"DETECTION OF CARBAPENEMASE PRODUCTION IN CLINICAL ISOLATES OF ENTEROBACTERIACEAE AT A RURAL TERTIARY HEALTH CARE CENTRE"

BY
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DISSERTATION SUBMITTED TO
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UNDER THE GUIDANCE OF

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SRI DEVARAJ URS MEDICAL COLLEGE, KOLAR
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LIST OF ABBREVIATIONS

MDR- Multi drug resistant

MIC- Minimal Inhibitory Concentration

MHT- Modified Hodge Test

CRE- Carbapenem Resistant Enterobacteriaceae

CPE- Carbapenemase-producing Enterobacteriaceae

PBPs -Penicillin binding proteins

PRPs -Penicillinase resistant penicillins

MRSA -Methicillin resistant *Staphylococcus aureus*

VRSA -Vancomycin resistant *Staphylococcus aureus*

ESBL -Extended spectrum beta lactamase

ICAAC -Interscience Conference on Antimicrobial Agents and Chemotherapy

SME -*S. marcescens* enzyme

IMI - imipenem hydrolyzing β-lactamase

NMC- not metalloenzyme carbapenemase

GES- Guiana extended-spectrum

KPC -*K. pneumoniae* carbapenemase

MBLs -Metallo betalactamases

EDTA- Ethylene diamine tetra acetic acid

IMP- Imipenemase

VIM -Verona integron-encoded metallo-b-lactamase

NDM- New Delhi metallo beta lactamase

SPM-1 -Sao Paulo MBL

GIM – 1-German imipenemase

SIM-1- Seoul imepenemase

OXA- oxacillin hydrolyzing Carbapenemases

CLSI -Clinical and Laboratory Standards Institute

DDST -Double Disc synergy test

CDT -Combined disc test

NHS -National health service

JAPI -Journal of Associations of Physicians in India

AST -Antibiotic susceptibility testing

ICU- Intensive Care Unit

PCR- Polymerase Chain Reaction

DAMA- Discharge against medical advice

SDH-sub dural haemorrhage

SAH- sub arachnoid haemorrhage

VAP -Ventilator Associated Pneumonia

QRD- Quinolone Resistant Determinant

ARD- Aminoglycoside Resistant Determinant

ABSTRACT

INTRODUCTION

Carbapenems are beta lactam antibiotics which are used as last resort drugs for multidrug resistant gram negative bacterial infections especially Extended spectrum and Broad spectrum beta lactamase producing strains. In the recent years rising resistance to carbapenems due to production of carbapenamases has been reported.

Carbapenemases are usually present in integrons on plasmids and pose a serious threat of massive dissemination among gram negative bacteria. The resistance is mainly due to mobile genes on the plasmids. *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, are among the most commonly isolated organisms of hospital acquired infections, they frequently show MDR phenotype including resistance to carbapenems. In the last few years this resistance pattern has been seen in Enterobacteriaceae.

The carbapenem resistance is a major public health concern since there are few antibiotics in reserve beyond carbapenems. Therefore, detection of Carbapenemase producing organisms especially among members of Enterobacteriaceae becomes important and also helps to limit the spread of resistance by implementing strict infection control measures. Hence this study was undertaken.

MATERIALS AND METHODS

A total of 1,245 isolates of Enterobacteriaceae were screened for carbapenem resistance using imipenem, meropenem and ertapenem discs and the resistance confirmed by determining MIC of ertapenem using E-strips. The carbapenemase production among members of Enterobacteriaceae was detected by Modified Hodge test.

A selected 60 isolates positive by MHT were subjected to multiplex PCR analysis to identify the drug resistant genes (blaNDM, blaVIM, blaIMP and blaKPC).

A total of 20 stool samples were collected from patients infected with carbapenemase producing isolates and were screened for CRE using selective MacConkey agar(0.5mg/L meropenem).

RESULTS

Among the 1,245 isolates of Enterobacteriaceae screened, 175(14.05%) of the isolates were found to be resistant to any one of the carbapenems. One hundred and eight isolates (61.71%) were found to be positive for carbapenemase production. Thus, the prevalence of CPE is 8.67%.

Majority of carbapenemase producing isolates were obtained from endotracheal secretions (15.85%) followed by pus (11.46%) and urine samples (5.88%).

K pneumoniae (15.89%) was the most common isolate found to be producing carbapenemase followed by *Enterobacter spp.* (10.7%) and *E.coli* (6.87%). Majority of the carbapenemase producing organisms were recovered from cases admitted at ICU(31%).

Among 108 carbapenemase producing Enterobacteriaceae majority of isolates were found to be sensitive to Chloramphenicol(76%) followed by Tetracycline(42%).

Of the 60 isolates subjected to multiplex PCR, 57(95%) were found to be harbouring one or more of carbapenemase producing genes. Fifty five of carbapenemase producing strains carried blaNDM, among which 10 isolates also harboured blaIMP, 3 harboured blaVIM and 2 isolates were positive for NDM, IMP and VIM. Additionally, blaVIM gene was detected in two strains.

Among the 97 patients infected with CPE, 81(83.5%) patients recovered, 11(11.3%) patients expired and 5(5.2%) patients went against medical advice. Among

11 patients who expired, majority of them were males and above 50 years of age (73%). The most common isolate was *K.pneumoniae* obtained from Endotracheal secretions of patients admitted in ICU.

Of the 20 stool samples collected from patients found to be infected with carbapenemase producing strains, 17(85%) yielded growth of carbapenem resistant strains and 10(59%) among them were carbapenemase producing strains.

CONCLUSION

Our study showed that the prevalence of CPE is 8.67%. Modified Hodge test was a useful method in the detection of carbapenemase producing isolates. As the treatment options for infections with CPE is limited, our study showed that cholramphenicol and tetracycline can be used as alternative drugs to treat such infections. Mortality seen in the ICU patients who succumbed to the illness had life threatening comorbidities in addition to infection with carbapenemase producing strains. Many patients infected with CPE were found to harbor carbapenem resistant strains in their gut flora.

KEY WORDS: Carbapenem Resistant Enterobacteriaceae (CRE), Carbapenemase, New-Delhi metallo beta lactamase (NDM).

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INTRODUCTION

In recent years, antimicrobial resistance in Enterobacteriaceae has become a cause of concern in many hospitals worldwide.[1] Among the wide spectrum of antibiotics, betalactams account for over 50% of all systemic antibiotics in use. As multidrug resistant organisms have increased, so has the use of broad spectrum agents to treat them. [2]

Carbapenems have a broad range of activity against gram positives, gram negatives and anaerobic bacteria.[3] Carbapenems (e.g., imipenem, meropenem, ertapenem, and doripenem) are effective in the treatment of severe gram-negative bacterial infections when resistance to other classes of antimicrobials is present.[4]

The most common cause of bacterial resistance to carbapenems is the production of carbapenemase. They have the capacity to hydrolyze all beta lactams including carbapenems with the exception of aztreonam.[5] Being plasmid mediated, they are easily transmitted among members of Enterobacteriaceae thus facilitating easy dissemination of resistance to betalactams.[6]

Among the Carbapenemase-producing Enterobacteriaceae (CPE), *Escherichia coli* and *Klebsiella pneumoniae* are the most notorious organisms causing infections.[7] *Acinetobacter baumannii* and *Pseudomonas aeruginosa,* are among the most commonly isolated organisms of hospital acquired infections, they frequently show multi-drug resistant (MDR) phenotype including resistance to carbapenems. In the last few years this resistance pattern has been seen in Enterobacteriaceae.[8]

Carbapenem resistance is a worldwide public health concern since there are few antibiotics in reserve beyond carbapenems. Therefore, detection of Carbapenemase producing organisms especially among members of Enterobacteriaceae is vital for optimal therapy and to limit the spread of resistance by implementing strict infection control measures.[9,10]

OBJECTIVES

1. To screen the Enterobacteriaceae isolates for Carbapenemase production.

2. To confirm the Carbapenemase production by using Modified Hodge Test.

3. To perform the Minimal Inhibitory Concentration for the strains using E-strips.

4. To suggest an alternative therapy for patients infected with such strains.

5. To follow up the final outcome of the patients.

REVIEW OF LITERATURE

Penicillin and other beta lactam antibiotics: Discovery, overuse, and development of resistance

Fifty years ago, the discovery of penicillin led to the beginning of the antibiotic era. In 1928, Alexander Fleming was working in the laboratory of St Mary's hospital, London. He observed that a mold had contaminated a plate on which Staphylococci was grown. In the vicinity of the mold the Staphylococci were not to be found; they had undergone lysis. The mold that caused lysis of bacteria on the plate was Penicillium. Fleming correctly thought that the mold had elaborated some substance which was inimical to the growth of bacteria. He also grew the fungus in broth cultures and showed it had inhibitory effect on many organisms. The antibacterial substance was called Penicillin.[11] Following the discovery of penicillin in 1928, paved the way for development of many other antibiotics. In the late 1940s and early 1950s new antibiotics were introduced including Streptomycin, Chloramphenicol, Tetracycline, Macrolide and glycopepetides (vancomycin). This was known as the golden age of antimicrobial chemotherapy.[12]

