems are considered as one of the important drugs used in treatment of drug resistant gram negative pathogens. Inappropriate usage of carbapenems has resulted in development of resistance to carbapenems also. During the past ten years the antibiotic resistance in Enterobacteriaceae has become a major concern[10].

Escherichia coli and klebsiella pneumonia causes a variety of nosocomial and community acquired infections[11]. It possesses betalactamases which mediates high level of resistance to betalactam antibiotics. It also has acquired resistance to cephalosporins through plasmids containing chromosomally encoded Amp c betalactamases[12]. The wide spread use of

carbapenems which are the only drug active against these resistant bacteria resulted in emergence of a new antibiotic resistant mechanisms[13]. Resistance to carbapenems is predominately mediated by metallobetalactomases, a class B type of betalactamases that are capable of recognising bivalent metal ions[14].

CLASSIFICATION OF BETALACTAMASES

Functional group	Subgroup	Molecular class	Main substrate	Peculiarities of beta-lactamase members
1	1	C	all groups of beta-lactam antibiotics except carbapenems	chromosome-encoded AmpC beta-lactamases, some plasmid-encoded AmpC beta-lactamases — are not inhibited by clavulanic acid
2	2a	A	penicillins	penicillinases of Gram-positive bacteria — are inhibited by clavulanic acid
	2b	A	penicillins, cephalosporins	broad spectrum beta-lactamases (TEM-1, TEM-2, SHV-1) — are inhibited by clavulanic acid
	2be	A	penicillins, cephalosporins, monobactams	extended spectrum beta-lactamases (ESBL) — are inhibited by clavulanic acid
	2br	A	penicillins	inhibitor-resistant beta-lactamases of TEM and SHV types
	2c	A	penicillins, carbenicillin	carbenicillin-hydrolyzing PSE type beta-lactamases
	2e	A	cephalosporins	inducible cephalosporins from *Proteus* spp. — are inhibited by clavulanic acid
	2f	A	penicillins, cephalosporins, carbapenems	serine carbapenemases — are inhibited by clavulanic acid
	2d	D	penicillins, oxacillin	OXA type beta-lactamases hydrolyzing oxacillin — are mainly inhibited by clavulanic acid
3	3a, 3b, 3c	B	most beta-lactams, including carbapenems	metallo-beta-lactamases — are not inhibited by clavulanic acid but are inhibited by EDTA
4	not determined		penicillins	penicillinases not belonging to other groups

EXTENDED SPECTRUM BETALACTAMASES

Emergence of resistance to betalactam antibiotics emerged even before the discovery of the first beta lactam penicillin. The first betalactamases was identified in Escherichia coli prior to the release of penicillin for medical practice[15]. The first plasmid mediated betalactamases in gram negative bacteria was TEM-1, which was originally isolated in a single strain of Escherichia coli, isolated from blood culture of a patient named Temonier in Greece[16]. SHV-1 is

chromosomally mediated betalactamase in Klebsiella pneumonia and plasmid mediated betalactamase in Escherichia coli[17].

A new family of plasmid mediated ESBL called CTX-M, which hydrolysed cefotaxime had arose and were classified in Ambler Class A and in 2be of Bush, Jacoby and Medeiros classifications. The serine residue present at position 237 in CTX-M plays an important role in the extended spectrum activity of the CTX-M type of betalactamases. The unique feature of this enzyme is being inhibited by betalactamases inhibitor Tazobactum[18.]

Next is the OXA ESBL, which confers resistance to Ampicillin and cephalothin. They are characterised by their high hydrolytic activity against oxacillin and are poorly inhibited by clavulanic acid[17]. Majority of OXA-type ESBLS confer resistance to ceftazidime; the OXA-17 betalactamases confers resistance to cefotaxime and ceftriaxone but only marginal protection against ceftazidime[19.]

Some uncommon ESBLs are found in *Pseudomonas aeruginosa*. They are PER-1 in isolates in Turkey, France and Italy; VEB-1, VEB-2 strains from South East Asia; GES-1, GES-2, IBC-2 isolates from South Africa, France and Greece[20]. PER-1 is also reported in multidrug resistant Acinetobacter species. Some of these enzymes are found in other Enterobacteriaceae as well[21].There are some uncommon ESBLs found only in Enterobacteriaceae, they are BES-1, IBC-1, SFO-1 and TLA-1[22].

DETECTION OF ESBLS

As per CLSI 2014 initial screening and confirmatory tests for production of ESBL in Escherichia coli and Klebsiella pneumoniae is done using disc diffusion and Broth micro dilution methods.

Initial screening test is done by disc diffusion on MHA with the following discs

TABLE-1

Drug sensitivity disc	Sensitivity zones
Cefpodoxime	≤ 17mm
Ceftazidime	≤ 22mm
Aztreonam	≤ 27mm
Cefotaxime	≤27mm
Ceftriaxone	≤ 25mm

Zones above may indicate ESBL production. The use of more than one antimicrobial agent for screening improves the sensitivity of ESBL detection.

In Broth micro dilution, the growth at or above the screening concentrations indicate ESBL production i.e. MIC ≥8ug/ml for Cefpodoxime, MIC ≥2ug/ml for Ceftazidime, Aztreonam, Cefotaxime and Ceftriaxone.

Phenotypic confirmation of the ESBL producing stains by disc diffusion is performed by testing both cefotaxime30ug and ceftazidime30ug alone and in combination with clavulanate 10ug.A ≥ 5mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanate

versus the zone diameter of the agent when tested alone is confirmatory of ESBL. In Broth microdilution method a ≥3 twofold concentration decrease in an MIC for Cefotaxime (0.25-64ug/ml) or Ceftazidime (0.25-128ug/ml) in combination with clavulanate versus the MIC of the antimicrobial agents when tested alone is confirmatory for ESBL production.

CARBAPENAMASES

The carbapenam antibiotic obtained from Thienamycin was produced by the soil organism streptomyces cattleya[23]. As these molecules were present in soil , it is a logic to expect that the organisms present in soil to produce some enzymes (carbapenamases) capable of degrading the betalactam molecules, produced by streptomyces cattleya for their survival[24]. In addition these chromosomal carbapenamases might have evolved initially as a mode of protection of bacterial cell wall against external dangers[25].

Carbapenems are the antimicrobials used as a last resort for the treatment of infections caused by ESBL or Plasmid mediated AmpC producing organisms. They are also resistant to other group of Antibiotics like Quinolones, Amino glycosides and cotrimoxazole[26.] Carbapenamases are heterogeneous mixture of betalactamases belonging to molecular Ambler Class A, Class B (Metallo betalactamases) and Class D (Oxacillinases).They have a common property of hydrolysing Imipenam, Meropenam together with other penicillin or cephalosporin Antibiotics[27].

Metallo betalactamases are the betalactamases with the ability to hydrolyse carbapenems and are resistant to commercially available betalactam inhibitors but susceptible to inhibition by metal ion chelators. These enzymes hydrolyse cephalosporins and penicillin's but lack the ability to hydrolyse Aztreonam[28].

The class B enzymes are plasmid mediated and integron located. It includes IMP-(active on imipenem),VIM-(Verona integron encoded Metallobetalactamase) ,GIM-(German imipenemase) ,SPM-1(Sao Paul imipenemase), SIM-1 (Seoul imipenemase) ,NDM-(New Delhi metallobetalactamases).

The metallobeta lactamases are sub classified into three subclasses based on their structural features, zinc affinities for the two binding sites and hydrolysis.

The sub classes B1 and B3 bind with two zinc ions to achieve maximal hydrolysis. But the enzymes in B2 subclass are inhibited if a second zinc ion is added to it. The enzymes of B2 subclass hydrolyses only the carbapenems in contrast to the broad spectrum hydrolysis exhibited by the B1 and B3 enzymes[29].

NEWDELHI METALLOBETALACTAMASES

Our target of interest NDM was first discovered in patients who had a history of travel to Indian subcontinent and hence named as New Delhimetallobetalactamase.NDM was isolated first in 2009 in a single isolate

of Klebsiella pneumonia, which were first recovered from a patient who returned back to Sweden after being treated in a hospital in New Delhi .During his stay in hospital his faecal sample also was positive for NDM-1 producing E.coli[30].

NDM hydrolyses all beta lactam antibiotics except Aztreonam and are susceptible to colistin and less consistently to Tigecycline[5]. The NDM producers has several factors which are alarming the public health. Firstly, the bla NDM gene which is identified in NDM is not present in a single species alone but presents in many unrelated species indicating that this gene is spreading in a very rapid rate. Secondly, it is not only present in Klebsiella pneumonia which is a nosocomial pathogen but also in Escherichia coli which is a community acquired pathogen. Thirdly Escherichia coli is the number one cause of diarrhoea in children in India and Pakistan and thus with an increased risk of releasing the resistant strains into the environment. The lack of sanitation facilities, unavailability of clean water, Temperate climate and finally over population are the factors that contribute to the spread of NDM in India and Pakistan and then internationally[31]. The Indian continent, the Balkan regions, and the Middle East were found to be the main reservoirs producing New Delhi Metallobetalactamase.

The presence of NDM producers in the patients who were hospitalised in United Kingdom were found to been previously treated and hospitalised in Indian subcontinent. The same observation was noted in France, Italy and Sultanate of Oman[32].

NDM-1 displays tighter binding to most of cephalosporins in particular to cefuroxime.NDM-1 at its active site has two metal ion binding sites: His and Cys sites. A 3D modelling of NDM-1 showed that two Zn ions were found at both of these His and Cys sites at a distance of 4.20 A[33]. On comparing IMP-1, VIM-2 and NDM-1 by an *in silico* approach it was found that NDM-1 might have greater drug profile and catalytic efficacy than IMP-1 and VIM-2 due to a larger pocket opening and a lower distance between zinc-1 ion and beta lactam oxygen of the carbapenam[34].

NDM-1 producers were mainly described in Urinary tract infections, peritonitis, septicaemia, pulmonary, soft tissue and device associated infections. In Enterobacteriaceae bla NDM-1 gene is located mostly on conjugative plasmids[32].

Emigration and migration of population is considered as main factor in spreading of antibiotic resistance of organisms and their globalisation. The evolving drug resistance among Enterobacteriaceae is mainly due the genes located on the plasmids, which spreads to different bacterial species through genetic recombination[8].

The genes that code for NDM-1 producing organisms are located on different positions of plasmids (a180kb plasmid for klebsiella pneumonia and 140 kb for Escherichia coli) [5]. Lateral gene transfer has lead to the rapid dissemination and spread of resistance among different bacterial species[35].

It was reported in a study that all patients who are positive for NDM-1 did not have a history of hospital admission in India[31].

The symptoms produced due to infection caused by NDM-1 producing bacteria depend on the site of infection. Blood, urinary tract, lungs and wounds are the common site of infection[34].

Only two classes of drugs like polymyxin (colistin) and glycylcyclines (tigecycline) showed good in vitro activity against NDM-1 producers. Successful treatment of pan resistant Enterobacteriaceae with combination therapy of colistin and tigecycline has been reported in similar studies. Combinations using Aztreonam or any other monobactum which are resistant to hydrolysis by metallolactamases are recommended in some studies. NDM-1 containing bacterial strain may also have other mechanism of resistance like ampC or ESBLs which may hydrolyze the Aztreonam. So inhibitors of these enzymes (like NXL104) should be the part of combination therapy. Fosfomycin is suggested for use in carbapenemase producing pan resistant Enterobacteriacea[36].

DNA mutations resulting in changes in the amino acid sequence of the carbapenemase have produced in an ever increasing range of subtypes or variants of each of type of carbapenemase.

Fifteen variants of NDM-1 have been identified. These variants were identified after sequencing and blasting. They differed from NDM-1 by various amino acid substitutions.

The first variant NDM-2 has been identified to have a C to G substitution at position 82 which resulted in substitution of a proline to an alanine residue at position 28. MIC values of Betalactams and carbapenems showed no difference between NDM-1 and NDM-2 producers.

NDM-2 has been identified in several Acinetobacter baumanii strains but not in Enterobacteriaceae. NDM-3 was described which differed from NDM-1 by a single nucleotide change at position 95 (Asp-Asn) this change did not modify the hydrolytic activities of the enzyme[37].

NDM-4 Varies by a amino acid alteration at position 154 (Met-Leu). But this amino acid substitution is responsible for increased hydrolytic activity of NDM-4 compared to NDM-1 towards Cefalothin, Ceftazidime, Imipenem, Meropenem but cefepime was less hydrolysed[38].

NDM-5 has substitution at position 154 (Met-Leu) similar to NDM-4 conferring maximum hydrolytic activity against carbapenems ,further has a second amino acid substitution at position 88 (Val-Leu) [39].

NDM-6 retains its uniqueness by a single amino acid substitution at 233 (Ala-Val), with no obvious modified hydrolytic activity of the enzyme[40.]

In NDM-7, bla NDM-7 gene differs from NDM-1 by two substitutions at position 130 (Asp-Asn) and 154 (Met-Leu).The Amino acid substitution at 154 increases the hydrolytic activity of the enzyme[41]. NDM-8 has amino acid substitutions at 130 (Asp-Gly) and 154 (Met-Leu) [42]. NDM-9 has single amino

acid substitutions E to K at 152 position (Gene Bank accession number KC999080)[35].

The NDM-1 primers used are NDM Forward: 5'- GGGCAGTCGCTTCCAACGGT and

NDM Reverse: 5'- GTAGTGCTCAGTGTCGGCAT (Sigma-Aldrich, Bengaluru), Which amplified an internal fragment of 475 bp of bla NDM-1 gene. The design of primers are based on the sequence of the bla $_{NDM-1}$ gene in the GenBank database under accession no HQ171206[43].

DETECTION METHODS OF CARBAPENAMASES

Different methods of detection are [33,44,45,46]

1. Modified Hodge test
2. Double disc synergy test
3. Combined disc or Disc enhancement test
4. Carba Np Test
5. Remodified Hodge test
6. E test

MODIFIED HODGE TEST

Escherichia coli ATCC 25922 was taken and made as a lawn culture in Muller Hinton Plate and dried , 3-5 colonies of the test organism grown

overnight on a blood agar plate was picked and inoculated from the edges of the Meropenam disc(placed in centre of plate) in the form of straight line. After overnight incubation there was evidence of Enhanced growth around the test. Enhanced growth was positive for Carbapenamases production resulting in Clover Leaf Pattern.

DOUBLE DISC SYNERGY TEST

With the test colonies a lawn culture was made on MHA plate. A 6mm Whatmann No filter paper disc with 10ul of EDTA was placed in the centre of the disc. Merapemem 10ug and ceftazidime 30ug discs were placed on either side at a distance of 10mm . On Incubation at 35^0c for 18-24hrs, any distortion or indentation towards the disc was considered as positive.

COMBINED DISC TEST

The test colonies were incubated and a lawn culture was made on MHA, two Meropenem (10ug) and two ceftazidime (30ug) discs were placed on it. Nearly 10ul of EDTA was added to one of each Meropenem and ceftazidime discs . The plates were placed at 35^0c for 24hrs.The zone size of greater than 5mm was considered as positive for metallobetalactamase production.

CARBA NP TEST

One calibrated loop of test organism was taken from a MHA plate .The colony was suspended in Tris Hcl lysis buffer 20mmol/L, vortexed for 1minute, incubated at room temperature for 30 minutes and centrifuged at

10,000 rpm for 5minutes. 30 ul of supernatant of centrifuged bacterial isolate was added to 100ul of a 1ml solution made of 3mg merapenem monohydrate, pH7.8, phenol red solution and 0.1mmol/l $Znso_4$ and Incubated at 37^0c for 2 hrs. The colour of wells changed from Red to yellow in all the test strains producing carbapenemases. The colour remained red for the negative strains.

REMODIFIED HODGE TEST

The ATCC Escherichia coli were inoculated in similar manner as the MHT. Ertapenem discs are placed far enough so that their inhibition zones do not overlap. One of the discs is labelled Zn and to which 140 ug of $Znso_4$ was added, the other disc was left blank. Two test isolates were streaked from both the merapenem discs to the periphery as mirror images. The plates were incubated at 35^0c for 18-24 hrs and read. In MBL production indentation was seen with MHT with merapenem disc only[47.]

E TEST

E test strip is a double-sided strip consisting of Meropenem (4 to 256 µg/ml) on one side and Meropenem plus EDTA (1 to 64 µg/ml) on the other. The E-test was placed on the lawn culture of test organism. The presence of MBL was indicated by a reduction of MRP MIC ≥3 twofold dilutions in the presence of EDTA (ratio of MRP/MRP+EDTA of ≥ 8) or the appearance of phantom zones, or deformation of ellipses[48].

Klebsiella pneumonia and Escherichia coli were the most commonly reported bacteria producing New Delhimetallobetalactamases-1 enzyme. A

number of countries including the United Kingdom, Italy and Oman have several case reports of infections caused by NDM-1. About 86.3% of these infections were from patients who had connection with the Indian subcontinent or Balkan countries[49.]