Penicillin belongs to group called beta-lactams, because all the beta lactam antibiotics share the same chemical structure, called Penicillin nucleus. The chemical structure was demonstrated by Dorothy Hodgkin.[13] The penicillins are made up of 3 components i.e a 5 membered thiazolidine ring, a 4 membered beta- lactam ring, and a side chain. The thiazolidine ring and beta lactam ring make up the penicillin nucleus, responsible for antimicrobial activity. By the changes in the lateral chains of the original beta-lactam, different semi-synthetic penicillins or cephalosporins have been obtained, either with broader antibacterial spectrum or with changes in their pharmacokinetic properties.[14] (fig 1)

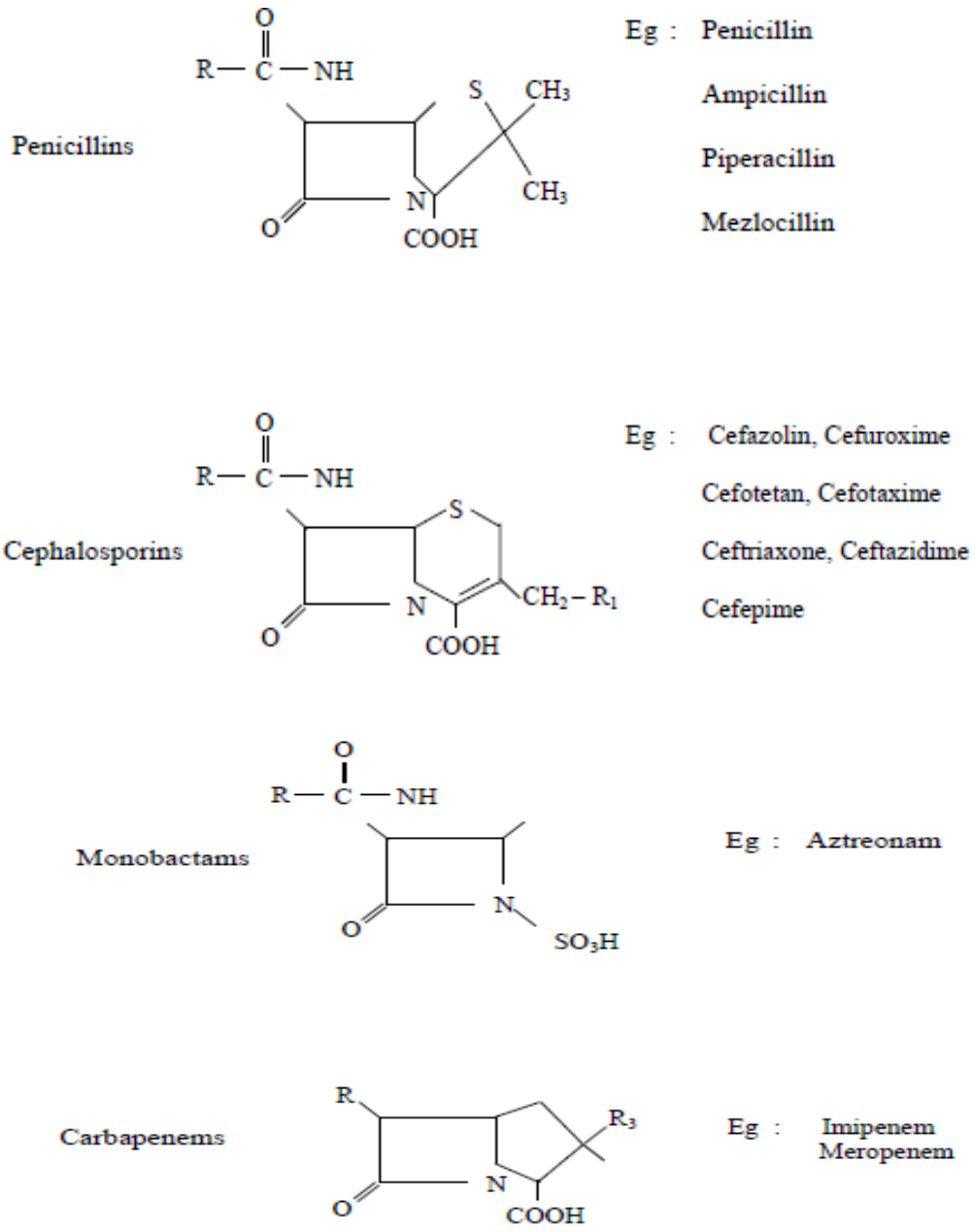


FIG 1: Structure of Beta lactam antibiotics

Action of penicillin and other beta lactams

The cell wall is an essential component of the bacterial cell structure. It provides rigidity and protects the cell from osmotic lysis. A change in the structure of the cell wall predisposes the organism to death by lysis. Cell wall is composed of highly crossed linked peptidoglycans, which are made up of alternating amino sugars: N-acetyl glucosamine and N-acetyl muramic acid held together by penta peptide side chains. The final step in the synthesis involves the removal of the terminal D-alanine from penta peptide chain by transpeptidases. The energy liberated is used in the formation of cross link with the adjacent peptidoglycan.[15] Penicillin interferes with the final step of cell wall synthesis by binding to transpeptidases,that is how they are referred to as the Penicillin binding proteins (PBPs). This results in inhibition of further elongation of peptidoglycan. The other beta- lactam antibiotics also work through the similar mechanism to bring about damage to the cell wall. PBPs vary in number and affinity in different bacteria, like *Staphylococci* and *E.coli* have 4 and 5 PBPs, respectively. This property is responsible for the varied spectrum exhibited by the beta lactams.[11]

Use and overuse of Penicillin

In 1930, Cecil George Paine, a pathologist used penicillin to treat sycosis barbae, but it failed. Later on in the same year it was used to treat Gonococcal infection in infants, which noted the first cure with pencillin.[16] In 1939, a marked progress was made in demonstrating the bactericidal activity of penicillin by Howard Florey and his team at the Sir William Dunn School of Pathology.[17] In 1940, began the use of penicillin for therapeutic purposes. It was very effective in the treatment of various infections like pneumonia, septicemia, skin and wound infections. During World War II, penicillin was used extensively which saved the lives of many

soldiers thereby decreasing the number of deaths and amputations caused by infected wounds. Due to the decreased supply and its rapid excretion in urine, it became common to collect the urine from patients being treated, so that the penicillin in the urine could be isolated and reused.[18] Another agent, *probenecid*, was eventually found to prolong the duration of penicillin in the human body. But, with the advent of a method for mass-production of the drug by Howard Florey and and his team in 1945, the issues were resolved. They were awarded the Nobel Prize in Medicine in1945 for the discovering the method of mass production.[19]

The indiscriminate use of penicillin following its mass production found that few microbes resisted it. There are many mechanisms that confer resistance to penicillins they include inactivation by enzymes that destroy the penicillin molecule, altered permeability to penicillins and altered penicillin binding proteins, but the most common being enzymatic degradation.[11] The presence of Penicillinase, the enzyme that degraded penicillin in bacteria was discovered by Abraham and Chain in 1940, even before penicillin was used in clinical practice. It was observed that some strains of *E.coli* produced it.[20] This issue was overshadowed. In 1944, a similar penicillinase was discovered in staphylococci.[21]

Penicillinases and penicillinase resistant penicillins

Penicillinases are the hydrolytic enzymes which cleave the beta lactam ring. Majority of *Staphylococci* and some strains of *Gonococci*, *B.subtilis*, *E.coli*, and *H.influenzae* produce penicillinases. The production of penicillinase varies in organisms. In the gram positive the penicillinase are very active and are produced extracellularly. As the enzyme diffuses into the surroundings, it confers protection on the organism producing it as well as the other susceptible organisms nearby. In gram

negative bacteria penicillinases are present in the periplasmic space.[14] To combat infections caused by penicillinase producing bacteria Penicillinase resistant penicillins (PRPs) were developed such as methicillin, oxacillin, cloxacillin and flucloxacillin. The discovery of PRPs was an important landmark in the history of antibacterial chemotherapy.[22] Methicillin was discovered by Beecham in 1959.[23] It was found to be resistant to the action penicillinases due to the presence of ortho-dimethoxyphenyl group attached to side chain carbonyl group of penicillin nucleus. It acts by inhibiting the cell wall synthesis as penicillin does. Methicillin was found to be unstable, this resulted in the discovery of more stable PRPs such as oxacillin, fluoxacillin and dicloxacillin.[24] Being resistant to penicillinases they were used for treating infections caused by staphylococci, which elaborate this enzyme. Over the past few decades, the incidence of resistance among gram positive bacteria has risen. In 1961, British scientists identified the first strain of *Staphylococcus aureus* that was resistant to methicillin. This was the so called birth of Methicillin resistant *Staphylococcus aureus* (MRSA).[25]