In a study conducted in a tertiary care hospital in North East India out of 270 isolates of Escherichia coli, fourteen were screened for carbapenamase production on basis of their reduction in susceptibility to Meropenam and Ertapenam. All the fourteen were found positive for blaNDM-1[50].In a study conducted on the prevalence of NDM-1 in multidrug resistant Enterobacteriaceae in India, Pakistan and UK in 2010. NDM-1 producing Escherichia coli and Klebsiella were isolated from Forty four cases in chennai33. In a study conducted in a tertiary care hospital Bangalore, out of a total of seventy four multidrug resistant Gram negative bacilli isolates, thirty four were positive for bla NDM-1 gene[51].

In a survey conducted among twenty four European countries to gather information about the spread of NDM-1 producing Enterobacteriaceae ,a total of seventy four cases were reported from thirteen countries from 2008-2010. these fifty five cases were recorded with travel history and thirty one with history of admission in India or Pakistan and five with previous history of being admitted in Balkan countries[52].

In a study conducted in NDM-1 producing isolates of Klebsiella pneumoniae in North East Asia, Out of 219 isolates, nineteen were screened for

carbapenamases production. Among them 57.89% were from urine 15.79% were from sputum 10.53% from blood and 15.79% were from pus. All the above screening positive isolates were found contain plasmid mediated NDM-1 gene by molecular methods[4].In a study conducted among Escherichia coli and Klebsiella pneumoniae isolates in a tertiary care hospital in south India ,75% of Klebsiella pneumoniae isolates were bla NDM-1 positive and 66% of Escherichia coli were bla NDM-1 positive.[53]

The following databasis is obtained from a review of Google maps on the world wide dissemination of New Delhi metallobetalactamases

In Afghanistan 2 isolates of NDM were first described in 2011 ; In Bangladesh 17 isolates were described in 2008 ; In Canada 18 cases were described in 2009-2010 ;In China 16 isolates described in 2009-2012 ;In France 4 isolates in2011 ;In India from Assam, Bangalore, Chennai, Guwahati, Haryana, Kolkata, Mumbai, New Delhi, Pune, Varanasi 374 cases were isolated in 2006-2007;Iran 1 isolate in 2011; Israel Jerusalem, 10 isolates in 2010 .In Japan 4 cases isolated in 2010; Kenya, 7 isolates from 2007-2009 ;

In South Korea 4 isolates were described in 2010 ;Spain , 1 case isolated in 2012 ; In Sweden Stockholm 4 cases were described in 2011 ; Switzerland Geneva 3 isolates in 2009-2010 . Taiwan Taipei 1 case in 2011.

In Thailand 6 isolates were described 2010 ; In Vietnam , 2 isolates were described in 2010 United Arab , 2 isolates in 2011 ;In United Kingdom ,in 10 cities 23 isolates were described in the year 2011[41].

Isolation of patients who are carriers from non carriers is the key component in preventing spread of carbapenamases in hospitals. Culture results alone cannot be solely relied to detect the carriers. Hence active surveillance should be carried out. The sites of Carbapenamase resistant Enterobacteriaceae includes lower G.I.T, the oropharynx, skin and urine. The primary surveillance site advocated by the US centers for Disease control and Prevention and European Society of Clinical Microbiology and Infectious disease is the rectal swab or stool[54].

Aim and Objectives

AIM OF THE STUDY

- To isolate carbapenam resistant Escherichia coli and klebsiella pneumoniae from various clinical specimens of a tertiary care hospital.
- Molecular detection of New Delhi metallobetalactamases in Escherichia coli and klebsiella pneumoniae.

OBJECTIVES

- To isolate the carbapenam resistant Escherichia coli and klebsiella pneumoniae from various clinical specimens.
- Comparison of phenotypic methods of identifying carbapenamases.
- Molecular detection of New Delhi Metallobetalactamases in Escherichia coli and klebsiella pneumoniae in clinical isolates of a tertiary care hospital.
- Screening of carbapenamases colonising in rectal samples of I.C.U Patients

Materials and Methods

After Ethical clearance from the institution, the study was conducted between April 2014 - August 2015. Out of Two thousand and forty two samples of Escherichia coli and thousand hundred and ninety two samples of Klebsiella pneumoniae, Five hundred and sixty samples of Klebsiella pneumoniae (47%) and hundred and forty eight isolates of Escherichia coli (7%) were resistant to carbapenems. A total of 100 strains from inpatients and out patients were studied randomly.

STUDY POPULATION / SAMPLING METHODS

Random sampling Method

SAMPLE SIZE

100 samples were selected randomly.

TYPE OF STUDY

Observational study.

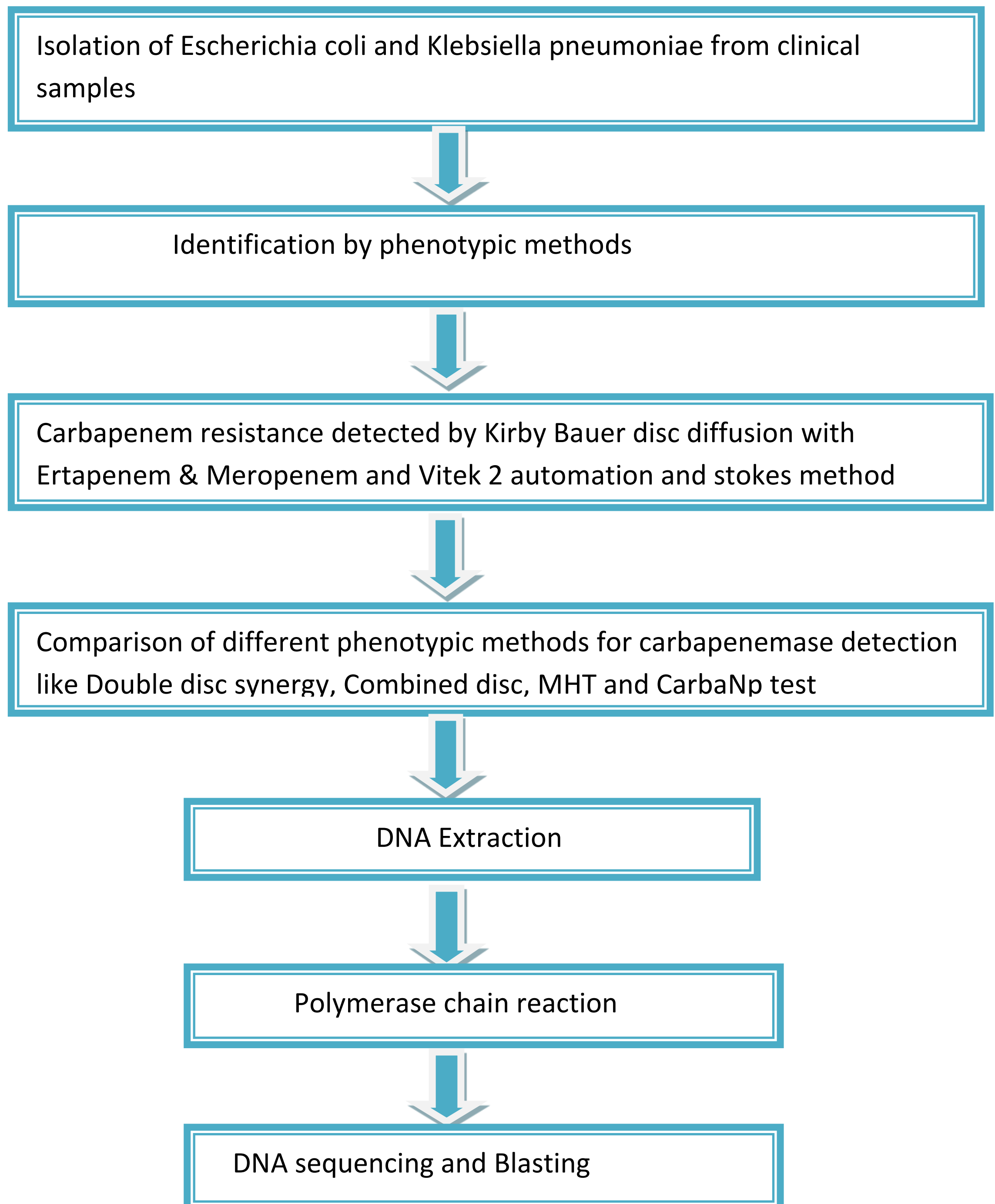
INCLUSION CRITERIA

Carbapenem resistant Escherichia coli and Klebsiella pneumoniae

EXCLUSION CRITERIA

Escherichia coli and Klebsiella pneumonia sensitive to Carbapenam.

STUDY DESIGN:



Collection of Rectal swabs of I.C.U patients who were on prolonged (>15days) or repeated Hospitalisation

Screening on CARBA CHROME AGAR to detect the presence of Carbapenamase producing Escherichia coli and Klebsiella pneumoniae

ISOLATION OF ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE

Gram staining of all the isolates of Escherichia coli and Klebsiella pneumoniae were performed. Gram negative rods were observed in the direct smear and the samples were streaked in the Blood agar, Mac conkey agar. Biochemical reactions like catalase, oxidase and IMViC were done.

SCREENING FOR CARBAPENAMASES:

The isolates were screened for the production of Carbapenam resistance using Ertapenem 10ug and Merapenam 10ug by standard Kirby bauer disc diffusion procedure and by strokes method.

As per CLSI 2014 Ertapenem non susceptibility is the most sensitive indicator of Carbapenem resistance. The zone diameter of inhibition < than 19-21mm for Ertapenem and < than 16-21mm for Merapenam indicates Carbapenemase production. Merapenam and Ertapenem were also tested with

automated identification system (VITEK 2) which analyses the susceptibility pattern based on Minimal Inhibitory Concentration.

COMPARISON OF DIFFERENT PHENOTYPIC METHODS FOR DETECTION OF CARBAPENEMASES [44,45,46]

DOUBLE DISC SYNERGY TEST

The colonies to be tested were inoculated in peptone water and incubated at 37^0C for 2 hours to get turbidity matching to Mac Farland 0.5. A lawn culture was made in Muller Hinton plate using a sterile cotton swab. A 6mm Whatmann No filter paper disc with 10ul of EDTA was placed in the center of the disc. Ertapenem 10ug and ceftazidime 30ug discs were placed 10mm apart. Incubated at 35^0c for 24hrs any distortion or indentation towards the disc with EDTA was considered as positive.

COMBINED DISC TEST

The test colonies were inoculated and incubated at 37^0c for two hours to match the Mac Farland turbidity of 0.5.A lawn culture was made in which two Ertapenem (10ug) and ceftazidime (30ug) discs were kept. About 10ul of EDTA was added to one of the discs. The plates were kept at 35^0c for 24hrs.The change in diameter of greater than 5mm was considered positive .

MODIFIED HODGE TEST

A 0.5 MacFarland standard suspension of Escherichia coli ATCC 25922 was taken and made as a lawn culture in Muller Hinton Plate. Allowed to dry

for 3-10 minutes. The Ertapenem disc was placed on the plate. Using 10 ul loops 3-5 colonies of the test organism grown overnight on a blood agar plate were picked and inoculated from the edges of the disc in the form of straight line .It is Incubated at 35^0c.After over night incubation there was evidence of Enhanced growth around the test .Enhanced growth with clover leaf pattern was considered as positive for Carbapenemase production.

CARBA NP TEST

One calibrated loop of test organism was taken from a MHA plate .The colony was suspended in Tris Hcl lysis buffer 20mmol/L, vortexed for 1minute and incubated at room temperature for 30 minutes.

This bacterial suspension was centrifuged at 10,000 rpm for 5minutes. 100ul of a 1ml solution made of 3mg Merapenam monohydrate, pH7.8, phenol red solution and 0.1mmol/l $Znso_4$ was prepared. 30 microliter supernatant of centrifuged bacterial isolate was added to 100ul of the above suspension in a 96 welled microtiter plate.

Incubated at 37^0c for 2 hrs. The colour of wells changed from Red to yellow in all the test strains producing carbapenemases. The colour remained red for the negative strains.

MOLECULAR DETECTION OF CARBAPENAMASES

EXTRACTION OF DNA

The isolates were incubated overnight at 35^0c in Luria broth .About 2-3 ml of inoculum was used for DNA extraction.

PREPARATION OF LURIA BROTH:

The Luria broth has the following ingredients

1. Tryptone 1.0% - 10g
2. Yeast extracts 0.5% - 5g
3. Nacl 0.5% - 5g
4. Water - 1000ml

The DNA was extracted by boiling method. The isolates which inoculated in Luria broth were centrifuged at 5000rpm for 10 minutes. After centrifugation the supernatant was discarded and the pellet was taken in a sterile eppendorph tube.

The pellets were resuspended in distilled water and kept in a water bath at 95^0c for 20 minutes. It is then cooled and centrifuged at 5000 rpm for 10 minutes. The supernatant is the extracted DNA and is stored at _20^0c.

POLYMERASE CHAIN REACTION

The extracted DNA was mixed with forward, reverse primers for NDM-1, master mix and PCR water and amplified in step one AB applied Bio system Real time PCR.

PCR cycle consists of the following steps.[55]

Initial denaturation: 94°C for 3 min,

Denaturation at 94°C for 30 s

Annealing at 60°C for 25 s 30 cycles

Extension at 72°C for 30 s,

Final extension at 72°C for 3 min.

The amplified products are stored at _20^0c[43]

TRIS BORIC ACETATE BUFFER PREPARATION

Tris base _ 10.78 g

Boric acid_ 5.50 g

EDTA_ 3.72g

1000 ml of milli Q water

This is a 10x solution.

To about 450 ml of Milli Q water, 50ml of above prepared Tris boric acetate buffer was added to obtain 1x solution.

PREPARATION OF ETHIDIUM BROMIDE

10 mg of Ethidium bromide powder was added to 1ml of sterile distilled water.

GEL PREPARATION AND GEL ELECTROPHORESIS

Agarose of 0.75g was added to 80ml of 1x solution .The mixture was mixed completely and melted for 2minutes in oven. A gel electrophoresis trough which can hold 80ml of water and 15 well combs was selected.

The melted agarose gel was cooled and Ethidium bromide (10mg/ml) 1ul was added to it. The mixture was poured in the trough and the agarose gel was allowed to set in after placing the comb.

The electrophoresis tank was taken and filled with buffer; the set agarose gel was kept in the tank after removing the comb. The amplified DNA of 5ul was added to 3ul of Methylene blue and pipetted into the well, with a positive and negative control.

The electrophoresis tank was connected to voltage meter. The current passed from cathode to anode. The temperature was set at 98°c and observed after 45minutes.After 45 minutes the gel was removed from Electrophoretic trough and placed in a gel Doc. The images of the gel were captured by Gel

doc and viewed. The presence of the NDM-1 gene was confirmed by the formation of bands at 475bp and a 100bp ladder was used for this purpose.

GENE SEQUENCING

The amplied products which were confirmed for the presence of NDM-1 were sent for gene sequencing to Eurofins Genomics Bangalore.

GENE BLASTING

The sequenced gene was blasted using BIO EDIT software and results were interpreted in the NCBI (National centre for Biotechnology Information) website.

SCREENING FOR THE PRESENCE OF CARBAPENAMASES IN I.C.U PATIENTS

The Rectal swab of 50 I.C.U patients who were hospitalised for more than 15 days were collected and plated on the Carba chrome plate which was commercially available. The plates were incubated at 37^0c for 18-24 hrs and observed for the presence of coloured colonies. Escherichia coli appeared purple coloured. Klebsiella pneumonia appeared green coloured[56.]

Results

Out of 2042 samples of Escherichia coli and 1192 samples of Klebsiella pneumoniae reported from April 2014 to August 2015, Five hundred and sixty samples of Klebsiella pneumoniae (47%) and Hundred and forty eight isolates of Escherichia coli (7%) were resistant to carbapenems.

Among them Forty two isolates of Escherichia coli and Fifty eight isolates of Klebsiella pneumoniae which were resistant to carbapenem were selected randomly for the study as shown in figure 1.

Cultural characteristics and Biochemical reactions of Escherichia coli and Klebsiella pneumoniae are shown in figures 2, 3, 4 and 5. Escherichia coli formed beta haemolytic colonies on blood agar and bright pink colonies on Mac conkey. The colonies were large, thick, greyish white, moist, smooth opaque or partially translucent. Escherichia coli were Catalase positive; oxidase negative; Indole and methyl red positive; citrate and Voges proskauer were negative; (IMViC ++--).

Klebsiella pneumonia formed non haemolytic grey moist colonies on blood agar and large dome shaped lactose fermenting mucoid colonies of varying degrees of stickiness in Mac conkey. The following biochemical reactions were observed. Catalase positive; oxidase negative; Indole and methyl red negative; ferments Glucose, lactose, sucrose were fermented with gas; citrate and Voges proskauer positive; (IMViC --++).

The isolates were taken from clinical samples like urine (msu and catheterized), tracheal aspirate, sputum, pleural fluid, BAL, Abdominal drain,

wound swab, wound tissues, CVP tip, peritoneal fluid, duodenal aspirate and Blood. They are shown in Figure 6. All the strains were reconfirmed as carbapenems resistant by strokes, Kirby bauer disc diffusion method and automatic broth dilution tests (Vitek-2) Figure-7and 8. All the strains were 100% resistant to Ceftazidime, Cefperazonesulbactum, Cefepime, Aztreonam, Imepenem, Meropenam, Gentamicin & ciprofloxacin.About 32% of the strains were sensitive to Amikacin. Cotrimoxazole was sensitive in 5% of isolates.All the isolates were sensitive to Tigecycline and Colistin (100%) ,as shown in table 3.