Methicillin resistant *Staphylococcus aureus* and Vancomycin resistant *Staphylococcus aureus*

The resistance mediated by MRSA is brought about by mecA gene located on Staphylococcal chromosomal cassette- genomic island. mecA gene encodes for PBP2a whose active site does not bind methicillin.[26] MRSA is troublesome especially in hospitals and crowded places (prisons, military recruits). Reports on cases of MRSA were first made in US then Canada.[25] A study in 2002 reported on the changing epidemiology of MRSA skin infections in San Fransisco County jail, where MRSA accounted for 70% of Staphylococcal aureus infection by 2002.[27] MRSA is of special concern with regard to treatment, as it is multidrug resistant. Vancomycin is

the drug of choice in such cases. With increase in MRSA and vancomycin being the drug of choice the Vancomycin resistant *Staphylococcus aureus* (VRSA) emerged as vancomycin was used extensively to treat MRSA.[28] In 2002, a report on VRSA was published, wherein it was isolated from the catheter tip of a diabetic, renal dialysis patient in Michigan. On DNA sequencing VRSA possessed a vanA gene, also found in Vancomycin resistant *Enterococci*(VRE).[29] The basis of this could be the horizontal gene transfer from VRE to *Staphylococcus aureus*, which was demonstrated earlier in 1992.[30] Until 2013, VRSA has been reported from US, Iran, India, Europe, and Latin America. This warranted the discovery of other beta lactams-cephalosporins and carbapenems.[29]

Cephalosporins and Extended spectrum beta lactamase (ESBL)

Cephalosporins form the largest and most diverse family of beta lactam antibiotics. They were first isolated from fungus cephalosporium now called Acremonium. It was discovered by Giuseppe Brotzu in 1948, from a sewer off the Sardinian coast, Italy.[11] It was found that the crude filtrates of the fungus could inhibit growth of staphylococci.[31]Further investigations on the crude filtrates by Abraham and Newton in England yielded cephalosporin P, N and C. However, these natural substances because of the lower potency could not be used as antimicrobial agents. It required few modifications to make them effective antibiotics.[32]A Cephalosporin molecule consists of beta lactam ring condensed with dihydrothiazine ring. The core is referred to as 7-aminocephalosporinic acid(7-ACA), derived from cephalosporin C which confers stability to acid hydrolysis and tolerance to action of beta lactamases.[11]Structural modifications of cephalosporin nucleus was done with a view to improve pharmacological properties and overcome resistance. Based on antimicrobial properties cephalosporins are grouped into generations. Presently, there

are five generations in cephalosporins. Cephalosporins have been used empirically for treating heterogenous group of infections. The introduction of third generation cephalosporins into clinical practice was to fight against ampicillin hydrolyzing TEM and SHV-1 beta lactamases.[33] In 1983, a β-lactamase capable of hydrolysing extended-spectrum cephalosporins was observed, in *Klebsiella pneumonia* from Germany. They also exhibit their spectrum of activity against oxyiminocephalosporins, these enzymes came to be known as extended spectrum β-lactamases (ESBLs).[34]

Carbapenems

Carbapenems are often used as a last resort in the treatment of multi drug resistant Gram negative infections such as bacterial meningitis, skin and intra-abdominal infections. The emergence of resistance to newer beta lactams led to the search for beta lactamase inhibitors to overcome the problem of resistance.[35] By 1976, the beta -lactamase inhibitors were discovered. They were olivanic acids, which were natural products produced by Gram-positive bacterium *Streptomyces clavuligerus*. They possess a unique backbone called "carbapenem backbone" (a carbon at the 1position, substituents at C-2, a C-6 ethoxy, and *sp2*-hybridizedC-3) with broad-spectrum beta lactam activity.[36] Further research in this area was abandoned due to their chemical instability and poor penetration into the cell wall. Following the discovery of olivanic acids two superior beta lactamase inhibitors were discovered: clavulanic acid from *S. clavuligerus*, and Thienamycin from *Streptomyces cattleya*. Thienamycin was the first carbapenem to be discovered and is considered the prototype.[37]

Carbapenems are defined based on chemical structure. They have a 4:5 fused beta lactam ring. The uniqueness lies in the substitution of carbon atom for sulfur

atom at C-1. The property of expressing its resistance to beta lactamases is due to the removal of hydroxyethyl side chain from the conventional structure of penicillin.[38] This discovery was first reported at the 16th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) meeting in 1976 at Chicago.[37] Thienamycin being a natural product and its bio-synthetic pathway known its yields were low. Over time, thienamycin gained importance as it was active against Gram-negative bacteria, including isolates of *Pseudomonas aeruginosa*, anaerobes, like *Bacteroides fragilis*, and Gram-positive bacteria, such as methicillin susceptible *Staphylococcus aureus* and streptococci.[39] Thienamycin was found to be unstable. However, the instability of the molecule inspired the search for more stable compounds.

The first derivative was the *N*-formimidoyl derivative, imipenem followed by a closely related carbapenem, Panipenem. In 1985, imipenem was the first carbapenem made available for the treatment of complex microbial infections.[40] Although, both these carbapenems were more stable than thienamycin they were found to be susceptible to deactivation by dehydropeptidase I (DHP-I), found in the human renal brush border. This necessitated the coadministration with an inhibitor, cilastatin.[35] In the course of discovery; many carbapenems with broader spectrum and stability were identified. They include meropenem, ertapenam doripenem and biapenem. The structure of the different carbapenems is shown in fig 2. A major breakthrough was modification at 1-β position of beta lactam ring by addition of a methyl group. This modification provided protection against hydrolysis by dehydropeptidase I.[37]In the subsequent two decades many carbapenems with the modification were identified. They included antipseudomonal carbapenems, anti-methicillin-resistant *S. aureus* (MRSA) carbapenems, and others.[41] Carbapenems act

by inhibiting bacterial cell wall synthesis and increase the permeability of the outer membrane of cells hence affecting their efflux system. They are highly toxic to the bacteria.[42]