The following phenotypic tests were compared.

1. Double disc synergy test
2. Combined disc test } figure-9
3. Modified Hodge test figure-10
4. Carba NP test figure-11

It was observed that 42% were positive by Double disc diffusion test,75% were positive by Combination disc test ,90% were positive by Modified Hodge test and 98% were positive by Carba Np test. The sensitivity and specificity of Carba Np test is 100 % and is better than Modified Hodge test (sensitivity 91% specificity 100%), Combined disc test (sensitivity 92% and specificity 50%) and double disc synergy test (sensitivity 42% and specificity 50%) It is shown in table 2.

All the strains were subjected to Molecular detection of NDM-1 type carbapenamases after extracting the DNA by boiling method in water bath, shown in Figure 12.

The DNA was amplified using ABI step one Real time PCR (figure 13).The amplified samples were subjected to Gel electrophoresis (figure-14) and the bands were viewed using Gel doc (figure -15).A total of eighty two (82%) strains were found to be NDM-1 positive (475bp) (figure-17).Gel pattern of NDM-1 is depicted in the figure 16 .Thirty seven (88%) of Escherichia coli and Forty five Klebsiella pneumoniae(77%) were NDM-1 producers. Urine samples were the maximum forty mid stream urine and four catheterized (53%).

Nine samples of amplified DNA (25ul) were sent randomly for sequencing at Euro fin genomics India pvt Ltd in Bangalore. Among them four were Escherichia coli and five were Klebsiella pneumoniae

The results send by the Eurofins Genomics were subjected to gene blasting using the BIO EDIT software and the sequence results were interpreted in NCBI (National centre for Biotechnology and Information) web site and shown in table-4 and figure 18-26.

.On interpretation it was found that PCR-1, PCR-2, PCR-4, PCR-6 and 8 had amino acid sequences of NDM-1 and PCR-3 was NDM-5 and PCR-5 was NDM-3 and PCR-7 and PCR-9 were NDM-4

The PCR-3 (NDM-5) showed a point mutation variant differing from NDM-1 having a Met to Leu (M-L) substitution at 154 and Val to Leu (V-L)substitution at 88.

PCR-5 (NDM-3) showed a point mutation variant differing from NDM-1 having substitution of Asp to Asn (D-N) at 95.

PCR-7 and PCR-9 (NDM-4) differs from NDM-1 by a single amino acid substitution (M-L) at 154 (Met to Leu).

The results of screening of carbapenamases producing Escherichia coli and Klebsiella pneumoniae using Carba chrome agar plate are shown in figure 27, 28.Out of fifty isolates four (8%) were found to be carbapenemases producers, three were Klebsiella pneumoniae (75%) and one was Escherichia coli (25%).

FIGURE-1 Percentage distribution of bacterial isolates used in study

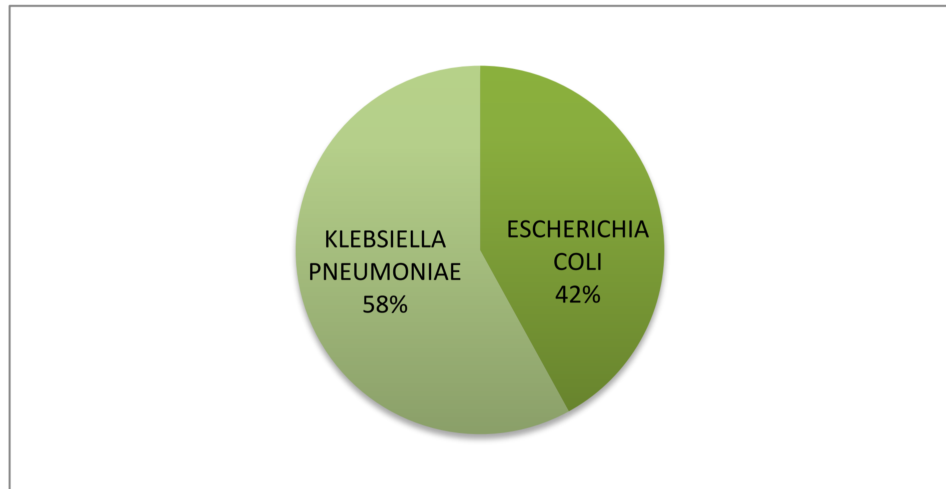


FIGURE-2 Mucoid lactose fermenting colonies of Klebsiella pneumoniae in Mac conkey agar

FIGURE-3 Flat lactose fermenting colonies of Escherichia coli in Mac conkey agar

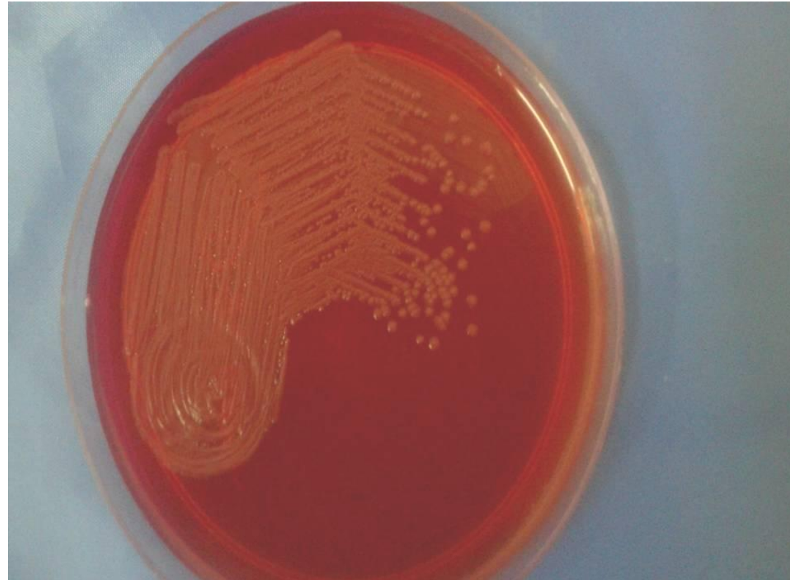
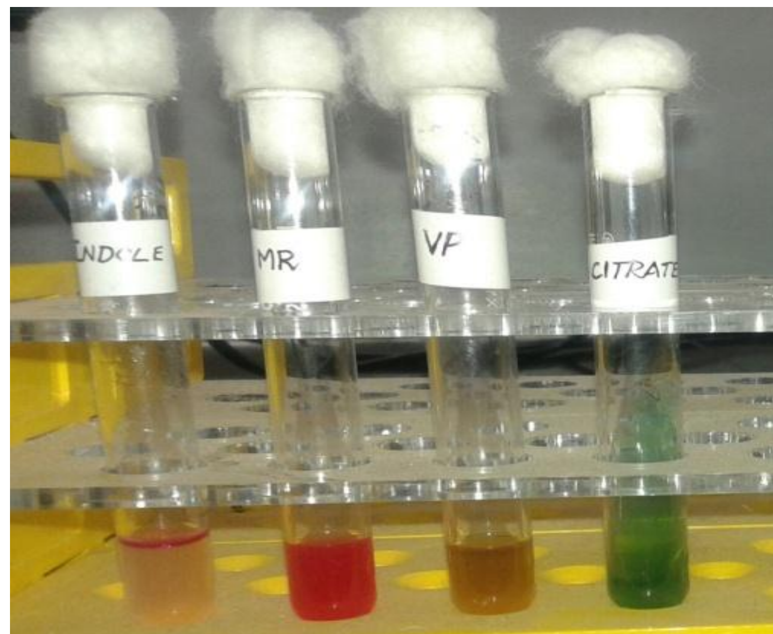


FIGURE-4 Biochemical reactions of Escherichia coli



L to R 1.Indole + ve 2.Methyl red test + ve, 3.Voges prausker test - ve, 4. Citrate test -ve.

FIGURE-5 Biochemical reactions of Klebsiella pneumoniae

L to R 1.Indole – ve, 2.Methyl red test - ve, 3.Voges prausker test + ve,

4.Citrate test +-.

FIGURE-6 Distribution of various samples used in the study

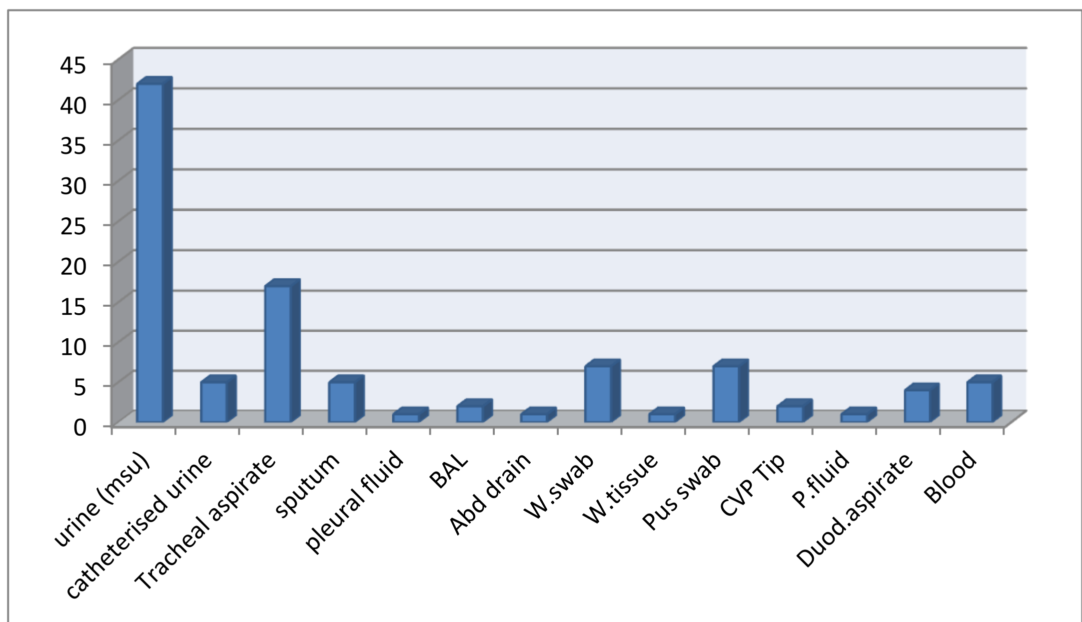


FIGURE-7 Carbapenem resistance screening by Ertapenem using

Kirby bauer disc diffusion method

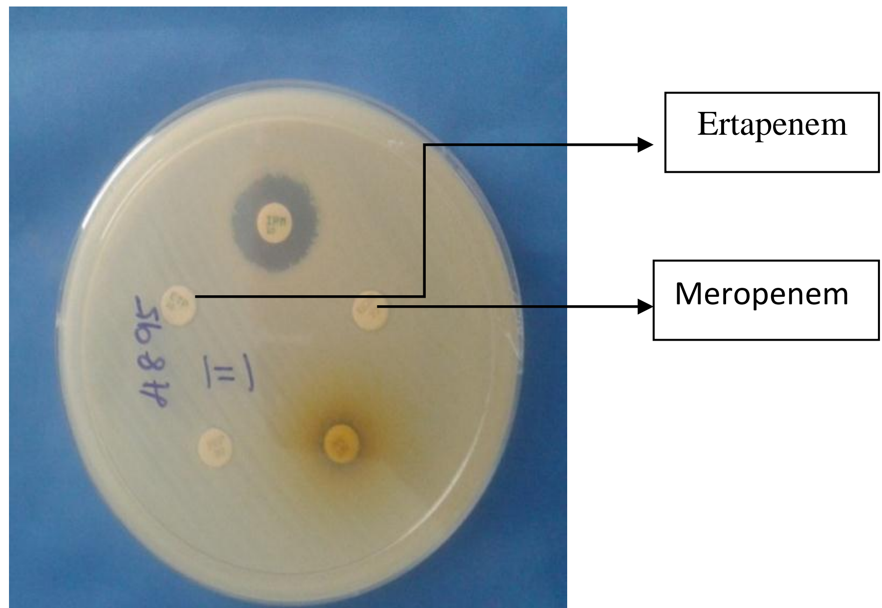


FIGURE-8 Carbapenem resistance screening by Ertapenem using stokes method

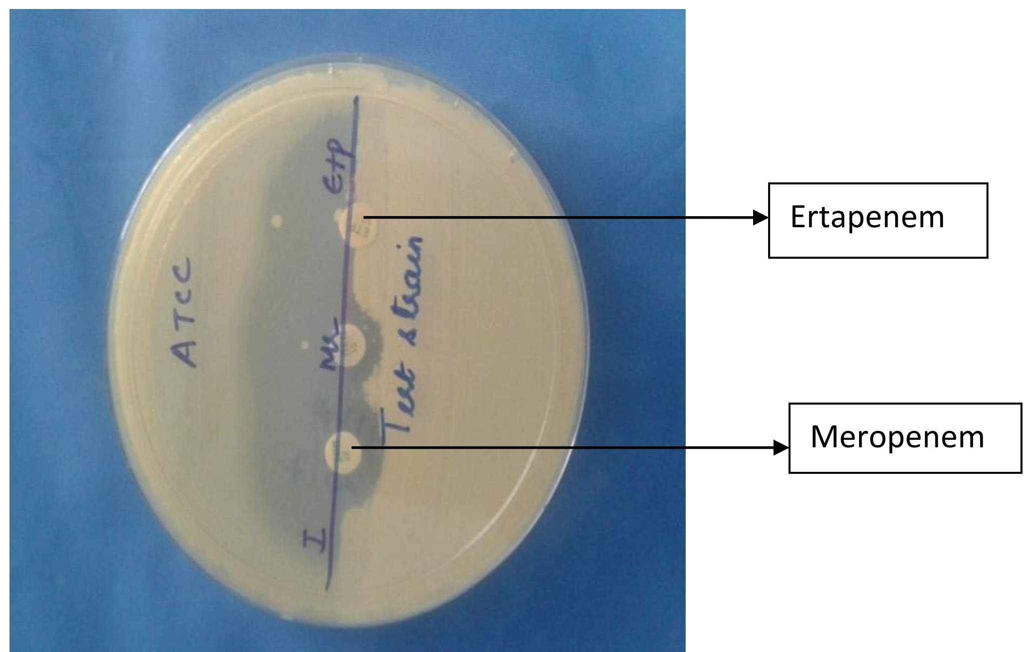


FIGURE-9 Detection of carbapenamase by double disc synergy test and combined disc test (arrow indicating synergy)