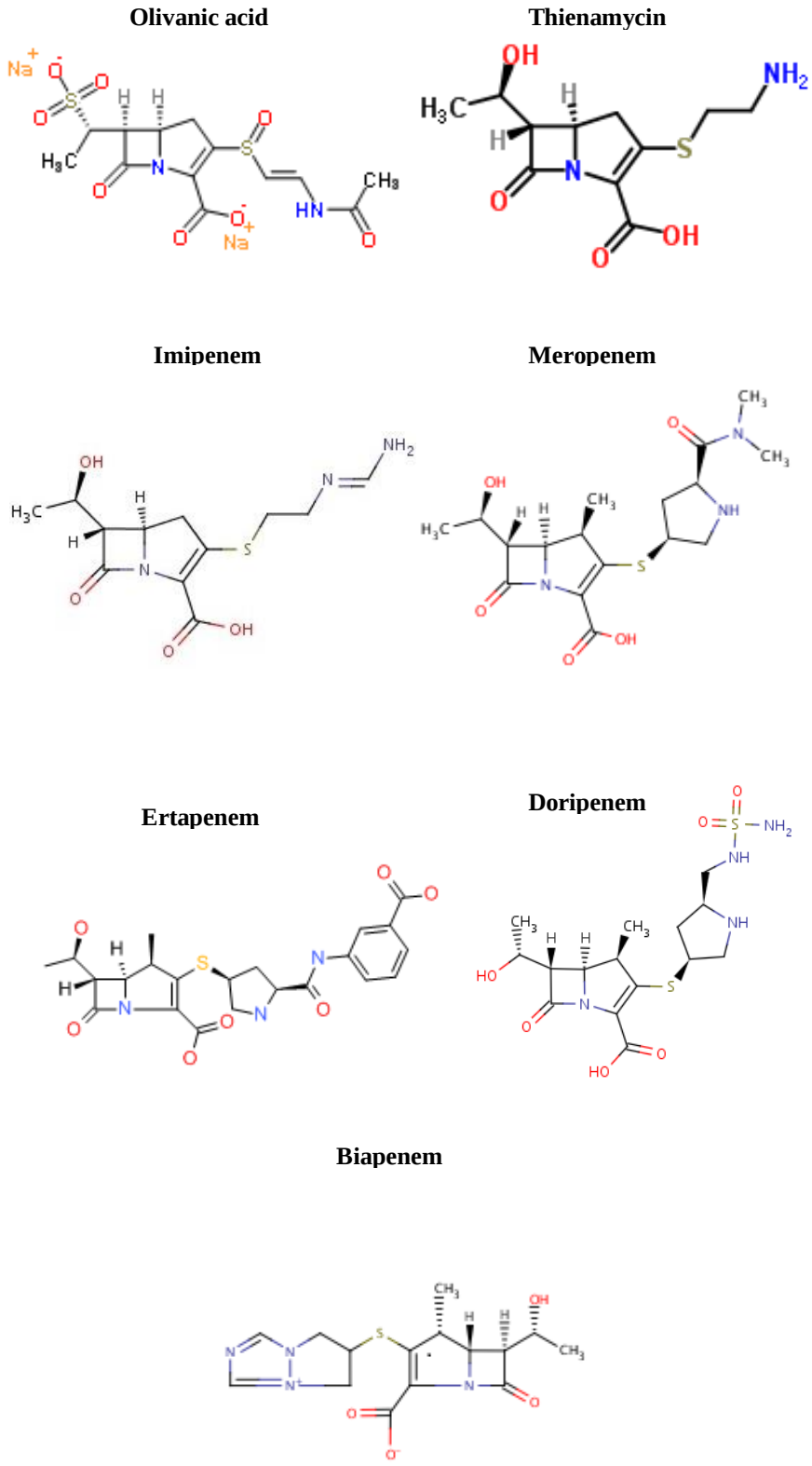


Fig 2: Molecular structures of various carbapenems

Carbapenems retained their activity against Enterobacteriaceae for around 20 years after imipenem was introduced in 1985, but resistance to carbapenems is now on the rise. The use of carbapenems to treat severe hospital acquired infections was thought to be a great advance. Less than a decade following its use, gram negative bacteria resistant to these agents had emerged.[43]It was noted in non-fermenting bacteria, *Pseudomonas* and *Acinetobacter spp*. In the last few years this resistance pattern has been seen among members of family Enterobacteriaceae, constituting an important public health problem.[8]

Carbapenemases

Carbapenem resistance can be broadly studied under two mechanisms – carbapenemase mediated resistance and non-carbapenemase mediated resistance. The former being the more common among the two.[44]

The carbapenemase production in bacteria began when some soil-dwelling bacteria (*Streptomyces sps*) or fungi in the environment started to produce beta lactams naturally. Other bacteria surviving in the same environment had to protect themselves by producing enzymes that degrade these cell-wall acting substances produced by other microorganisms. During the course of evolution, some environmental bacteria (*B. cereus, B. anthracis*) started producing metallo-enzymes that provided them selective advantage for growth. In these bacteria, genes encoding for the enzymes appear to be a natural component of their respective chromosomes.[43]Until the early 1990s, chromosomally encoded enzymes were described which were species-specific. Over the time, these genes have escaped from the chromosome into mobile genetic elements (integron, plasmids, transposons).[45]

Carbapenemases are considered the most versatile family of β-lactamases with a very broad spectrum of activity.[46] Many of the carbapenemases are able to

hydrolyze a large number of β-lactam antibiotics which include the penicillins, cephalosporins, carbapenems, and monobactams and are resistant to commercially available β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam.[6] Owing to their clinical significance, carbapenemases receive a lot of attention but an equal amount of importance is also given to the structural/functional relationship to device newer pharmacological substances.[47]

Classification of carbapenemases

The carbapenemase enzymes are encoded by bla genes. A large variety of carbapenemases has been identified among members of family Enterobacteriaceae.[48] Based on Ambler's molecular classification the carbapenemases have broadly been grouped under class A, B, and D.[49] Molecular classes A and D contain beta-lactamases with serine in their active site while group B contains zinc in their active sites therefore called metallo beta lactamases.[42] The classification is shown in mind map. (Fig 3)

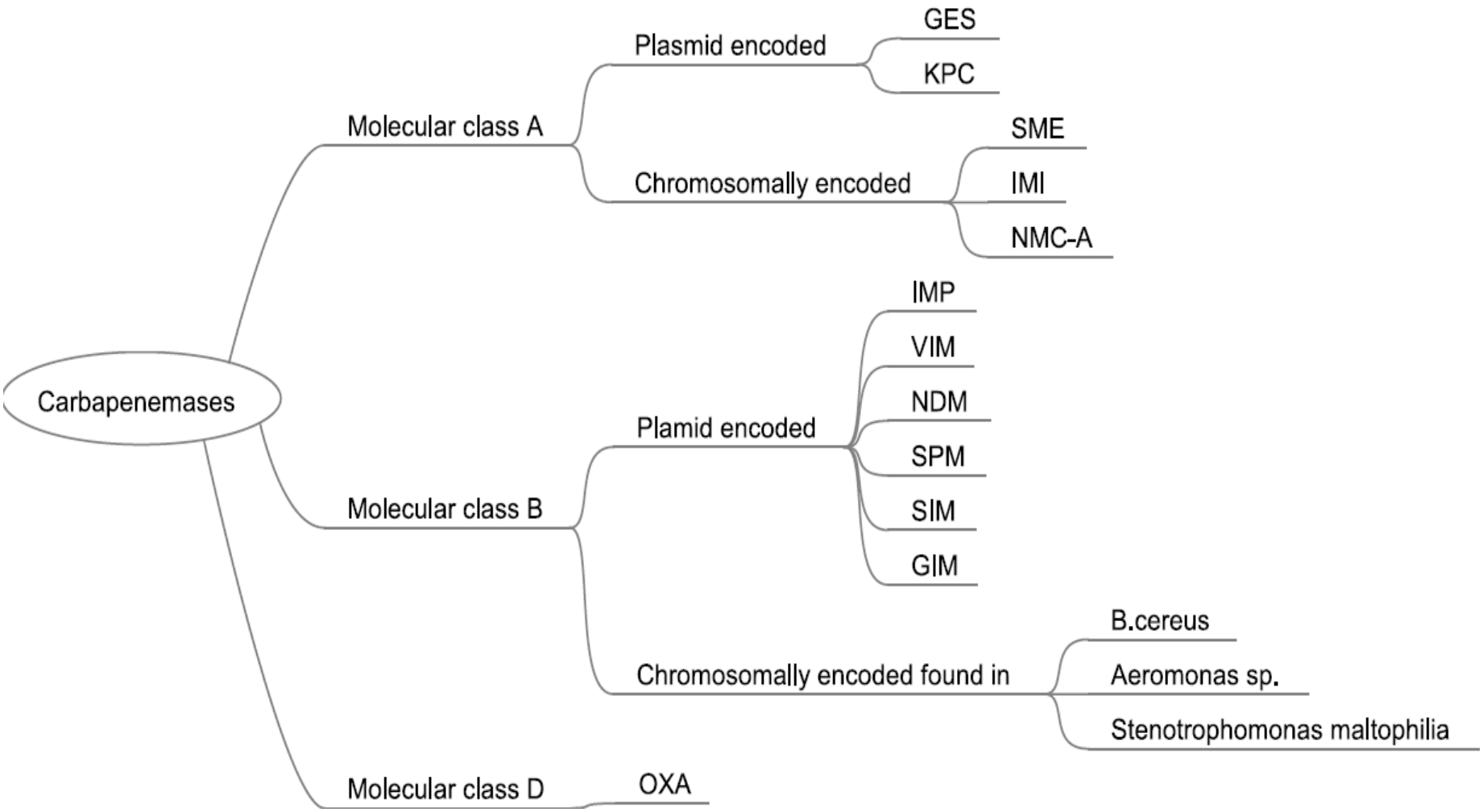


Fig 3: Classification of carbapenemases

I) Molecular class A

Characteristic properties of these enzymes include presence of serine at active-site. They are resistant to penicillins, cephalosporins, aztreonam and carbapenems.[50]They are inhibited by Clavulanic acid and sulbactam. These enzymes are placed under functional 2f subgroup. Earlier class A carbapenemases were mostly chromosomal (SME, IMI, NMC) but in the recent past plasmid mediated carbapenemases (KPC, GES) have increased.