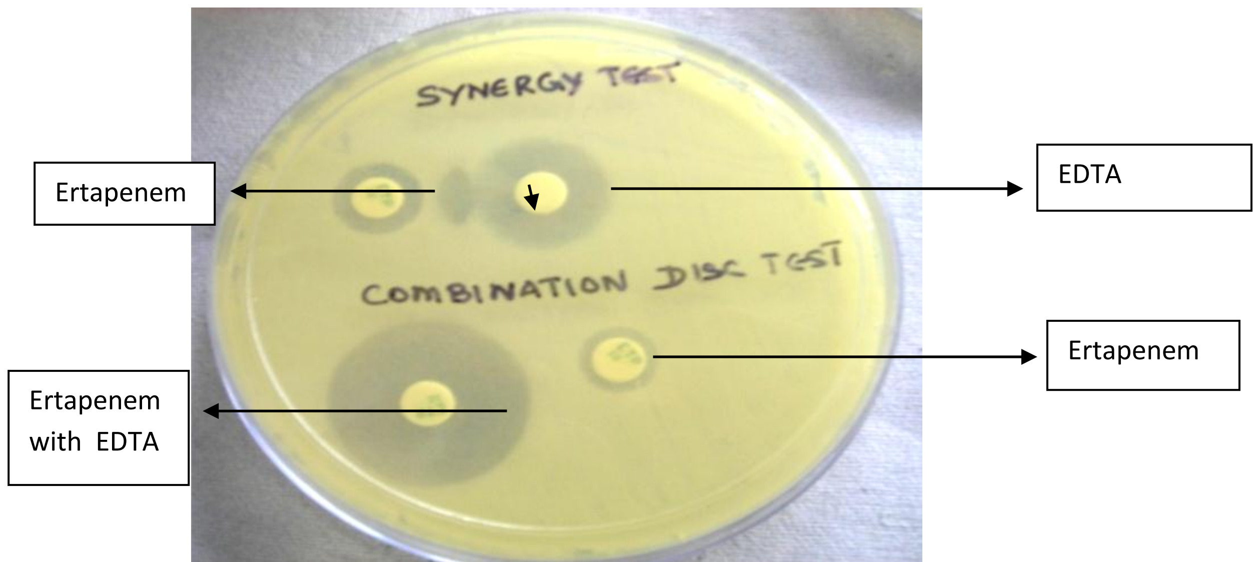


FIGURE-10 Detection of carbapenamase by Modified hodge test

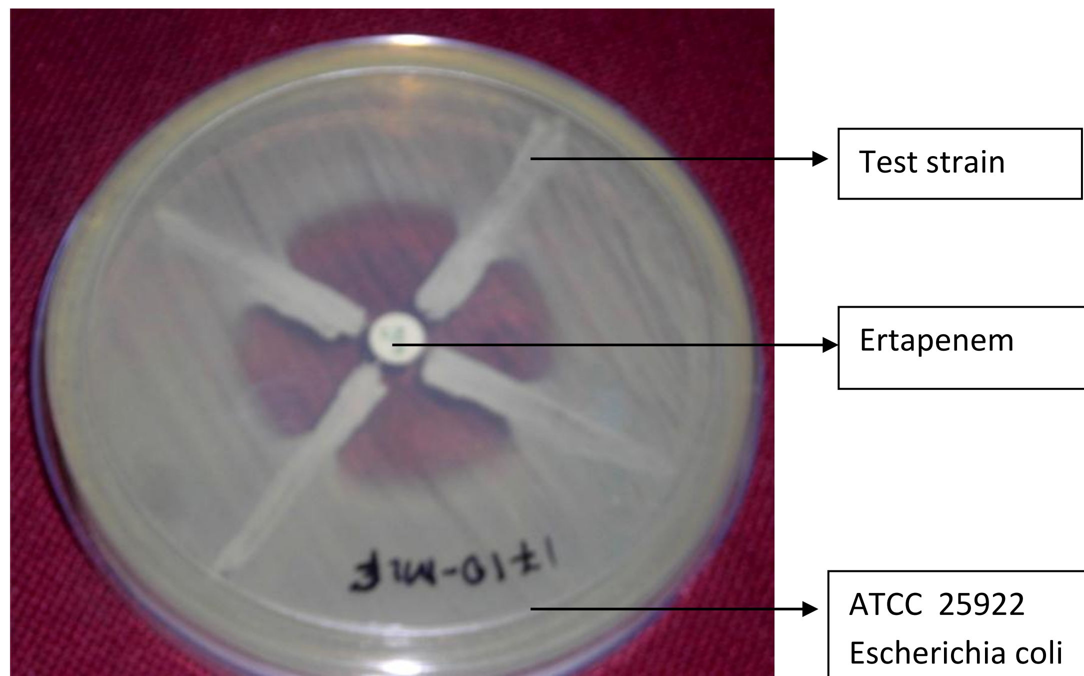
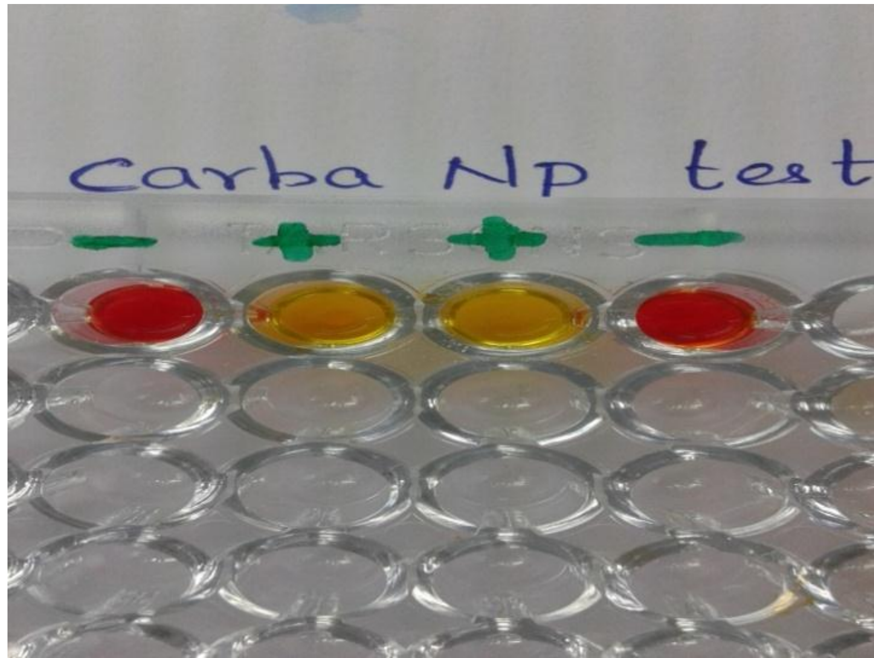


FIGURE-11 Detection of carbapenamase by Carba Np test



Positive- Yellow colour; Negative- Red colour

FIGURE-12

WATER BATH

FIGURE-13 Real time pcr machine (ABI-step one)

FIGURE-14 Gel electrophoresis of amplified DNA of NDM gene

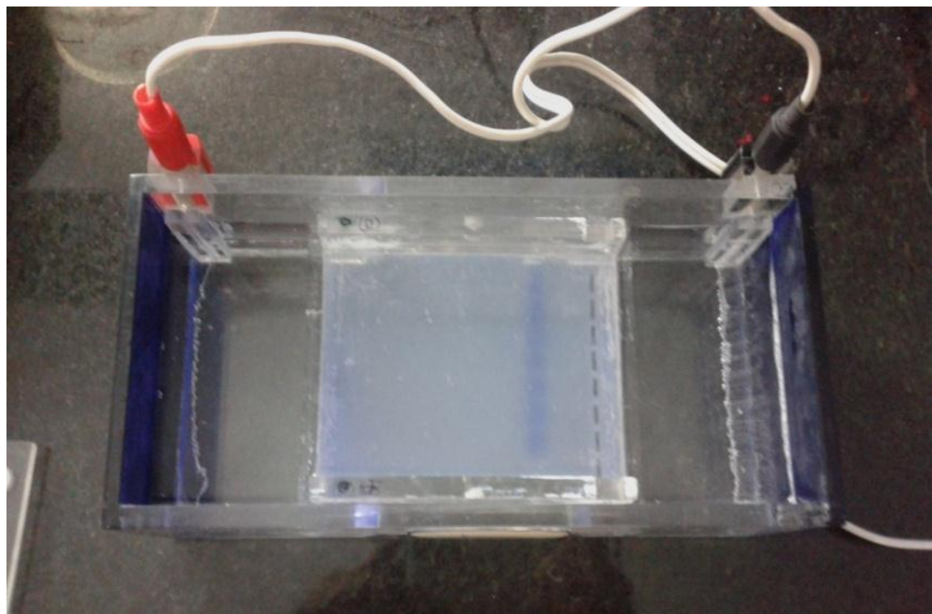
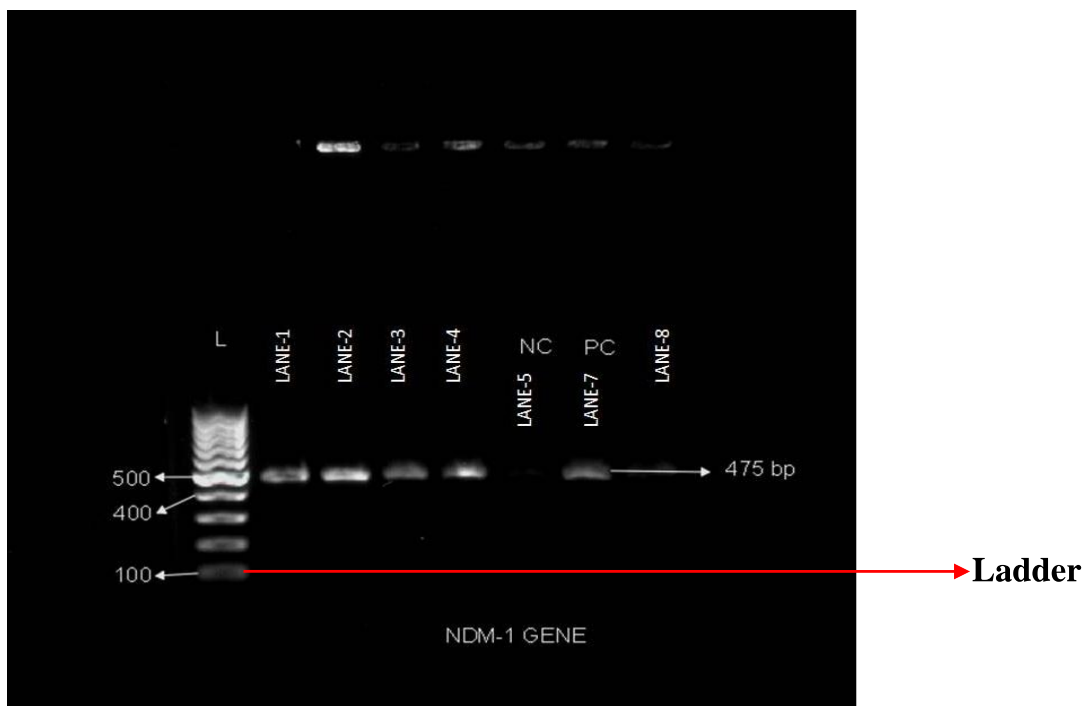


FIGURE-15 Gel doc EZ imager (Bio Rad , USA)

FIGURE-16 Gel electrophoretic analysis of amplified NDM-1 gene products



LANE-1&2- Escherichia coli (+ve for NDM-1);

LANE 3&4 –Klebsiella pneumoniae(+ve for NDM-1)

FIGURE-17 prevalence of NDM-1 carbapenamase in our study (82%)

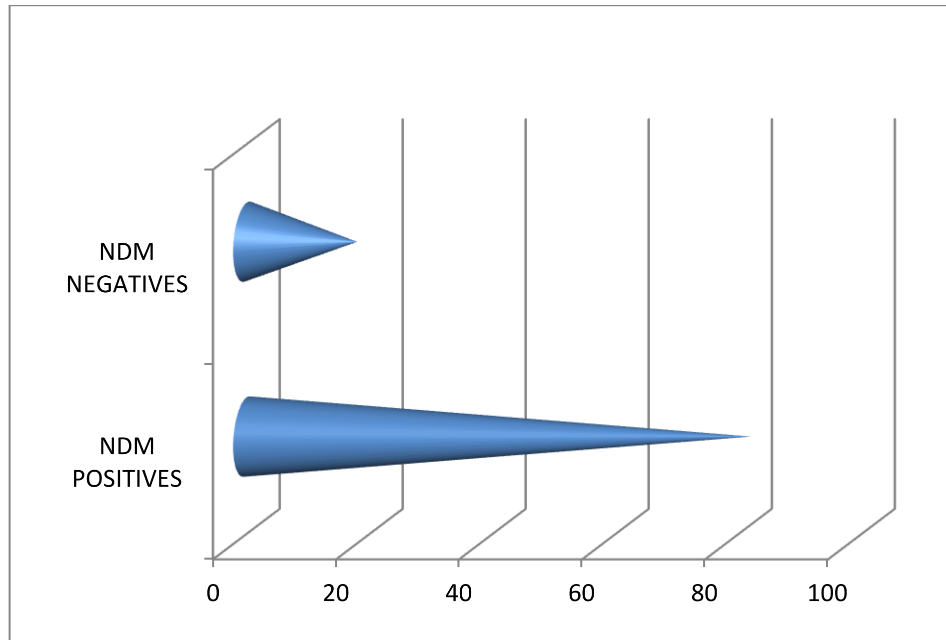
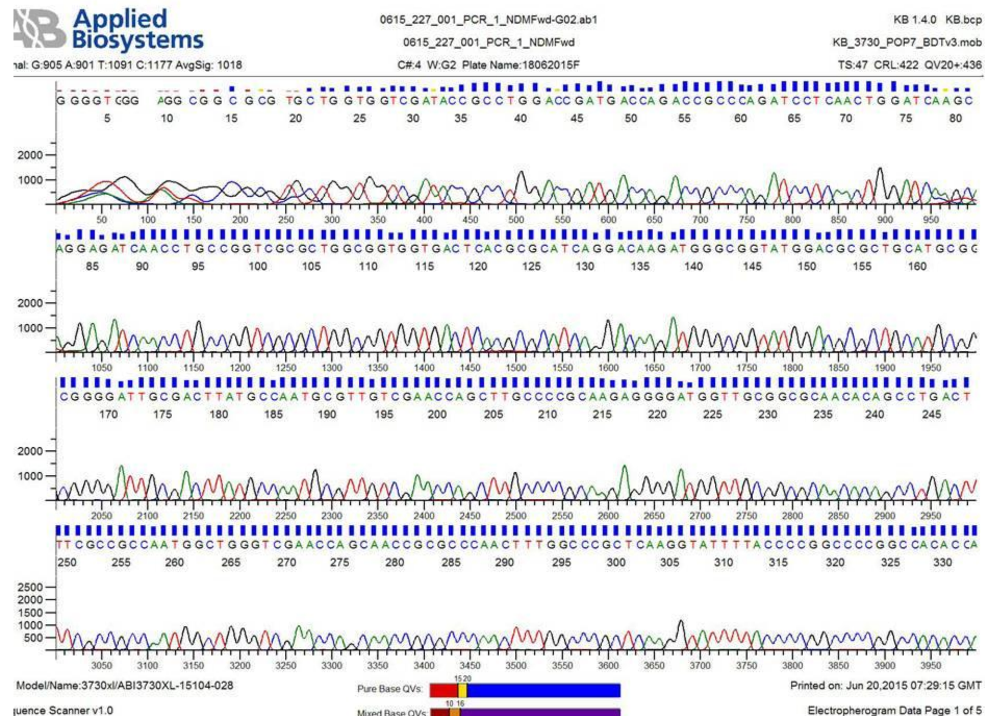


FIGURE -18 DNA sequencing results of amplified NDM-1 carbapenamase gene from Klebsiella pneumoniae

Isolate-1



>0615_227_14_PCR_1

AACGATTGGCCTTGCTGTCCTTGATCAGGCAGCCACCAAAAGCGATGTCG
GTGCCGTCGATCCCAACGGTGATATTGTCACTGGTGTGGCCGGGGCCGGG
GTAAAATACCTTGAGCGGGCCAAAGTTGGGCGCGGTTGCTGGTTCGACCC
AGCCATTGGCGGCGAAAGTCAGGCTGTGTTGCGCCGCAACCATCCCCTCT
TGCGGGGCAAGCTGGTTCGACAACGCATTGGCATAAGTCGCAATCCCCGC
CGCATGCAGCGCGTCCATACCGCCCATCTTGTCCTGATGCGCGTGAGTCAC
CACCGCCAGCGCGACCGGCAGGTTGATCTCCTGCTTGATCCAGTTGAGGA
TCTGGGCGGTCTGGTCATCGGTCCAGGCGGTATCGACCACCAGCACGCGG
CCGCCATCCCTGACGATC

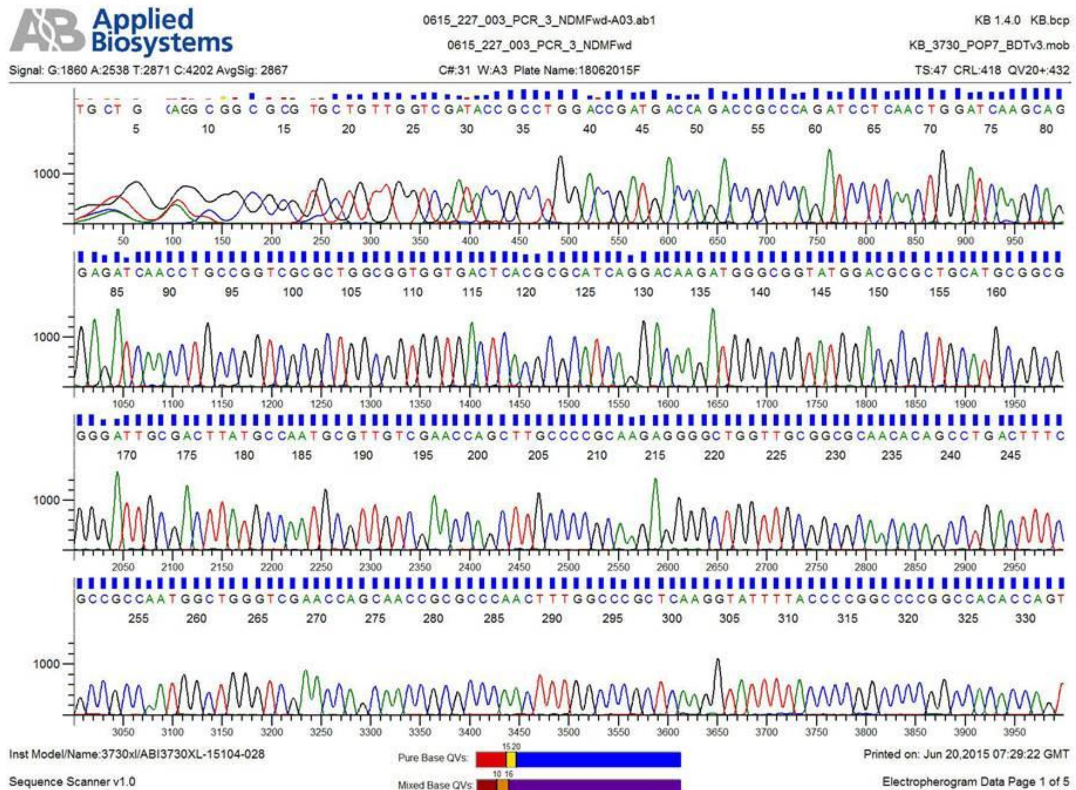
AMINOACID BLAST(aminoacid sequence of above product)

>0615_227_14_PCR_1

IVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTHAHQDKMGG
MDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFG
PLKVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDS

FIGURE-19 DNA sequencing results of amplified NDM-1 carbapenamase gene from Klebsiella pneumoniae

Isolate-2



>0615_227_14_PCR-2

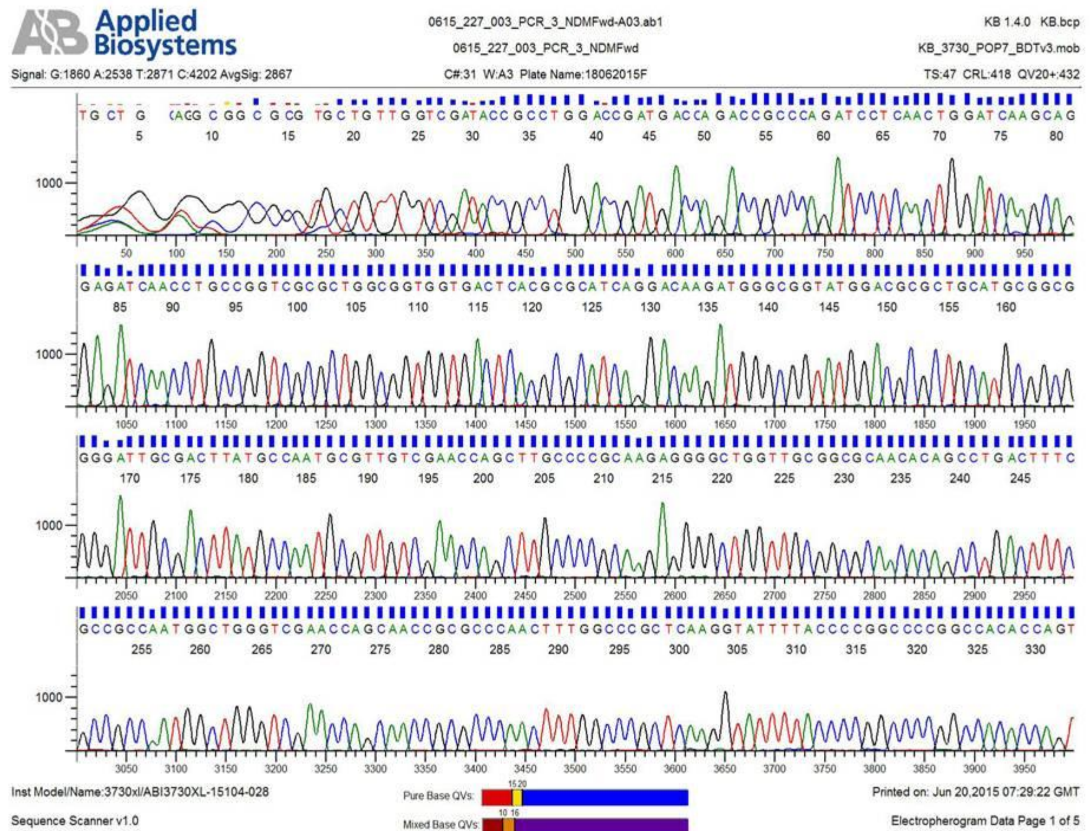
GGCTTGCTGTCCTTGATCAGGCAGCCACCAAAAGCGATGTCGGTGCCGTC
GATCCCAACGGTGATATTGTCACTGGTGTGGCCGGGGCCGGGGTAAAATA
CCTTGAGCGGGCCAAAGTTGGGCGCGGTTGCTGGTTCGACCCAGCCATTG
GCGGCGAAAGTCAGGCTGTGTTGCGCCGCAACCATCCCCTCTTGCGGGGC
AAGCTGGTTCGACAACGCATTGGCATAAGTCGCAATCCCCGCCGCATGCA
GCGCGTCCATACCGCCCATCTTGTCCTGATGCGCGTGAGTCACCACCGCCA
GCGCGACCGGCAGGTTGATCTCCTGCTTGATCCAGTTGAGGATCTGGGCG
GTCTGGTCATCGGTCCAGGCGGTATCGACCACCAGCACGCGGCCG

AMINOACID BLAST

>0615_227_14_PCR_2
GRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTHAHQDKMGGMDALH
AAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPLKVFY
PGPGHTSDNITVGIDGTDIAFGGCLIKDSK

FIGURE -20 DNA sequencing results of amplified NDM-1 carbapenamase gene from Klebsiella pneumoniae

Isolate-3



>0615_227_14_PCR-3

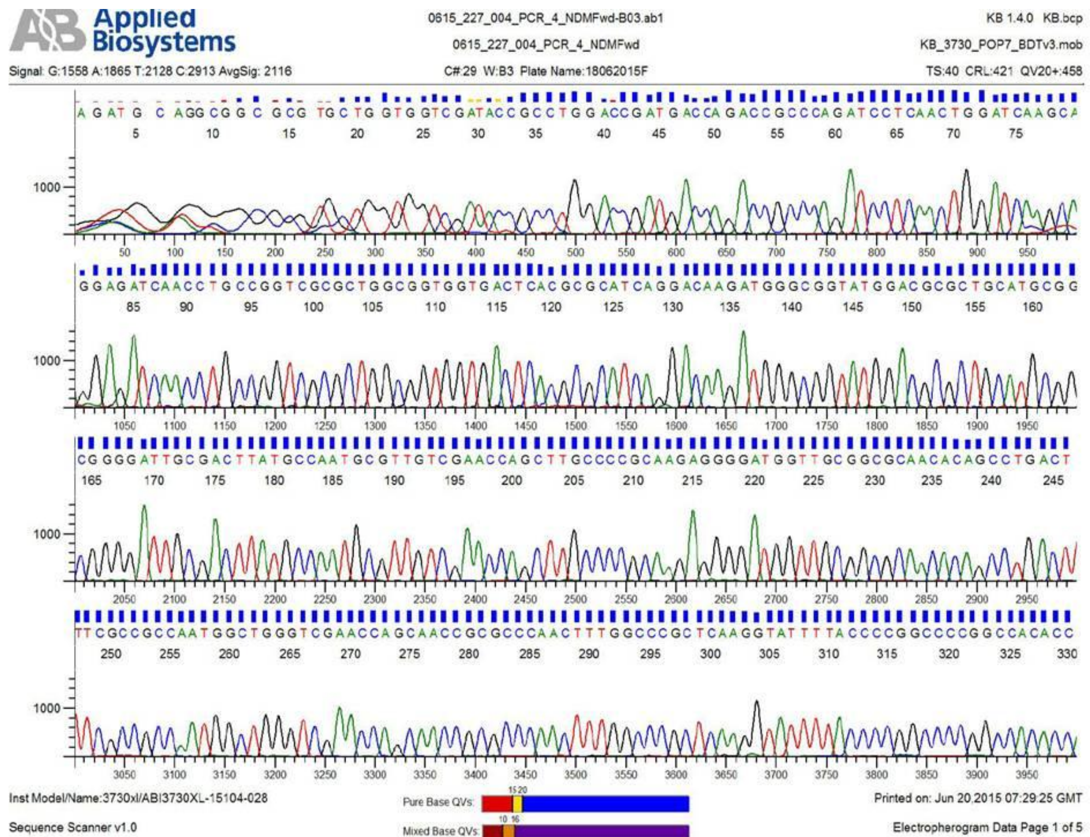
TTTTGCTGTTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAGATCC
TCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTG
ACTCACGCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGC
GGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAG
AGGGGCTGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCTGG
GTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTACCC
CGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGCACCG
ACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCC

AMINOACID BLAST

>0615_227_14_PCR_3L**L**VDTAWTDDQTAQILNWIKQEINLPVALAVVTHAHQ
DKMGGMDALHAAGIATYANALSNQLAPQEG**L**VAAQHSLTFAANGWVEPAT
APNFGPLKVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKA

FIGURE-21 DNA sequencing results of amplified NDM-1 carbapenamase gene from Klebsiella pneumoniae

Isolate-4



>0615_227_14_PCR_4

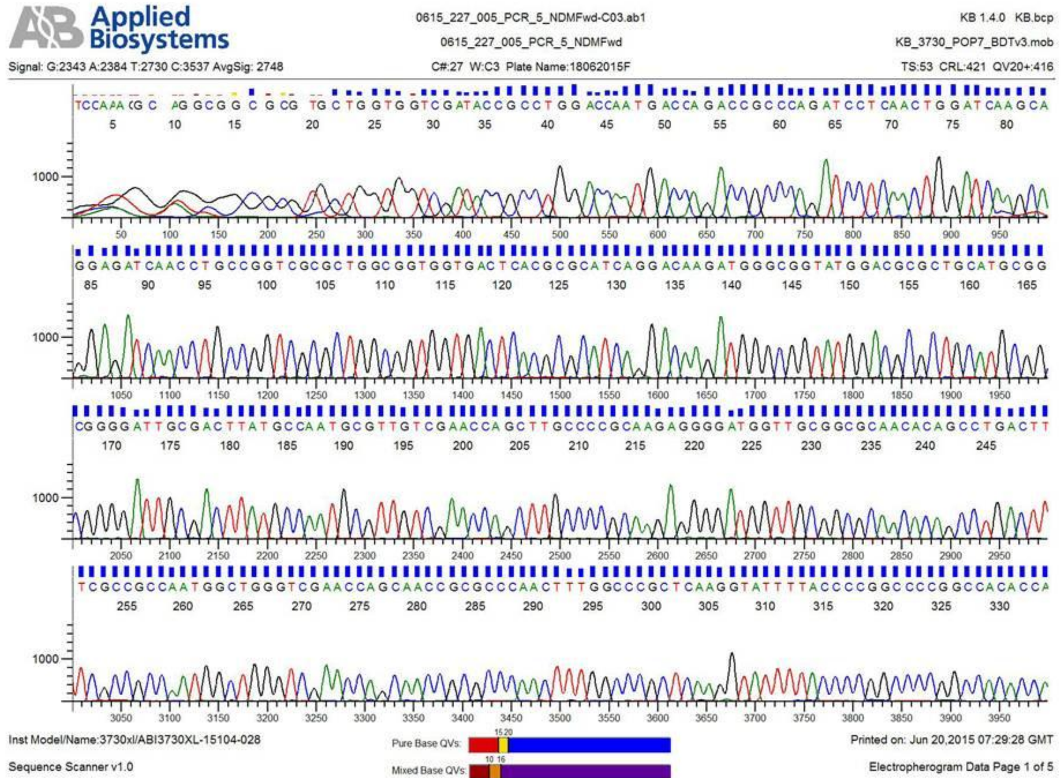
GGGGCAGTTTGGCTCCAACGGTTTGATCGTCAGGGATGGCGGCCGCGTGC
TGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAGATCCTCAAC
TGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCA
CGCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGG
ATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGG
GATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCG
AACCAGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTACCCCGGC
CCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGCACCGACAT
CGCTTTTGGTGGCTGCCTGATCAAGGACA

AMINOACID BLAST

>0615_227_14_PCR_4GQFGSNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQ
EINLPVALAVVTHAHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAH
SLTFAANGWVEPATAPNFGPLKVFYPGPGHTSDNITVGIDGTDIAFGGCLIKD

FIGURE-22 DNA sequencing results of amplified NDM-1 carbapenamase gene from Escherichia coli

Isolate-5



>0615_227_14_PCR-5

CTGGTGGTCGATACCGCCTGGACCAATGACCAGACCGCCCAGATCCTCAA
CTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTC
ACGCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGG
GATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGG
GGATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTC
GAACCAGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTACCCCGG
CCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGCACCGACA
TCGCTTTTGGTGGCTGCCTGATCAAGGACAGC

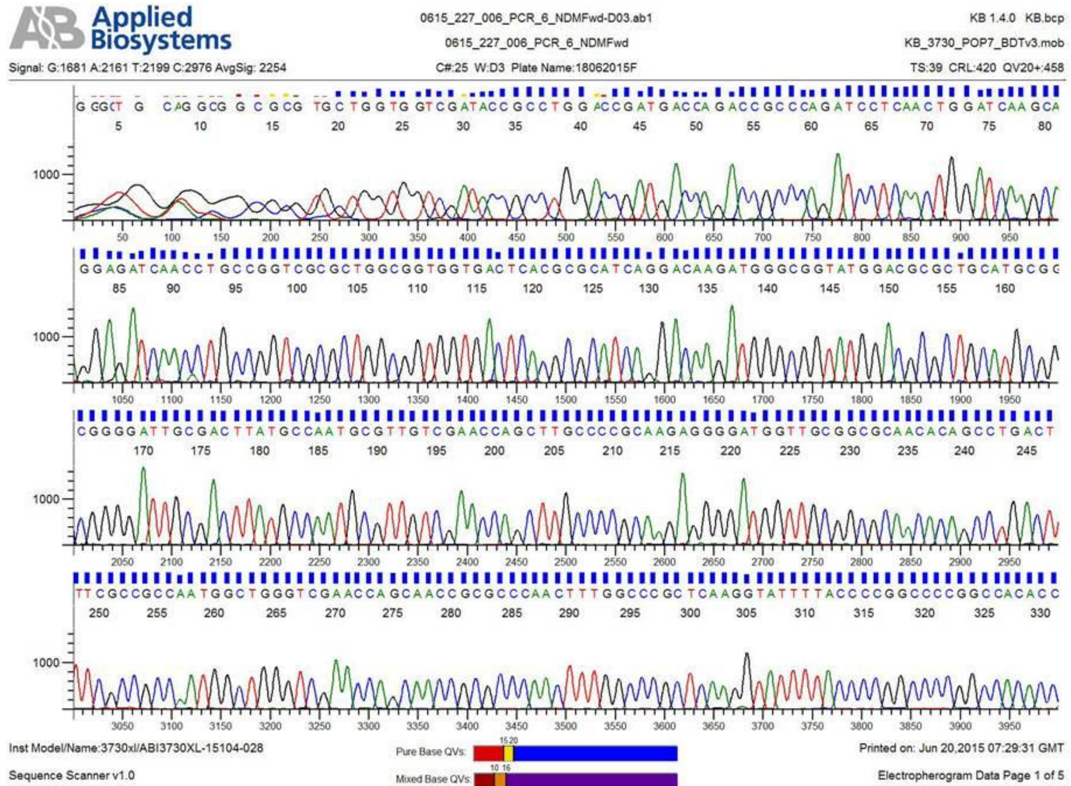
AMINOACID BLAST

>0615_227_14_PCR_5

LVVD**TAWTN**DQTAQILNWIKQEINLPVALAVVTHAHQDKMGGMDALHAAG
IATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPLKVFYPGP
GHTSDNITVGIDGTDIAFGGCLIKDS

FIGURE-23 DNA sequencing results of amplified NDM-1 carbapenamase gene from Klebsiella pneumoniae

Isolate-6



>0615_227_14_SEQUENCE_6

TTGCTGTCCTTGATCAGGCAGCCACCAAAAGCGATGTCGGTGCCGTCGAT
CCCAACGGTGATATTGTCACTGGTGTGGCCGGGGCCGGGGTAAAATACCT
TGAGCGGGCCAAAGTTGGGCGCGGTTGCTGGTTCGACCCAGCCATTGGCG
GCGAAAGTCAGGCTGTGTTGCGCCGCAACCATCCCCTCTTGCGGGGCAAG
CTGGTTCGACAACGCATTGGCATAAGTCGCAATCCCCGCCGCATGCAGCG
CGTCCATACCGCCCATCTTGTCCTGATGCGCGTGAGTCACCACCGCCAGCG
CGACCGGCAGGTTGATCTCCTGCTTGATCCAGTTGAGGATCTGGGCGGTCT
GGTCATCGGTCCAGGCG

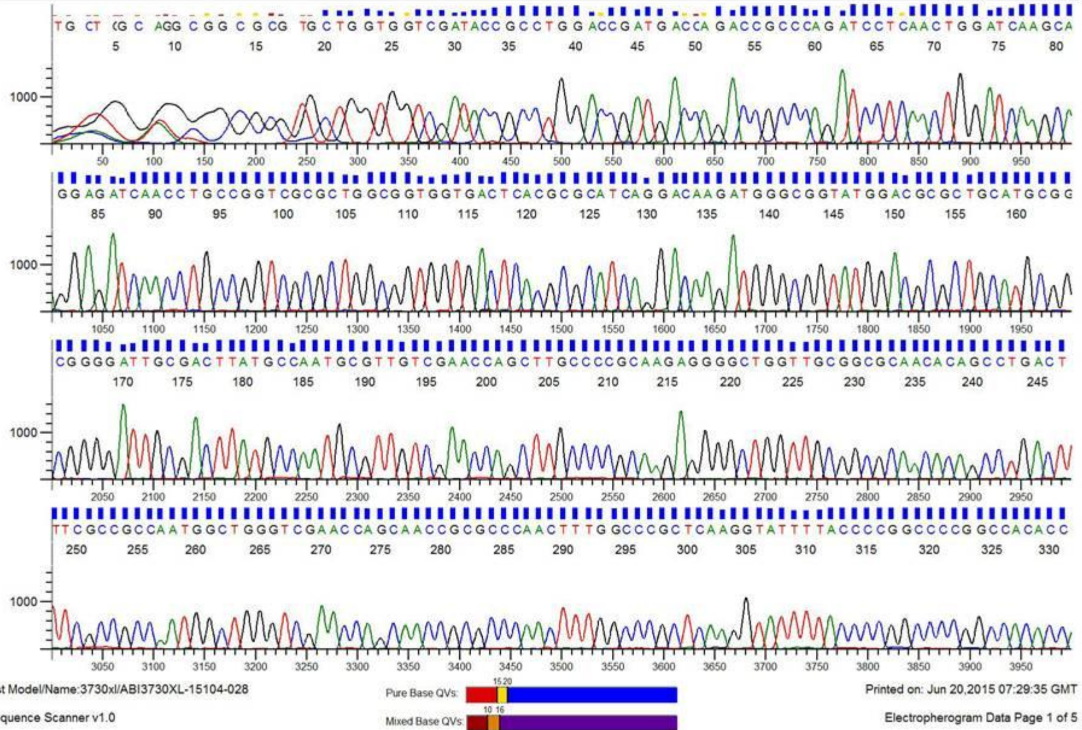
AMINOACID BLAST

>0615_227_14_PCR_6

AWTDDQTAQILNWIKQEINLPVALAVVTHAHQDKMGGMDALHAAGIATYA
NALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPLKVFYPGPGHTSD
NITVGIDGTDIAFGGCLIKDSK