Chromosomally encoded: SME, NMC, IMI

The production of these enzymes is induced by Imipenem and Cefoxitin

1) **SME** (for *S. marcescens* enzyme) enzyme was first discovered in *S. marcescens* from England in 1982. There are three variants that differ by one to two amino acids (SME-1 to SME-3). SME enzymes have been isolated in the U.K. and the U.S.A.[50]
2) **IMI** (for imipenem hydrolyzing β-lactamase): This enzyme was first discovered in an *Enterobacter cloacae* isolate in United States during 1984. Since then these have rarely been observed in clinical isolates of *E. cloacae* in United States, France and Argentina.[45]
3) **NMC** (for not metallo enzyme carbapenemase): NCM-A enzyme was isolated from *E. cloacae* isolate in France during 1990. NMC-A enzymes have been isolated from France, Argentina, and U.S.A. [45,50]

The NMC-A and IMI have amino acid identity of 97% and are related to SME- by ~70% amino acid identity.[45]

Plasmid encoded: GES and KPC

The plasmid-encoded molecular class A carbapenemases include GES (Guiana extended-spectrum) and KPC (*K. pneumoniae* carbapenemase) enzymes.[51]

1) Guiana extended-spectrum (GES)

The first GES enzyme was isolated in 1998 from an infant with a *K. pneumoniae* infection in French Guiana. Currently 17 GES (GES-1 to GES-17) variants have been identified. Two enzymes with similar profile that were described as IBC-1 and IBC-2 (for integron borne cephalosporinase) have now been renamed as GES-7 and GES-8 respectively. GES enzymes have not spread rapidly but are more frequently associated with small outbreaks caused by *P. aeruginosa* and *K. pneumoniae.* Originally this family of enzymes was termed ESBLs due to their broad spectrum of activity against extended-spectrum cephalosporins. GES enzymes are active against penicillins, cephalosporins, carbapenems (although weak), and Aztreonam, but may be inhibited by Clavulanic acid and Tazobactam but at a lower level. They have been found from different geographical locations including Greece, France, Portugal, South Africa, Brazil, Argentina, Korea, and Japan.[45,50,52]

2) Klebsiella pneumoniae carbapenemase (KPC)

Klebsiella pneumoniae carbapenemases are among the most common carbapenemases encountered within the family Enterobacteriaceae worldwide. This was first observed in *K. pneumoniae* from North Carolina in 1996. Currently there are 13 variants of KPC (KPC-1 to KPC-13).[51]KPC-2 and KPC-3 account for a majority of subtypes distributed worldwide among Enterobacteriaceae, *P. aeruginosa*, and *Acinteobacter* spp.[50] Although more commonly seen in *K. pneumoniae,* they have been observed in *K. oxytoca, E.*

cloacae, E. coli, P.mirabilis, S.marcesens and Pseudomonas aeruginosa. Considering the fact that KPC have a potential for dissemination, it is interesting to note that they are not routinely detected by laboratory susceptibility screening.[53]

I) Molecular class B- Metallo betalactamases (MBLs)

Metallo beta lactamases (MBLs) belong to Ambler class B beta lactamases which require metal cation (Zn+) for β-lactam hydrolysis.[54] MBLs can hydrolyze penicillins, cephalosporins and carbapenems: they cannot hydrolyse aztreonam. MBLs are not susceptible to clavulanic acid and sulbactum but can be inhibited by chelating agents like EDTA.[55] Class B beta lactamases are further subdivided into three groups, B1, B2, and B3, based on homology in the sequence of amino acids and structural analysis. They belong to group 3 of functional classification.

Chromosomally encoded MBLs

These were the first MBLs discovered. They were detected in *Bacillus cereus* (BCI, BCII), *Aeromonas* spp (CphA), and *Stenotrophomonas maltophilia* (L1). Chromosomally encoded MBLs were inducible on exposure to β-lactams.[43]

Transferable MBLs

There has been a drastic increase in the detection and spread of the acquired or transferable families of these metallo enzymes. Plasmid mediated MBLs include IMP, VIM, NDM, SPM, GIM, and SIM. Among them the most commonly encountered are NDM, IMP, and VIM. [56]

1) IMP (for Imipenemase)

Acquired carbapenem resistance was first detected in *P. aeruginosa* from Japan in 1988. There are 33 IMP variants which have spread worldwide. The variants of IMP carbapenemase isolated from various organisms are shown in table 1. It has

been reported to be endemic in Japan, Taiwan, East of China, and more recently from Greece, although outbreaks and single reports have been reported in many other countries. IMP-1 was first detected in 1999 from *K. pneumoniae* while analyzing a blood sample of a leukemic patient in Singapore. A new carbapenem hydrolyzing metallo- beta lactamase, IMP-6 was identified which showed a point mutation responsible for a high degree of resistance to meropenem when compared to other carbapenems.[42,44]

Table 1: Variants of IMP carbapenemase isolated from various organisms

Enzymes	**Organisms**
IMP-1	*S.marcescens*
IMP-2(variant IMP-8)	*K.pneumoniae*
IMP-3	*Shigella flexneri*
IMP-4	*Citrobacter youngae*
IMP-6	*S.marcescens* and *A.baumanii*

2) VIM (for Verona integron-encoded metallo-b-lactamase)

VIM carbapenemase was first detected in P.aeruginosa isolate from Verona, Italy, in 1997.[42]There are total of about 33 VIM – type MBLs.[54] VIM-2 is the MBL most often reported world wide.It has been detected in more than 23 species across 40 countries. VIM-2 is known to be endemic in Southern Europe (Greece, Spain, Italy) and Southeast Asia (South Korea, Taiwan).[44]

The variants blaVIM and blaIMP are incorporated into the variables regions of integrons. The likely possibilities for the dissemination of MBL genes in distinct plasmids are:

i) reshuffling of MBL cassettes among plasmid borne integrons and

ii) *en bloc* mobilization of MBL gene-containing structures through transposition and/or recombination events. These events are non-mutual.[55]

In contrast, *bla*NDM genes are not associated with integrons.

3) NDM (for New Delhi metallo beta lactamase)

NDM was first reported in 2009 from *K.pneumoniae* isolate obtained from a Swedish national of Indian origin who had acquired UTI and was treated for the same in India. The isolate was identified on his return to Sweden and was found to possess a metallo-beta lactamase gene which had not been reported earlier.[58]There are 5 variants of NDM (NDM1-NDM5). NDM-1 are different when compared to other MBLs, although they do have few similarities, especially to VIM-1/VIM-2 with 32.4% identity.[43] The NDM-1 has spread from India, Pakistan and Bangladesh to UK, US, France, Kenya, Japan, Canada, Belgium, the Netherlands, Taiwan, Singapore, Sultanate of Oman, and Australia. A significant risk factor identified among patients infected with NDM-1–producing bacteria has been admission to hospitals in the Indian subcontinent.[10]Various organisms that produce NDM are: *K.pneumoniae*, *K.oxytoca, E.coli, C.freundii, Morganella morganii, Providencia spp., Proteus spp*., *E.cloaceae*.[45] Most NDM-1 positive Enterobacteriaceae are detected in India, Pakistan and UK.[10]Although, now its occurrence is established world-wide.

Coexpression of other unrelated resistance genes, such as other carbapenemases (OXA-48 type, VIM type), AmpC cephalosporinases, ESBLs, resistance to aminoglycosides (16S RNA methylases), macrolides (esterases) has been

observed in most of the NDM positive isolates which poses a significant threat in clinical settings.[44]

This broad spectrum of resistance to various antibiotics, carried on plasmids is a worrisome issue in the clinical scenario especially in developing countries like India, which already has a high level of antibiotic resistance. Organisms producing NDM carbapenemase are sensitive to colistin, tigecycline and to a lesser extent to fosfomycin.[59]

4) SPM-1 (Sao Paulo MBL)

A new family of MBL with 35.5% amino acid identity to IMP-1 was first isolated in *P.aeruginosa* strain in Sao Paulo, Brazil and is associated with high mortality rates. Genetic analysis showcased that it was not part of an integron but instead was associated with a new type of transposable structure with potential recombinase and promoter sequences.[43]

5) GIM – 1 (German imipenemase)

GIM-1 was first identified in Germany in 2002 from *P. aeruginosa* isolates. It has 30%, 43%, 29% homology to VIM, IMP and SPM respectively and is found within an integron on plasmid. GIM – 1 demonstrates a hydrolytic profile similar to that of IMP – 1 but is comparatively a weaker enzyme.[43,54]

6) SIM-1 (Seoul imepenemase)

SIM-1 is the latest member of the MBL family which was isolated from Seoul, Korea. It has the closest aminoacid identity with the IMP family. It was discovered during a large scale screen of imipenem resistant *Psedomonas spp*. and *Acinetobacter spp*.[43]

7) Other MBLS

DIM-1 and AIM-1 have not been identified so far in enterobacterial strains. Recently, the beta-lactamase KHM-1 was identified in Japan from a *Citrobacter freundii* clinical isolate that had been recovered in 1997.[44]

II) Molecular Class D

Class D are OXA (for oxacillin hydrolyzing) Carbapenemases. The first OXA enzyme with carbapenemase activity was identified in an isolate of *A. baumannii* in 1985 from a patient in Scotland. This enzyme was called ARI-1(Acinetobacter resistant imipenem-1) which was later renamed OXA-23.[44]

There are more than 200 types of OXA enzymes reported till date and many of them are unable to hydrolyze the extended-spectrum cephalosporins.[51] However, a small number can confer resistance to extended-spectrum cephalosporins(OXA-163)and low levels of resistance to the carbapenems.[48] Coexpression of OXA enzymes with ESBLs and porin resistance factors confers high level resistance to carbapenems.[59] The important OXA carbapenemases in the year of discoveries, organism harbouring them, their genetic location in the bacteria and the geographical distribution has been shown in table 2.