FIGURE-24 DNA sequencing results of amplified NDM-1 carbapenamase gene from Escherichia coli

Isolate-7



>0615_227_14_PCR-7

GCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAGAT
CCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGG
TGACTCACGCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCG
GCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCGCA
AGAGGGGCTGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCT
GGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTAC
CCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGCAC
CGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCA

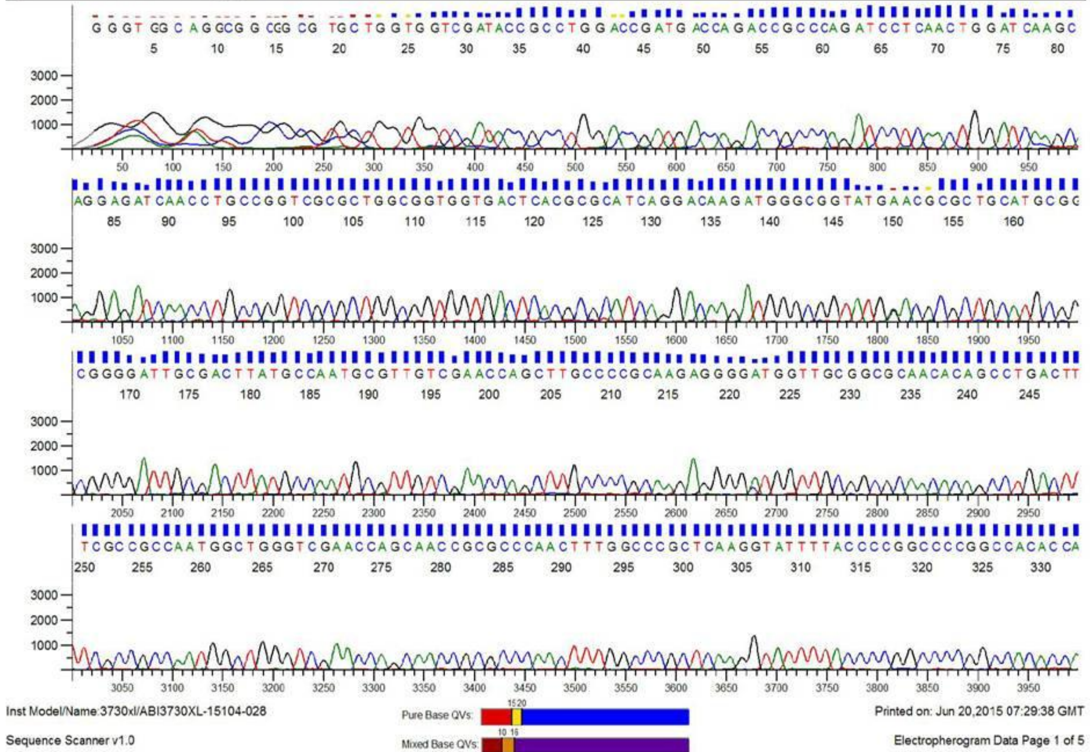
AMINOACID BLAST

>0615_227_14_PCR_6

VLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTHAHQDKMGGMDALHAA
GIATYANALSNQLAP**QEGL**VAAQHSLTFAANGWVEPATAPNFGPLKVFYPG
PGHTSDNITVGIDGTDIAFGGCLIKDSKA

FIGURE-25 DNA sequencing results of amplified NDM-1 carbapenamase gene from Escherichia coli

Isolate-8



>0615_227_008_PCR_8_NDMFwd

GCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAGAT
CCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGG
TGACTCACGCGCATCAGGACAAGATGGGCGGTATGAACGCGCTGCATGCG
GCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCGCA
AGAGGGGATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCT
GGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTAC
CCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGCAC
CGACATCGC**TTTTGGTGGCTGCCTGATCAA**

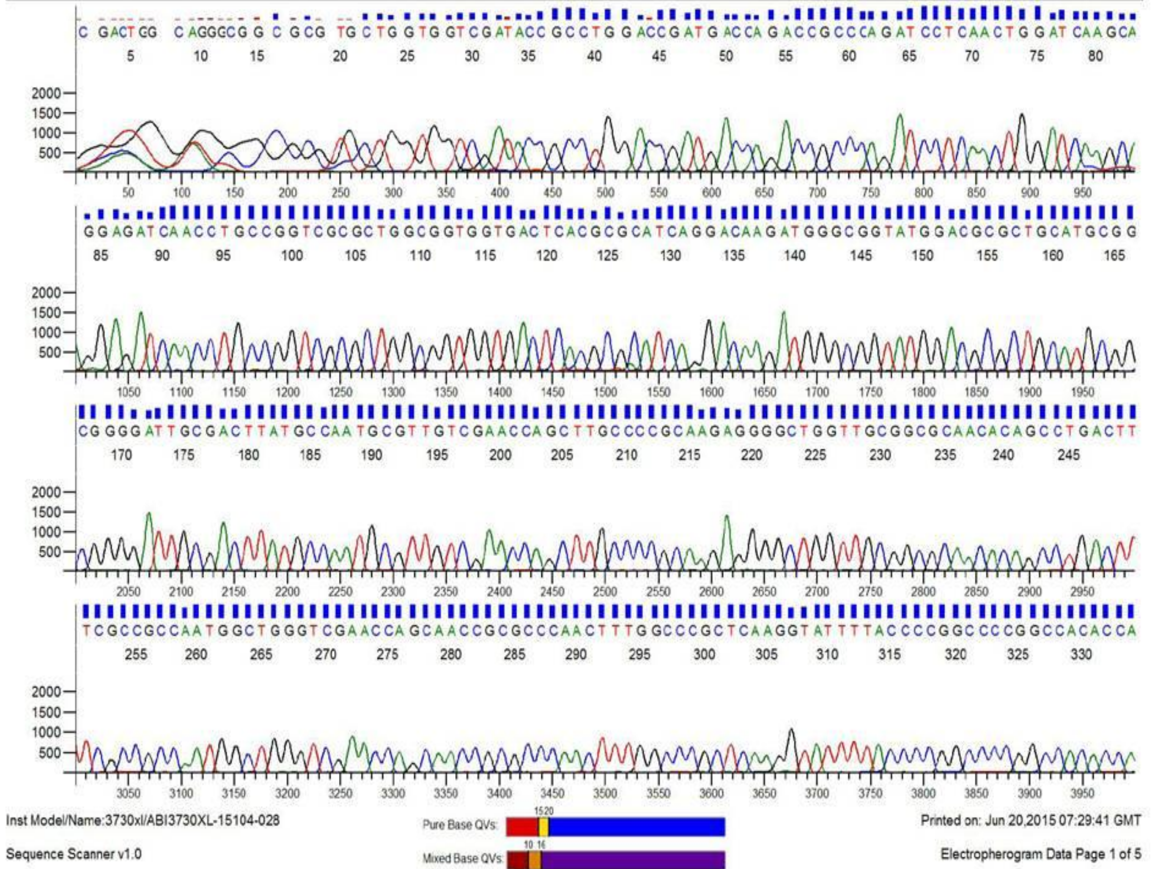
AMINOACID BLAST

>0615_227_008_PCR_8_NDMFwd

VLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTHAHQDKMGGMNALHAA
GIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPLKVFYPG
PGHTSDNITVGIDGTDIAFGGCLI

FIGURE-26 DNA sequencing results of amplified NDM-1 carbapenamase gene from Escherichia coli

Isolate-9



>0615_227_009_PCR_9_NDMFwd

GCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAGATC
CTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGT
GACTCACGCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCG
GCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCGCA
AGAGGGGCTGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCT
GGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTAC
CCCGGCCCC**GGCCA**

AMINOACID BLAST

>0615_227_009_PCR_9_NDMFwd

VLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTHAHQDKMGGMDALHAA
GIATYANALSNQLAPQEGLVAAQHSLTFAANGWVEPATAPNFGPLKVFYPGP
G

FIGURE-27 Results of carrier rate of carbapenamase positive isolates from I.C.U patients

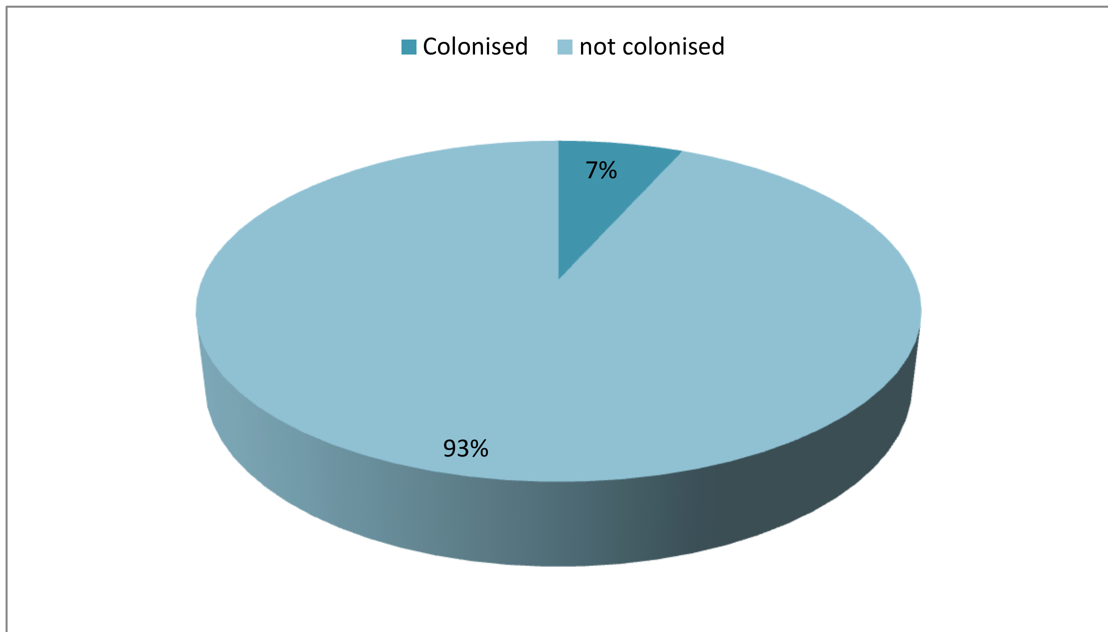


FIGURE-28 carba chrome agar screening of faecal carriers of carbapenam resistant bacteria

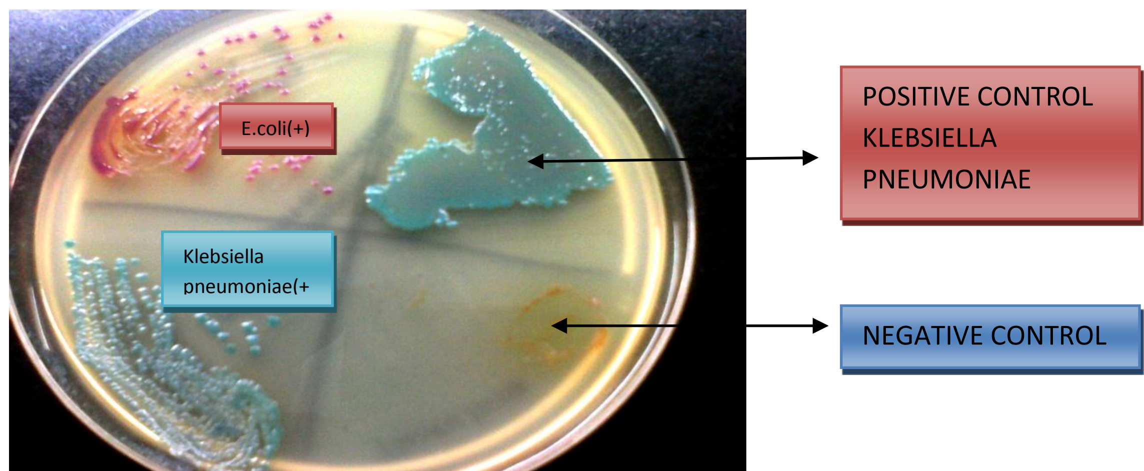


TABLE- 2 Comparison of phenotypic methods

S. NO	NAME OF THE TEST	POSITIVES (%)	NEGATIVES (%)	SENSITIITY (%)	SPECIFICITY (%)
1	Double disc synergy test	42	58	42	50
2	Combined disc test	75	25	92	50
3	Modified hodge test	90	10	91	100
4	Carba NP test	98	2	100	100

TABLE-3 Antibiotic susceptability pattern of carbapenam resistant bacteria isolates(no:100)

S.NO	DRUG	SENSITIVE (%)	RESISTANCE (%)
1	Ceftazidime	Nil	100%
2	Cefoperazone sulbactum	Nil	100%
3	Cefepime	Nil	100%
4	Aztreonam	Nil	100%
5	Imipenem	Nil	100%
6	Meropenem	Nil	100%
7	Amikacin	32%	68%
8	Gentamicin	Nil	100%
9	Ciprofloxacin	Nil	100%
10	Tigecycline	100%	Nil
11	Colistin	100%	Nil
12	Cotrimoxazole	5%	95%

TABLE-4 –DNA sequencing results of NDM-1 carbapenamase genes studied

ISOLATE	AMINOACID SUBSTITUTION	INTERPRETATION
SEQ-I	Aminoacid sequences of NDM-1	NDM-1
SEQ-2	Aminoacid sequences of NDM-1	NDM-1
SEQ-3	Met to Leu (M-L) substitution at 154 and Val to Leu (V-L)substitution at 88.	NDM-5
SEQ-4	Aminoacid sequences of NDM-1	NDM-1
SEQ-5	Substitution of Asp to Asn (D-N) at 95.	NDM-3
SEQ-6	Aminoacid sequences of NDM-1	NDM-1
SEQ-7	Single amino acid substitution (M-L) at 154 (Met to Leu).	NDM-4
SEQ-8	Aminoacid sequences of NDM-1	NDM-1
SEQ-9	Single amino acid substitution (M-L) at 54 (Met to Leu).	NDM-4

Isolate-1,2,3,4,6- Klebsiella pneumoniae

Isolate-5,7,8,9-Escherichia coli

Discussion

Gram negative bacteria belonging to the family of Enterobacteriaceae are the most frequently isolated organisms from clinical specimens. They are incriminated in virtually any type of infectious diseases and recovered from any specimen received in the laboratory.

Escherichia coli and Klebsiella pneumoniae cause sepsis, endotoxic shock, urinary tract infections, meningitis in neonates and pneumonia in immune compromised .They are normally treated based on the antibiotic sensitivity, site of infection and co morbidity condition .Infections of *Escherichia coli* are treated with Ampicillin, TMP/SMZ, doxycycline, cephalosporins and fluroquinolones. Uncomplicated cystitis requires TMP/SMZ and nitrofurantoin where as complicated cystitis responds to Ceftriaxones and quinolones.

Klebsiella infections are intrinsically resistant to Ampicillin and Ticarcillin. Nitrofurantoin is poorly active against them. In Severely ill patients with Klebsiella infections, the treatment options include third generation cephalosporins, quinolones and amino glycosides.[2]

Escherichia coli and Klebsiella infections initially responded to betalactam antibiotics, but there was a dramatic increase in resistance to beta lactam antibiotics due to betalactamases produced by them. This resistance was superseded by use of second and third generation cephalosporins and combination of betalactam antibiotics with betalactamases inhibitors.

Further with the emergence of ESBLs and Amp C resistance, carbapenems became the drug of choice.[3] There has been a rapid increase in carbapenems resistance in Enterobacteriaceae in the recent years. The carbapenems resistance may be due to carbapenamases production, Efflux pump and Amp C enzyme production with membrane impermeability. However, carbapenamase (betalactamases) were found to be the predominant mechanism of this resistance.

Ambler classified betalactamases as class A, class B, class D based on amino acid sequences. Carbapenamase resistance is associated with class A,B and class D. Class A and D contain betalactamases with serine in their active site; Class A hydrolyses all betalactams and is inhibited by clavulanic acid and Tazobactum. Class B also called as metallobetalactamases hydrolyses all beta lactam except Aztreonam but are inhibited by EDTA. Class D enzymes hydrolyse carbapenems, weakly hydrolyse broad spectrum cephalosporins and are poorly inhibited by clavulanic acid and EDTA[57.]