OXA-48 is of major concern within the Enterobacteriaceae family as its hydrolytic efficiency against imipenem is approximately 10-fold higher than those of the Acinetobacter OXAs.[51,60] The first OXA-48 producer was identified from K. pneumoniae recovered in Turkey in 2003. Since then nosocomial outbreaks have been reported from Turkey and countries of Southern Europe and Africa.[44] Recently enterobacterial isolates from the Indian subcontinent were found to have a point mutant derivative of OXA 48, named OXA 181 which has identical hydrolytic property to OXA-48. OXA-48 and -181 originated from *Shewenella*, a genus lacking

clinical importance.[61] A study conducted between 2010- 2012 in Singapore showed that 24.5% of *K.pneumoniae* were OXA-181 carbapenemase producers the second most detected following NDM-1.[62]

Table 2: The important OXA carbapenemases in the year of discoveries, organisms harbouring them, the genetic location in the bacteria and the geographical distribution [51]

Enzyme group	Year	Organism	Geographic distribution	Genetic location
OXA 23/27	1985	A.baumanii, P.mirabilis	Europe, USA, Middle East	Plasmid, chromosomal
OXA 51/66/69	1993	A.baumanii	World wide	chromosomal
OXA 24/40	1997	A.baumanii	Europe, USA	Plasmid, chromosomal
OXA 48	2001	*K.pneumoniae*	Middle East, Europe, Argentina, India	Plasmid
OXA 58	2003	A.baumanii	Europe, USA, Middle East, South America	Plasmid
OXA 143	2004	A.baumanii	Brazil	Plasmid

As the above table shows, both chromosomal and plasmid located genes were found till 1997. But, 2001 onwards the genes were increasingly being carried on the plasmids.

The table 3 summarizes the important carbapenemases with regard to the hydrolytic profile, inhibitory pattern and their potential to cause outbreaks

Table 3: The classification, hydrolytic profile, inhibitory pattern of carbapenemases and outbreak potential of the strains producing them. Modified from 42,43

	Molecular class	A					B					D
	Functional group	2f					3					2df
	Enzymes	NMC	IMI	SME	**KPC**	GES	**IMP**	**VIM**	GIM	SPM	**NDM**	**OXA**
[a]Hydrolysis profile	Penicillin	+	+	+	+	+	+	+	+	+	+	+
	Early cephalosporins	+	+	+	+	+	+	+	+	+	+	+
	Extended spectrum cephalosporins	±	±	±	±	+	+	+	+	+	+	±
	Aztreonam	+	+	+	+	-	-	-	-	-	-	-
	Carbapenems	+	+	+	+	±	+	+	+	+	+	±
[b]Inhibition profile	EDTA	-	-	-	-	-	+	+	+	+	+	-
	Boronic Acid	+	+	+	+	+	-	-	-	-	-	-
	Clavulanic acid	+	+	+	+	±	-	-	-	-	-	±

[a]Hydrolysis profile: + strong hydrolysis, ± variable hydrolysis, -no hydrolysis

[b]Inhibition profile: +reported inhibition, ± variable inhibition, -no inhibition

Marked in bold indicates the potential to cause outbreaks

In summary, all the carbapenemases irrespective of the group they belong to hydrolyse penicillins and PRPs. They also hydrolyse all early cephalosporins. The extended spectrum cephalosporins are hydrolysed by enzymes in group B; the group A and D enzymes are variable in their ability to hydrolyse the extended spectrum cephalosporins. Those carbapenemases which fall in group A hydrolyse aztreonam but those that fall under group B and D do not hydrolyse aztreonam. Though carbapenemases are named because of their hydrolytic activity on carbapenems, some of the enzymes such as GES and OXA show variability even regard to the hydrolysis of carbapenems. Clavulanic acid inhibits the carbapenemases belonging to group A. Phenyl boronic acid can also inhibit these enzymes. EDTA inhibits the carbapenemases belonging to group B. Infections caused by bacteria harbouring KPC, IMP, VIM, NDM, and OXA have potential to cause outbreaks.

Risk factors for infections with carbapenemase producing organisms

Carbapenemase producing bacteria have been observed to be endemic in many areas of the world. They have produced outbreaks under certain situations. The factors which could help in establishing an infection include: [63]

1) Age
2) Prolonged ICU stay
3) Severity of underlying illness
4) Stem cell/ organ transplantation

In a case-control study conducted to evaluate the risk factors for KPC bacteremia in hospitalized patients found that advanced age, mechanical ventilation and ciprofloxacin exposure were independently associated with increased risk for KPC bacteremia.[64]

Detection of carbapenemase production

Accurate identification of Carbapenemase Producing Enterobacteriaceae (CPE) in the clinical laboratory is the first step to proper treatment and prevention of further spread. The detection of carbapenemase producers is based first on Antibiotic susceptibility testing results obtained by diffusion methods, or by automated systems (e.g.Phoenix, Vitek, Microscan).[65] However, it is important to highlight that reference MIC determination methods such as broth microdilution and agar dilution are more sensitive than the disk diffusion, the Etest (bioMerieux) or automated systems.[66] Carbapenemases display significant genetic diversity, thus their detection becomes difficult. In addition to this there are other factors which also contribute towards this problem. They include:

- CPE often coexpress genes that determine resistance to other antibiotics.
- The resistance pattern varies with individual carbapenems. Thus, an enzyme may hydrolyse ertapenem but not meropenem
- Some of the KPC harbouring organisms show MIC values not indicative of resistance when in reality they are. As a result, they might go undetected for long periods, accounting for the apparently low prevalence of CPE.[66,67]

To overcome this problem the Clinical and Laboratory Standards Institute (CLSI) in 2010, recommended lowering of the breakpoints for better detection. The previous and revised MIC breakpoints for carbapenems formulated by CLSI is represented in table 4.[48,67]

Table 4: Representation of previous and revised Clinical and Laboratory Standards Institute (CLSI) interpretive criteria for Carbapenems [68]

Previous breakpoints MIC (µg/ml)				**Revised breakpoints MIC (µg/ml)**		
Agent	**Susceptible**	**intermediate**	**Resistant**	**Susceptible**	**Intermediate**	**Resistant**
Ertapenem	≤2	4	≥8	≤0.25	0.5	≥1
Imipenem	≤4	8	≥16	≤1	2	≥4
Meropenem	≤4	8	≥16	≤1	2	≥4
Doripenem	-	-	-	≤1	2	≥4

For making a choice on the treatment, application of the CLSI breakpoints is all that is needed. The special tests are suggested for epidemiological purposes.[69]

Methods for detection of carbapenemase activity

The various methods involved in the detection of carbapenemase production has been presented in the mind map. (Fig 4)