These carbapenamases are classified under 2f and 3 groups in Bush jacoby and Medeiros[71] classification. The class B enzymes are plasmid mediated and integron located. It includes IMP-(active on Imepenam), VIM-(Verona integron encoded metallobetalactamases), GIM-(German imipenemase), SPM-1 (Sao Paul imipenemase), SIM-1 (Seoul imipenamase), NDM- (New Delhi metallobetalactamase). But for NDM-1, others are rarely reported in Enterobacteriaceae.

Among the newly emerged carbapenamases, New Delhi Metallo betalactamases (NDM-1) represents a war between the bugs and drugs.NDM-1 producing organisms are called super bugs[4]. NDM-1 was first reported in 2009 in Klebsiella pneumoniae and Escherichia coli. Both of them were recovered from a Swedish patient of Indian origin who was previously admitted in a hospital in New Delhi, India [4]. Thereafter, NDM-1 has increasingly been reported from India and from several other parts of the world.

Besides this, most of these NDM-1 positive bacteria are also resistant to a wide variety of Antimicrobials like aminoglycosides,flouroquinolones, macrolides and sulphonamides and carry several additional resistance mechanisms, leaving only few or no therapeutic options[58].

In our study period carbapenem resistance was observed in 47% of Klebsiella pneumoniae and 7% of Escherichia coli in our hospital.

As per CLSI 2014 Ertapenem is a good marker for detection of Carbapenems resistance as others do not predict this resistance well. We randomly selected 42 Escherichia coli followed by 58 klebsiella pneumoniae which were carbapenems resistant for molecular analysis. All these strains were resistant to Ceftazidime, Cefperazonesulbactum, Cefepime, Aztreonam, Imepenem, Meropenam,Gentamicin & ciprofloxacin but 32% were sensitive to Amikacin and 5% were sensitive to cotrimoxazole . All of the above strains were susceptible to colistin and Tigecycline.

Most of Carbapenem resistant strains were from urine samples (47%). A similar study showed 57.89% carbapenamases producing Klebsiella pneumoniae in urine samples[4]. Khanduri et al detected in his study that among 50 isolates of carbapenem resistant Enterobacteriaceae 44% were Klebsiella pneumoniae and 20% were Escherichia coli[74]

The various methods of carbapenem resistance detection are double-disc synergy , Combined disc ,Modified Hodge and Carba Np test.

Double disc synergy test was done with Ertapenem, ceftazidime and disc with EDTA in between .Any distortion of zone towards EDTA disc was considered positive. Combination disc test was done with Ertapenem and Ertapenem with EDTA. An increase in zone diameter with combination disc of more than or equal to 5mm was considered as positive. Clover leaf pattern on the Muller hinton plate with in a lawn ATCC Escherichia coli and test strain streaked perpendicular to it indicates positivity. Carba Np test was interpreted as positive if the test strain turns orange to yellow colour on incubation with carbapenems and phenol red as indicator.

Carba Np test is found to be the most specific and sensitive method to detect carbapenamases production. In our study also we found that Carba Np test is most specific (100%) and sensitive (100%) in detecting carbapenamases and it was better than Modified Hodge test (sensitivity 91% specificity 100%),Combination disc test (sensitivity 92% and specificity 50%) and Double disc synergy test (sensitivity 42% and specificity 50%).

In another study on comparison of various phenotypic tests (DDST, CDT, MHT). Modified Hodge test was comparatively more specific and sensitive than Double disc synergy test and combination disc test[59]. Amjad et al observed carbapenamases in 69% of isolates (Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumanii, Citrobacter diversus, and Enterobacter agglomerans) by Modified Hodge test[60].

In a study on comparison of carbapenemase detection using a novel chromogenic rapid test—the Carba NP test and the modified Hodge test (MHT).Sensitivities were found to be comparable (CNP, 100%, versus MHT, 98%; P 0.08), but Carba Np was more specific (100% versus 80%; P < 0.0001) and faster[61].

In a similar study Carba NP test detected all carbapenemase producers regardless of the type of the carbapenemase and of their mucoid phenotype with 100% sensitivity and 100% specificity[62].

However molecular tests are the most accurate and usually taken as Gold standard. In our study we used the following primers to detect NDM-1 by conventional PCR

NDM Forward: 5'-GGGCAGTCGCTTCCAACGGT and

NDM Reverse: 5'- GTAGTGCTCAGTGTCGGCAT

Amplification, sequencing and subsequent blasting were performed to identify the subtypes. Eighty-two (82%) strains were found to be NDM-1

positive (475bp) of which Thirty seven (45%) were Escherichia coli and Forty five were Klebsiella pneumoniae (54%). The presence of NDM-1 has also been reported in Bangladesh, Australia, Netherlands, USA, Canada, Japan, and China indicating that "blaNDM-1is widely spread all over the world[49.]

In a similar study from south India, NDM-1 was detected in 75% of K. pneumoniae and 66% of E. coli by PCR and the remaining isolates were assumed to posses other mechanisms of resistance like efflux pumps, other carbapenamases, Amp C production and membrane impermeability[53.]

Fomda et al in his study conducted in Jammu and Kashmir observed that out of 15 class B metallobetalactamase producers, nine carried bla NDM-1 gene of which 2 were Escherichia coli and 2 were Klebsiell[63].

DNA and protein sequencing were started in the 1970s, when the virus Lambda (50,000 nucleotides) was sequenced by Sanger *et al*.DNA sequencing was initially carried out for small genomes such as viruses and organelles, and complete sequencing of a bacterial genome were started later on with the improvements in sequencing technologies. Besides epidemiological surveillance sequencing has helped to identify new drug targets, which can be used in the design of novel antibiotics[74]. DNA mutations has resulted in changes in the amino acid sequences and produced an ever increasing range of subtypes or variants in each of carbapenamase.

Fifteen variants of NDM-1 have been identified. These variants were identified after sequencing and blasting. They differ by various aminoacid

substitutions. NDM-4 was identified in Escherichia coli from a patient hospitalized in India, possessed a higher carbapenemase activity compared with NDM-1[43]. Ndm-4 producers were found in Africa specifically, in the highly populated city of Douala, which provided an environment that may promote the dissemination of this strains[64] .In another study six NDM-4-producing E.coli isolates were obtained from two patients admitted to an Italian hospital[65].

NDM-5 was recovered from a Patient in the United Kingdom in *Escherichia coli* ST64 who had a previous history of hospitalization in India[39].

NDM-7 was isolated from wound, throat and rectal swabs in a patient in Germany.[67]New Delhi metallo-β-lactamase-3 (NDM-3) was identified in a multidrug-resistant *Escherichia coli* isolate, obtained from the feces of a patient in Japan[68]. In another study NDM-5, NDM-6,NDM-7 were isolated in Enterobacteriaceae in India.The prevalence of NDM-5 to NDM-7 varients was significantly higher in Escherichia coli and particularly higher in urine samples.[69]

We found NDM-1 in 5 strains (56%),NDM-4 in 2 strains (22%) NDM-3and NDM-5 in one strain each (11%).Colonization with potential pathogens is almost always an important cause for the development of nosocomial infections. But only a minority of colonized patients eventually develops clinical infection. It is influenced by various factors like pathogen virulence and host defence mechanisms, medical procedures, and exposure to antibiotics.

Identifying the risk factors for subsequent infections among colonized patients may promote control of modifiable risk factors and direct empirical antimicrobial therapy when necessary. A SUPERCARBA medium with zinc ,cloxacillin and a carbapenems molecule which detects carbapenemase producer (including OXA-48 producers) and other chrome medium (CHROMagar KPC; CHROMagar, Paris) are used for screening carbapenemases[72].

However, PCR for bla KPC represents the best screening test for KPCs in rectal swabs. It has significantly higher sensitivity and less hands-on time[73].

We found that carbapenems resistant carrier state was 8% in our study .Among them three were Klebsiella pneumoniae and one was Escherichia coli. A carrier state of 10-30% was reported from various studies in various parts of India and other countries[70]. The less incidence in this study is probably due to the infection control measures like effective decontamination of equipments, periodic assessment, routine hand hygiene and training given to the medical and nursing staff.

The spread of Enterobacteriaceae harbouring carbapenemases is a clinical and public health problem. Strict contact isolation and physical separation of carriers from non-carriers are key components in eliminating CRE in acute care hospitals. Sites of CRE carriage include the lower gastrointestinal tract, the oropharynx, skin and urine.

The primary surveillance screening site, which has been advocated by the US Center for Disease Control and Prevention CDC and the European Society of Clinical Microbiology and Infectious Diseases, is the stool or rectal swab[70].

Further characterization of the 18 strains (5 were Escherichia coli and 13 Klebsiella pneumoniae which were negative for NDM-1 gene) for the mechanism of resistance was not done in our study. These isolates probably have other carbapenemases like VIM or it may have efflux changes or Amp C beta lactamase production with outer membrane impermeability.

Presently NDM-1 producing bacterial infections can be managed with Colistin and Tigecycline combination. Colistin should not be given alone as it provokes resistance in vivo due to hetero resistant strains. Fosfomycin may also serve as an option for combination therapy against Multidrug resistant Gram negatives. Falagas et al observed by Meta analysis of 17 studies that 90% of NDM-1 positive isolates were susceptible to Fosfomycin. Detection of NDM-1 like carbapenemases in members of Enterobacteriaceae will help in the management and prevention of spread of such bacteria in hospital and community which in turn will save the patients and valuable drugs.

Summary

- There has been a rapid increase in carbapenem resistance of particularly NDM-1 carbapenamase in Escherichia coli and klebsiella pneumoniae ,the most common cause of bacterial infections .Present work was under taken to study the NDM-1 carbapenamase resistance in bacteria
- A total of Two thousand and forty two samples of Escherichia coli from same number of patients and thousand hundred and ninety two samples of Klebsiella pneumoniae from same number of patients were isolated between April 2014 and August 2015.
- Carbapenems resistance was observed in five hundred and sixty samples of Klebsiella pneumoniae (47%) and hundred and forty eight isolates of Escherichia coli (7%) using Ertapenem and Meropenem susceptibility.
- Forty two isolates of Escherichia coli and fifty eight isolates of Klebsiella pneumoniae were selected randomly from above carbapenems resistant strains for further characterisation.
- Various phenotypic methods of detection of carbapenemases like Double -disc synergy, combined disc test, Modified Hodge test and Carba Np test done of which Carba Np test was 100% sensitive and 100% specific.
- Modified Hodge test was 91% sensitive and 100% specific; combined disc test was sensitive 92% and 50% specific; Double disc synergy test was 42% sensitive and 50%.
- Molecular detection of NDM-1 type carbapenemases was done after extracting the DNA by boiling method in water bath. DNA was

amplified using ABI step -1 Real time PCR. The primers targeting NDM-1 were

  - NDM Forward: 5'-GGGCAGTCGCTTCCAACGGT and
  - NDM Reverse: 5'- GTAGTGCTCAGTGTCGGCAT

- A total of eighty two (82%) strains were found to be NDM-1 positive yielding 475bp amplicon.
- Nine samples of amplified DNA (positive for NDM-1) were sent for sequencing at Euro fin genomics India pvt Ltd in Bangalore.
- The sequenced fragments were subjected to gene blasting using the BIO EDIT software and the sequence results were interpreted as per NCBI data base. Out of 9 samples, five were NDM-1, two were NDM-4, and remaining two were NDM-3, and NDM-5.
- Further characterization of the 18 strains (five were Escherichia coli and thirteen Klebsiella pneumoniae which were negative for NDM-1 gene) for the mechanism of resistance was not possible as a part of our study. These isolates probably had other carbapenemases or they may had efflux changes or Amp C beta lactamase production with outer membrane protein permeability.
- Screening of rectal swabs in 50 I.C.U patients for carbapenemases producing Escherichia coli and Klebsiella pneumoniae using Carba chrome agar plate identified 8% as carriers. Among them three were Klebsiella pneumoniae and one was Escherichia coli .

Conclusion

Indiscriminate use of antibiotics led to the development of drug resistance to almost all antibiotics discovered. The latest among them is the development of carbapenams resistance through NDM-1 especially in the members of Enterobacteriaceae, which are the commonest etiological agents in various infections. Being a plasmid coded drug resistance it is spreading with enormous speed not only within the country but also in various nations across the globe. Appropriate detection of such emerging mechanism of resistance goes in a long way in preventing unnecessary mortality and morbidity among patients.

Bibliography

1. Tille, Patricia. *Bailey & Scott's diagnostic microbiology*. Elsevier Health Sciences, 2013. 13 edition p 308-310.

2. Longo, Dan, Anthony Fauci, Dennis Kasper, and Stephen Hauser. *Harrison's Principles of Internal Medicine 18th edition*. McGraw-Hill Professional, 2011. 18 th edition p 1251-54.

3. Nosocomial burn wound infections. In: Hospital Epidemiology and Infection Control, 4th ed. Mayhall CG, ed. Lippincott Williams and Wilkins, Philadelphia, 2004.495-497.

4. BorA, Arijit, and GiASuddin AhMed. "Detection of NDM-1 in Clinical Isolates of Klebsiella Pneumoniae from Northeast India." *Journal of Clinical and Diagnostic Research* 6, no. 5 (2012): 794-800.

5. Yong D, Toleman MA, Giske CG et al. Characterization of a new metallo-b-lactamase gene, blaNDM-1, and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrob Agents Chemother 2009; 53: 5046–54.

6. Washington Winn.Jr.Stephen Allen William Janda Elmer Koneman Garj Propcop. Paul Schreckenberger Gail Woods, Konemans Color Atlas and Textbook of Diagnostic Microbiology 6th edition. pg.235-240

7. Martin J. Wood, Robert C. Moellering, Jr.Microbial Resistance: Bacteria and MoreClin Infect Dis. (2003) 36 (Supplement 1): S2-S3 doi:10.1086/344655.

8. Huo, Teh-Ia. "The first case of multidrug-resistant NDM-1-harboring Enterobacteriaceae in Taiwan: here comes the superbacteria!." *Journal of the Chinese Medical Association* 73.11 (2010): 557-558.

9. Zarfel G, Hoenigl M, Leitner E, et al. Emergence of New Delhi Metallo-β-Lactamase, Austria. *Emerging Infectious Diseases*. 2011;17(1):129-130. doi:10.3201/eid1701.101331.

10. Chen R, Tong Q, Zhang Y, et al. Loop-mediated isothermal amplification: rapid detection of *Angiostrongylus cantonensis* infection in *Pomacea canaliculata.Parasites & Vectors*. 2011;4:204. doi:10.1186/1756-3305-4-204.

11. Podschun R, Ullmann U. *Klebsiella* spp. as Nosocomial Pathogens: Epidemiology, Taxonomy, Typing Methods, and Pathogenicity Factors. *Clinical Microbiology Reviews*. 1998;11(4):589-603.

12. Payne, David J., Neil Woodford, and Sabastain GB Amyes. "Characterization of the plasmid mediated β-lactamase BIL-1." *Journal of Antimicrobial Chemotherapy* 30, no. 2 (1992): 119-127.

14. Rajput, Anil, Bhavin Prajapati, Bimal Chauhan, Atit Shah, Toral Trivedi, and Mina Kadam. "Prevalence of Metallo-betalactamases

(MBL) producing Pseudomonas aeruginosa in a Tertiary care Hospital." *Indian. J. Basic. Appl. Med. Res* 1, no. 4 (2012): 304-308.

15. Abraham, Edward P., and Ernst Chain. "An enzyme from bacteria able to destroy penicillin." *Nature* 146.3713 (1940): 837.

16. Medeiros, antone a. "β-Lactamases." *British Medical Bulletin* 40.1 (1984): 18-27.

17. Bradford, Patricia A. "Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat." *Clinical microbiology reviews* 14.4 (2001): 933-951.

18. Bonnet, R. "Growing group of extended-spectrum β-lactamases: the CTX-M enzymes." *Antimicrobial agents and chemotherapy* 48, no. 1 (2004): 1-14.

19. Danel, F., Hall, L. M., Duke, B., Gur, D., & Livermore, D. M. (1999). OXA-17, a further extended-spectrum variant of OXA-10 β-lactamase, isolated from Pseudomonas aeruginosa. *Antimicrobial agents and chemotherapy*, *43*(6), 1362-1366.

20. Weldhagen, Gerhard F., Laurent Poirel, and Patrice Nordmann. "Ambler class A extended-spectrum β-lactamases in Pseudomonas aeruginosa: novel developments and clinical impact." *Antimicrobial agents and chemotherapy* 47, no. 8 (2003): 2385-2392.

21. Yong, D., Shin, J. H., Kim, S., Lim, Y., Yum, J. H., Lee, K., ... & Bauernfeind, A. (2003). High prevalence of PER-1 extended-spectrum β-lactamase-producing Acinetobacter spp. in Korea. *Antimicrobial agents and chemotherapy*, *47*(5), 1749-1751.

22. Matsumoto, Yoshimi, and Matsuhisa Inoue, "Characterization of SFO-1, a plasmid-mediated inducible class A β-lactamase from Enterobacter cloacae."*Antimicrobial agents and chemotherapy* 43.2 (1999): 307-313.

23. Kahan, J. S., F. M. Kahan, R. Goegelman, S. A. Currie, M. Jackson, E. O. Stapley, T. W. Miller et al. "Thienamycin, a new. BETA.-lactam antibiotic. I. Discovery, taxonomy, isolation and physical properties." *The Journal of antibiotics* 32, no. 1 (1979): 1-12.