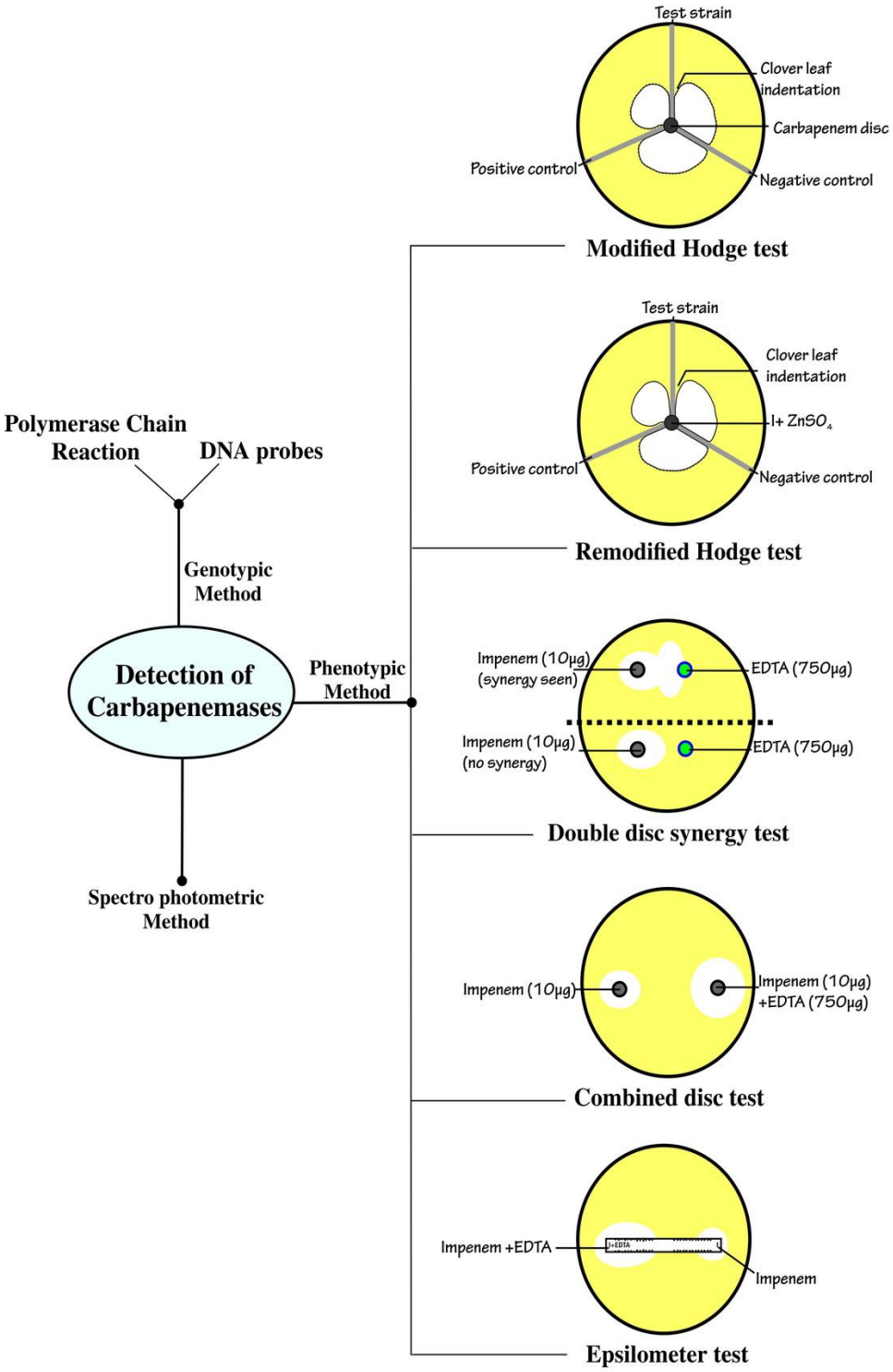


Fig 4: Methods of detection of carbapenemase production

Phenotypic tests for detection of carbapenemases

i) Modified Hodge Test (MHT)

The modified Hodge test (MHT) is a phenotypic test that has been recommended by the CLSI for the detection of carbapenemase production.[60,66] This method only detects the carbapenemase production, but does not differentiate between serine and metallo beta lactamases produced by the organisms.[42] As per CLSI recommendations, if the MHT gives a positive result, a comment on the carbapenemase production should be made in the microbiology report to inform the clinicians and infection control staff.[43] In this method multiple isolates (up to eight) can be tested on a single Mueller–Hinton agar plate. However, it lacks specificity (e.g. false positive results when ESBL or AmpC producers are associated) and sensitivity (e.g. false negative due to weak carbapenemase activity of NDM and VIM producers). This test may be useful for detecting KPC and OXA-48 type carbapenemases.[66]

In this test, 1 in 10 dilution of 0.5McFarland standard suspension of *E.coli* ATCC 25922(indicator strain) is inoculated onto Muller-Hinton Agar (MHA) and is allowed to dry for 3-5 minutes. A carbapenem disc is placed at the centre. The test strains along with positive and negative controls are streaked in a straight line from the edge of the disk to edge of the plate. The plate is incubated overnight. The presence of a distorted zone of inhibition (clover leaf shape) is interpreted as positive for carbapenemase production.[70]

ii) Re-modified Hodge test

It is based on the fact that the metallo-beta lactamases have Zn^{+} in their active sites. The catalytic activity of these enzymes is enhanced by addition of zinc. The procedure is similar to MHT. The indicator strain is inoculated onto MHA and 2 points are marked ~ 3cm apart. On one, carbapenem disc is placed and on the other a disc

containing 10 µg of 50mM of $ZnSo_4$ + Imipenem (10 µg) is placed. The test strain is streaked from the edge of the disc to edge of the plate and incubated overnight. Enhancement of growth of the indicator strain indicates presence of carbapenemases. Advantage of this test is that enzymes that have very weak carbapenemase activity (OXA-23, GES-5, GES-6) can be detected by this method.[6]

iii) Double Disc synergy test (DDST)

This test is used for the detection of MBLs. The test is based on the principle of specific inhibition of MBLs by chelating agents (ethylene-diamine-tetra-acetic acid (EDTA). These compounds deprive the MBL of zinc cations, making them unavailable to carbapenems. In DDST a carbapenem disc is placed at a distance of 20-30mm from a plain disc containing given amount of an MBL inhibitor (most commonly EDTA). Enhancement of zone around the carbapenem disc is considered positive for MBL production. As the enzyme produced by the bacteria is inactivated by chelation of the zinc ion, carbapenem is unaffected and manifests the killing of the bacteria as an enhanced zone.[60]

iv)Combined disc test (CDT)

CDT was first described by Yong et al. It is helpful in the detection of MBLs. In this method, test organisms are inoculated on to plates of MHA. Two carbapenem discs are placed on the plate and appropriate amounts of EDTA solution is added to one of them. The plate is incubated for 16 to 18 hr at 35^0C. An increase in the zone diameter around the carbapenem disc with inhibitor by $\geq$ 7mm than the carbapenem disk alone indicates it is positive for carbapenemase production. Phenotypic detection of KPC production is done in a similar manner wherein boronic acid is used. Boronic acid inhibits KPC-type betalactamases mainly in *K.pneumoniae* isolates.[71] So far,

inhibition tests are not available for the detection of OXA-48 carbapenemase producers.[66]

v) The Epsilometer test (E-test) -MBL strip method

The E test MBL strip contains a double sided seven dilution range of Imipenem (4 to256 μg/ml) and Imipenem + EDTA (1 to 64 μg/ml). A reduction in the MIC of imipenem of more than or equal to 3 dilutions in the presence of EDTA is interpreted as a positive test.[42]

vi) Rapid test using chromogenic media

Currently, there are no screening media to detect all classes of carbapenemases with high sensitivity and specificity. Commercially available chromogenic media, CHROMagar-KPC (CHROMagar; BBL) Brilliance CRE agar (Thermo Fisher Scientific) and SUPERCARBA help in the detection of carbapenemase production.[60]

Genotypic methods for detection of carbapenemases

Molecular techniques remain the gold standard for the identification of carbapenemase genes.[66] Many clinical laboratories employ PCR-based methods to overcome the problems associated with phenotypic detection methods. In addition, these methods also are able to detect OXA-48 carbapenemase, for which no specific tests are available.[60] The use of simplex, Multiplex, and real-time PCR assays targeting single and multiple carbapenemase genes, respectively have proved to be effective with excellent sensitivity and specificity.[69] The continuous globalization of carbapenemases has led to the development of a variety of other molecular methods capable of detecting all types of carbapenemases. They are DNA probing, DNA sequencing, Microarray technology etc.[59]

However, the main drawbacks of molecular-based technologies for detection of carbapenemases are their cost, the necessity of trained staff, and inability to

detect any novel carbapenemase gene. Often, these methods are beyond the reach of smaller laboratories which receive lesser financial support. Thus, there is a need for rapid, inexpensive, sensitive, and specific tests for detection of carbapenemase producing bacteria.[48]

Spectrophotometric methods

Spectrophotometric assays are reference methods that detect the hydrolytic property of carbapenemases on the carbapenems. They can be performed in presence or absence of inhibitors (EDTA). They use crude cell extracts or enzyme preparations to examine the carbapenemase activity. Measurement of hydrolysis is undertaken at 299 nm. The different carbapenemases cannot be differentiated on spectrophotometric methods.[60]

Indian scenario

Multidrug resistant gram negative bacteria producing various beta lactamases especially carbapenemases have been increasingly reported from India as in other parts of the world. There are many studies on carbapenem resistant non-fermenting gram negative bacilli (Pseudomonas, Acinetobacter) from India. However the studies on the CRE are limited. Since the emergence of NDM – 1 in India and other parts of South East Asia the scenario has changed and a number of studies are being published based on this new MBL enzyme.

The SENTRY Antimicrobial Surveillance Program conducted a surveillance in India during 2006 – 2007. An investigation into the occurrence of CRE by this group, recovered 39 carbapenem resistant isolates of which 26(67%) were carbapenemase producing isolates obtained from New Delhi, Mumbai and Pune. Of the 26 isolates 58%, 38% and 8% accounted for NDM, OXA-181 and VIM type carbapenemases respectively. This report predates the publication by Yong et al

(2009) [74] indicating the circulation of CRE strains in India, as early as 2006.[72] A study published from Delhi in 2008, among patients admitted to ICU showed that carbapenemase producing E coli and K pneumoniae accounted for an impressive 13% and 51% of infections respectively.[73] All these observations predate the discovery of NDM by Yong et al.