24. Materon, Isabel C., Anne Marie Queenan, Theresa M. Koehler, Karen Bush, and Timothy Palzkill. "Biochemical characterization of β-lactamases Bla1 and Bla2 from Bacillus anthracis." *Antimicrobial agents and chemotherapy* 47, no. 6 (2003): 2040-2042.

25. Queenan, Anne Marie, and Karen Bush. "Carbapenemases: the versatile β-lactamases." *Clinical microbiology reviews* 20.3 (2007): 440-458.

26. Pitout, Johann DD, and Kevin B. Laupland. "Extended-spectrum β-lactamase-producing Enterobacteriaceae: an emerging public-health concern." *The Lancet infectious diseases* 8.3 (2008): 159-166.

27. Rasmussen, Beth A., and Karen Bush. "Carbapenem-hydrolyzing beta-lactamases. " *Antimicrobial agents and chemotherapy* 41.2 (1997): 223.

28. Kuwabara, S., and E. P. Abraham. "Some properties of two extracellular beta-lactamases from Bacillus cereus 569/H." *Biochem. J* 103 (1967): 27C-30C.

29. Frère, Jean-Marie, Moreno Galleni, Karen Bush, and Otto Dideberg. "Is it necessary to change the classification of β-lactamases?." *Journal of Antimicrobial Chemotherapy* 55, no. 6 (2005): 1051-1053

30. Rolain, J. M., P. Parola, and G. Cornaglia. "New Delhi metallo-beta-lactamase (NDM-1): towards a new pandemia?." *Clinical Microbiology and Infection* 16, no. 12 (2010): 1699-1701.

31. Karthikeyan, Kumarasamy, M. A. Thirunarayan, and Padma Krishnan. "Coexistence of blaOXA-23 with blaNDM-1 and armA in clinical isolates of Acinetobacter baumannii from India." *Journal of antimicrobial chemotherapy*65.10 (2010): 2253-2254.

32. Kumarasamy, Karthikeyan K., et al. "Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study." *The Lancet infectious diseases* 10.9 (2010): 597-602.

33. Wang JF, Chou KC. Metallo-*β*-lactamases: structural features, antibiotic recognition, inhibition, and inhibitor design. *Current Topics in Medicinal Chemistry*. 2013; 13(10):1242–1253.

34. Pal A, Tripathi A. An *in silico* approach for understanding the molecular evolution of clinically important metallo-*β*-lactamases. *Infection, Genetics and Evolution*. 2013; 20:39–47.

35. Wang, Xiaojuan, Henan Li, Chunjiang Zhao, Hongbin Chen, Jingbo Liu, Zhanwei Wang, Qi Wang et al. "Novel NDM-9 metallo-β-lactamase identified from a ST107 Klebsiella pneumoniae strain isolated in China." *International journal of antimicrobial agents* 44, no. 1 (2014): 90-91.

36. Charan, Jaykaran, Summaiya Mulla, Sridhar Ryavanki, and Naresh Kantharia. "New Delhi Metallo–beta lactamase–1 containing Enterobacteriaceae: origin, diagnosis, treatment and public health concern." *Pan African Medical Journal* 11, no. 1 (2012).

37. M. Kaase, P. Nordmann, T. A. Wichelhaus, S. G. Gatermann, R. A. Bonnin, and L. Poirel, “NDM-2 carbapenemase in Acinetobacter baumannii from Egypt,” Journal of Antimicrobial Chemotherapy, vol. 66, no. 6, pp. 1260–1262, 2011.

38. Nordmann, Patrice, Anne E. Boulanger, and Laurent Poirel. "NDM-4 metallo-β-lactamase with increased carbapenemase activity from

Escherichia coli." *Antimicrobial agents and chemotherapy* 56, no. 4 (2012): 2184-2186.

39. Hornsey, Michael, Lynette Phee, and David W. Wareham. "A novel variant, NDM-5, of the New Delhi metallo-β-lactamase in a multidrug-resistant Escherichia coli ST648 isolate recovered from a patient in the United Kingdom." *Antimicrobial agents and chemotherapy* 55.12 (2011): 5952-5954.

40. Williamson, Deborah A., et al. "Identification and molecular characterisation of New Delhi metallo-β-lactamase-1 (NDM-1)-and NDM-6-producing Enterobacteriaceae from New Zealand hospitals." *International journal of antimicrobial agents* 39.6 (2012): 529-533.

41. Göttig, Stephan, et al. "Detection of NDM-7 in Germany, a new variant of the New Delhi metallo-β-lactamase with increased carbapenemase activity." *Journal of Antimicrobial Chemotherapy* 68.8 (2013): 1737-1740.

42. Tada, Tatsuya, et al. "NDM-8 metallo-β-lactamase in a multidrug-resistant Escherichia coli strain isolated in Nepal." *Antimicrobial agents and chemotherapy* 57.5 (2013): 2394-2396

43. Deshpande P, Rodrigues C, Shetty A, Kapadia F, Hedge A, Soman R. New Delhi Metallo-beta lactamase (NDM-1) in Enterobacteriaceae: Treatment options with carbapenems compromised. J Assoc Physicians India 2010;58:147-9.

44. Wayne, P. A. "National committee for clinical laboratory standards." *Performance standards for antimicrobial disc susceptibility testing* 12 (2002).

45. Galani, Irene, et al. "Evaluation of different laboratory tests for the detection of metallo-β-lactamase production in Enterobacteriaceae." *Journal of antimicrobial chemotherapy* 61.3 (2008): 548-553.

46. Nordmann, Patrice, Laurent Poirel, and Laurent Dortet. "Rapid detection of carbapenemase-producing Enterobacteriaceae." *Emerging infectious diseases* 18.9 (2012): 1503.

47. Rai S, Manchanda V, Singh N P, Kaur I R. Zinc-dependent carbapenemases in clinical isolates of family Enterobacteriaceae. Indian J Med Microbiol 2011;29:275-9

48. Singh, Rajkumar Manojkumar, Soma Sarkar, Puranjay Saha, and Manideepa Sengupta. "carbapenemase producing enterobacteriaceae among urinary isolates: scenario from a tertiary care hospital in eastern india." 2014Month :FebruaryVolume :Vol3Issue :Issue 6Page :1323-1333

49. Berrazeg, M., S. Diene, L. Medjahed, P. Parola, M. Drissi, Didier Raoult, and J. Rolain. "New Delhi Metallo-beta-lactamase around the world: an eReview using Google Maps." *Euro Surveill* 19, no. 20 (2014): 19.

50. Bora, A., G. U. Ahmed, N. K. Hazarika, K. N. Prasad, S. K. Shukla, V. Randhawa, and J. B. Sarma. "Incidence of blaNDM-1 gene in Escherichia coli isolates at a tertiary care referral hospital in Northeast India." *Indian journal of medical microbiology* 31, no. 3 (2013): 250.

51. Anjana Shenoy K, Jyothi E K, Ravikumar R. Phenotypic identification & molecular detection of blandm-1 gene in multidrug resistant Gram-negative bacilli in a tertiary care centre. Indian Journal of Medical Research. 2014 Apr;139 (4): 625-631.

52. Struelens, M. J., D. L. Monnet, A. P. Magiorakos, and O. Santos. "'Connor F, Giesecke J; European NDM-1 survey participants. New Delhi metallo-beta-lactamase 1-producing Enterobacteriaceae: emergence and response in Europe." *Euro Surveill* 15, no. 46 (2010): 19716.

53. Nagaraj, S., S. P. Chandran, P. Shamanna, and R. Macaden. "Carbapenem resistance among Escherichia coli and Klebsiella pneumoniae in a tertiary care hospital in south India." *Indian journal of medical microbiology* 30, no. 1 (2012): 93.

54. Carmeli, Y., M. Akova, G. Cornaglia, G. L. Daikos, J. Garau, S. Harbarth, G. M. Rossolini, M. Souli, and H. Giamarellou. "Controlling the spread of carbapenemase-producing Gram-negatives: therapeutic approach and infection control." *Clinical Microbiology and Infection* 16, no. 2 (2010): 102-111.

55. Jeremiah, S. S., V. Balaji, S. Anandan, and R. D. Sahni. "A possible alternative to the error prone modified Hodge test to correctly identify the carbapenemase producing Gram-negative bacteria." *Indian journal of medical microbiology* 32, no. 4 (2014): 414.

56. Vrioni, Georgia, et al. "Comparative evaluation of a prototype chromogenic medium (ChromID CARBA) for detecting carbapenemase-producing Enterobacteriaceae in surveillance rectal swabs." *Journal of clinical microbiology* 50.6 (2012): 1841-1846.

57. Bush, Karen, and George A. Jacoby. "Updated Functional Classification of Β-Lactamases" *Antimicrobial Agents and Chemotherapy* 54.3 (2010): 969–976. *PMC*. Web. 3 Sept. 2015.

58. Krishna BV. New Delhi metallo-beta-lactamases: a wake-up call for microbiologists. Indian J Med Microbiol. 2010;28(3):265-6.

59. Balan, K., P. Sireesha, and C. R. Setty. "Study to detect incidence of carbapenemase among Gram negative clinical isolates from tertiary care hospital." *J of Dental and Medical Science* 1, no. 6 (2012): 8-12.

60. Amjad A, Mirza I, Abbasi S, Farwa U, Malik N, Zia F. Modified Hodge test: A simple and effective test for detection of carbapenemase production. *Iranian Journal of Microbiology*. 2011;3(4):189-193.

61. Vasoo, Shawn, et al. "Comparison of a novel, rapid chromogenic biochemical assay, the Carba NP test, with the modified Hodge test for

detection of carbapenemase-producing Gram-negative bacilli." *Journal of clinical microbiology* 51.9 (2013): 3097-3101.

62. Dortet, L., Poirel, L., & Nordmann, P. (2014). Further Proofs of Concept for the Carba NP Test. *Antimicrobial Agents and Chemotherapy*, *58*(2), 1269. doi:10.1128/AAC.01825-13

63. Fomda BA, Khan A, Zahoor D. NDM-1 (New Delhi metallo beta lactamase-1) producing Gram-negative bacilli: Emergence & clinical implications. Indian J Med Res 2014;140:672-8

64. Dortet, Laurent, Laurent Poirel, Nadia Anguel, and Patrice Nordmann. "New Delhi Metallo-β-Lactamase 4–producing Escherichia coli in Cameroon." *Emerging infectious diseases* 18, no. 9 (2012): 1540.

65. Coppo, E., Del Bono, V., Ventura, F., Camera, M., Orengo, G., Viscoli, C., & Marchese, A. (2014). Identification of a New Delhi metallo-beta-lactamase-4 (NDM-4)-producing Escherichia coli in Italy. *BMC microbiology*, *14*(1), 148.

67. Cuzon, Gaelle, Remy A. Bonnin, and Patrice Nordmann. "First identification of novel NDM carbapenemase, NDM-7, in Escherichia coli in France." *PloS one* 8, no. 4 (2013).

68. Tada, T., Miyoshi-Akiyama, T., Shimada, K., & Kirikae, T. (2014). Biochemical Analysis of Metallo-β-Lactamase NDM-3 from a Multidrug-Resistant Escherichia coli Strain Isolated in

Japan. *Antimicrobial Agents and Chemotherapy*, *58*(6), 3538–3540. doi:10.1128/AAC.02793-I

69. Rahman, Mohibur, et al. "Prevalence and molecular characterisation of New Delhi metallo-β-lactamases NDM-1, NDM-5, NDM-6 and NDM-7 in multidrug-resistant Enterobacteriaceae from India." *International journal of antimicrobial agents* 44.1 (2014): 30-37.

70. Schechner, V., et al. "Asymptomatic rectal carriage of blaKPC producing carbapenem-resistant Enterobacteriaceae: who is prone to become clinically infected?." *Clinical Microbiology and Infection* 19.5 (2013): 451-456.

71. Bush, K., Jacoby, G. A., & Medeiros, A. A. (1995). A functional classification scheme for beta-lactamases and its correlation with molecular structure.*Antimicrobial Agents and Chemotherapy*, *39*(6), 1211–1233.

72. Nordmann, P., M. Gniadkowski, C. G. Giske, L. Poirel, N. Woodford, and V. Miriagou. "Identification and screening of carbapenemase - producing Enterobacteriaceae." *Clinical Microbiology and Infection* 18, no. 5 (2012): 432-438.

73. Singh, Kamaljit, et al. "Rectal screening for Klebsiella pneumoniae carbapenemases: comparison of real-time PCR and culture using two selective screening agar plates." *Journal of clinical microbiology* 50.8 (2012): 2596-2600.

74. Sanger, Frederick, Steven Nicklen, and Alan R. Coulson. "DNA sequencing with chain-terminating inhibitors." *Proceedings of the National Academy of Sciences* 74, no. 12 (1977): 5463-5467.

75. Khanduri, Naresh, Biswaroop Chatterjee, and Rajiv Kumar Agarwal. "Experience with a Commercial Assay for Detecting Different Carbapenemases in Enterobacteriaceae." Int.J.Curr.Microbiol.App.Sci (2015)Special Issue-1: 150-156

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

April 14, 2014

To
Dr M Uma Maheswari
Postgraduate
Department of Microbiology
PSG IMS & R
Coimbatore

The Institutional Human Ethics Committee, PSG IMS & R, Coimbatore -4, has reviewed your proposal on April 4, 2014 in its expedited review meeting held at IHEC Secretariat, PSG IMS&R, between 10.00 am and 11.00 am, and discussed your study proposal entitled:

"Molecular detection of New Delhi metallo betalactamases in escherichia coli and klebsiella infections in clinical isolates of a tertiary care hospital"

The following documents were received for review:

1. Duly filled application form
2. Proposal
3. Informed Consent forms
4. Permission letter from Head of the Department
5. CV
6. Budget

After due consideration, the Committee has decided to approve the above study.

The members who attended the meeting, at which your proposal was discussed, are listed below:

Name	Qualification	Responsibility in IHEC	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
Dr P Sathyan	DO, DNB	Clinician, Chairperson	Male	No	Yes
Dr S Bhuvaneshwari	M.D	Clinical Pharmacologist Member - Secretary	Female	Yes	Yes
Dr Sudha Ramalingam	M.D	Epidemiologist Alt. Member - Secretary	Female	Yes	Yes
Dr Y S Sivan	Ph D	Member –Social Scientist	Male	Yes	Yes

The approval is valid for one year.

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

We request you to intimate the date of initiation of the study to IHEC, PSG IMS&R and also, after completion of the project, please submit completion report to IHEC.

This Ethics Committee is organized and operates according to Good Clinical Practice and Schedule Y requirements.

Non-adherence to the Standard Operating Procedures (SOP) of the Institutional Human Ethics Committee (IHEC) and national and international ethical guidelines shall result in withdrawal of approval (suspension or termination of the study). SOP will be revised from time to time and revisions are applicable prospectively to ongoing studies approved prior to such revisions.

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Yours truly,

Dr S Bhuvaneshwari
Member - Secretary
Institutional Human Ethics Committee

Digital Receipt

This receipt acknowledges that Turnitin received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

Submission author:	201314404.m.d Microbiology DR.M...
Assignment title:	TNMGRMU EXAMINATIONS
Submission title:	MOLECULAR DETECTION OF NEW..
File name:	thesis_on_sep_13.docx
File size:	106.15K
Page count:	89
Word count:	11,398
Character count:	71,550
Submission date:	15-Sep-2015 07:42AM
Submission ID:	568413746

MOLECULAR DETECTION OF NEWDELHI METALLOBETALACTAMAES IN ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE IN CLINICAL ISOLATES OF A TERTIARY CARE HOSPITAL

Dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University

In partial fulfillment of the regulations

For the award of the degree of

M.D. MICROBIOLOGY

Branch - IV

DEPARTMENT OF MICROBIOLOGY,
PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH,
PEELAMEDU, COIMBATORE-641004,
TAMILNADU, INDIA.

Copyright 2015 Turnitin. All rights reserved.

201314404.m.d Microbiology DR.M.UMA MAHESWARI User Info Messages Student English Help Logout

Class Portfolio Peer Review My Grades Discussion Calendar

NOW VIEWING: HOME > THE TAMIL NADU DR.M.G.R.MEDICAL UTY 2014-15 EXAMINATIONS

Welcome to your new class homepage! From the class homepage you can see all your assignments for your class, view additional assignment information, submit your work, and access feedback for your papers.

Hover on any item in the class homepage for more information.

Class Homepage

This is your class homepage. To submit to an assignment click on the "Submit" button to the right of the assignment name. If the Submit button is grayed out, no submissions can be made to the assignment. If resubmissions are allowed the submit button will read "Resubmit" after you make your first submission to the assignment. To view the paper you have submitted, click the "View" button. Once the assignment's post date has passed, you will also be able to view the feedback left on your paper by clicking the "View" button.

Assignment Inbox: The Tamil Nadu Dr.M.G.R.Medical Uty 2014-15 Examinations

	Info	Dates	Similarity	
TNMGRMU EXAMINATIONS	ⓘ	Start 01-Sep-2014 11:27AM Due 30-Oct-2015 11:59PM Post 30-Oct-2015 12:00AM	19%	Resubmit View

Copyright © 1998 – 2015 Turnitin, LLC. All rights reserved.

Usage Policy Privacy Pledge Helpdesk Research Resources