In 2009 Yong et al isolated a carbapenemase producing *K.pneumoniae* and showed that it possessed genes of a unique sequence. It was recovered from a Swedish patient of Indian origin who had visited New Delhi, India and acquired urinary tract infection there. This carbapenemase was named NDM-1 after New Delhi, India from where the patient had acquired the infection during his visit.[74] In the SMART (Study for Monitoring Antimicrobial Resistance Trends) conducted in 2009, found that 28% of isolates belonging to family Enterobacteriaceae had carbapenemase gene with $bla_{\text{NDM-1}}$ being present in 14%, bla_{KPC} in 9%, bla_{VIM} in 2% and $bla_{\text{OXA 48}}$ in 1% of the isolates.[75]

In 2009, Kumaraswamy et al investigated the prevalence of NDM-1 in MDR Enterobacteriaceae causing infections in patients from India, Pakistan and UK. Their study showed that there were 44(1%) isolates from Chennai, 26(13%) from Haryana, 37(1%) from UK and 73 isolates from other parts of India and Pakistan that were NDM-1 producers. They also found that 17/29 UK patients had history of travel to India and 14 being admitted in Indian hospitals. The paper had also cautioned that the treatment of hospital acquired infections caused by NDM positive organisms in India and Pakistan during cosmetic surgery would cost more money to National health service (NHS) of UK. Thus, the proposals to get cosmetic surgeries done in India to reduce expenditure by NHS was to be discouraged.[10] Following this lancet publication

which stated that the NDM gene originated in India and it was the poor antibiotic policy of the Indian hospitals that was responsible for the spread of this infection, it was not taken well by the Indian government and The Ministry of Health strongly refuted the claim of the Lancet editor.[76] The article in Journal of Associations of Physicians in India (JAPI) was partly in agreement with the view that the antibiotic policy in India needs to be supervised and monitored closely for the emergence of new multidrug resistant strains.[77] A debate with regard to the naming of the carbapenemase after New Delhi, India without any scientific data to support its origin was also made.[78]

The British Journal, Lancet had initially refused to apologize for the naming of the resistant strain as NDM after the protest from the Indian Centre for Disease Control. However, in January 2011, Richard Horton the editor apologized and was in agreement that naming the strain after New Delhi was an error that could have been avoided.[79] Many officials stated that this was a conspiracy to damage medical tourism in India.

An environmental study from Delhi by Walsh et al in 2010, showed the presence of bla- NDM -1 in samples collected from soil, sewer and drinking water. This study highlighted the wide spread distribution of NDM in the environment and the need to bring about improvements in sanitary conditions3. Subsequently there were many reports published from India with regard to the detection of NDM producing organisms.[80] In the same year Deshpande et al studied 24 carbapenemase producing Enterobacteriaceae, of which 22(91.6%) were positive for bla NDM.[81] In a study from NorthEast India (2010), a report on the incidence of NDM-1in *E.coli* isolates obtained from various samples was to the tune of 5.18%. They also found that NDM producing *E.coli* were isolated from non-ICU cases (64.28%).[82] During the

same year, a multicentric study from Chennai, Bangalore and Mumbai reported 13 of 252 isolates of Enterobacteriaceae (5.2%) were found to possess blaNDM-1 gene.[83]

A study in 2010, observed that the prevalence of MBL was 61.7% among 102 CRE isolates detected by various phenotypic methods.[6] In 2011, a study from Chandigarh found 26 isolates of 330(5.75%) CRE were MBL producers with no detection of class A carbapenemases.[84] A very recent study carried out in the ICU and wards of Sir Ganga Ram Hospital, Delhi showed overall prevalence of NDM-1 in *E.coli* isolates was 8.1%.[85] A study from Bangalore reported an incidence of 75% and 13% for blaNDM and blaVIM respectively among *K.pneumoniae* isolates. The incidence of blaNDM and blaVIM among *E.coli* isolates was found to be 66% and 13% respectively.[86]

Treatment

Treatment options are limited for infections caused by organisms producing carbapenemases. They usually show decreased susceptibility due to carriage of resistance genes to other classes of antibiotics including Fluoroquinolones, Aminoglycosides and Cotrimoxazole. However, if they are found sensitive invitro they can be used providing an option for therapy. Organisms harbouring genes encoding NDM type carbapenemase are found to be resistant to all clinically available aminoglycosides. This is due to coproduction of 16srRNA methylase.[60] In a few situations beta lactam antibiotics can be used to treat infections. They include: Aztreonam, ceftazidime and carbapenems. The sensitivity pattern depends on the type of carbapenemase produced. If the enzyme carried by the organism belongs to group B carbapenemase especially NDM, Aztreonam can be used as the organism producing this enzyme are susceptible to aztreonam.[43] Ceftazidime can still be used to treat infections caused by organisms producing OXA type carbapenemase as the resistance

to Ceftazidime is variable in these organisms producing this group of carbapenemases.[42] If there is coproduction of ESBL or AmpC beta lactamase along with carbapenemase production the above drugs will not be useful. Carbapenems may still be effective against some isolates wherein low level resistance to carbapenems is seen as evidenced by MIC.[87] Though the group A carbapenemases are susceptible to the action of clavulanic acid, combination of clavulanic acid and carbapenems are not used. No studies are available to support this. Recently it has been evidenced that this combination has been effective in treating Mycobacterium tuberculosis as studied in animal models.[88]

In the present situation it is thought that drugs useful in treating infections caused by gram negative bacteria are Colistin, Polymixin B, Tigecycline and sometimes fosfomycin.[59] Polymixins (colistin and polymixin B) are cyclic polypeptide antibiotics possessing bactericidal activity. Colistin and polymixin B were isolated from bacteria *Bacillus colistinus* and *Bacillus polymyxa*, respectively. Polymixins were discovered fifty years ago, but were rarely used because of the associated side effects such as, neurotoxicity and nephrotoxicity.[43]The emergence of MDR gram negative has led to the reintroduction of polymixins as therapeutic agents.[59]Treatment failure with colistin has been reported to occur during therapy due to development of resistance. Therefore, combination of colistin and tigecycline is required to prevent emergence of resistance.[43] Tigecycline is a tetracycline analogue that has been reported to be active against carbapenemase producing bacteria. The drug attains low levels in urine or blood therefore, is not advised for treating UTI, bacteremias, pneumonia and CNS infections. Fosfomycin was discovered in 1968. It acts by inhibiting early steps of cell wall synthesis. It is useful in the treatment of UTI. Fosfomycin can be administered both parenterally and orally. Based on the currently

available knowledge from published literature combination therapy of an aminoglycoside or colistin with a carbapenem is probably the most effective option. Furthermore, combinations of tigecycline with colistin or aminiglycosides are other therapeutic options.[59]

In order to target carbapenemase producing organisms there have been many newer drugs under development. The novel agents against CPE, their chemical structure and the enzymes they target are shown in table 5

Table 5: Representation of newer drugs for CPE, chemical structure and their target enzymes[60]

Drug	**Chemical structure**	**Target enzyme**
Penem derivatives	Heterocyclic methylidene penems	Serine beta-lactamases
1-beta-carbapenems	Methyl group at C1 of carbapenem nucleus	KPC
Sulfones	C6 substituted penicillin sulfones	KPC
Succinic acid	Succinic acid derivatives	IMP
Thiols	Thiamandelic acid	VIM, IMP
Avibactam	Diazabicyclo octanone	KPC

METHODOLOGY

Source of clinical Material

Clinical samples from patients received for culture and sensitivity at Department of Microbiology, from R L Jalappa Hospital attached to Sri Devaraj Urs Medical college, Kolar, were included in the study.

Inclusion criteria

All isolates of Enterobacteriaceae from various clinical samples such as blood, urine, pus, body fluids from patients admitted to R L Jalappa Hospital were included in the study.

Method of processing of samples

The various clinical samples received from patients were cultured and isolates were identified by standard methods. A total of 1245 organisms belonging to family Enterobacteriaceae were isolated from these samples. They were screened for possible carbapenem resistance by Kirby Bauer disc diffusion method[89] using Meropenem(10µg), Ertapenem(10µg) and Imipenem(10µg) discs (Himedia, Mumbai). A total of 175 isolates showed a zone of inhibition of ≤ 23mm for the above mentioned discs and were considered resistant to carbapenem according to CLSI guidelines, 2012.[68] To confirm carbapenem resistance the minimal inhibitory concentration for ertapenem using E-strips was determined and were subjected to Modified Hodge test for carbapenemase detection.

Among the 108 isolates showing MIC levels diagnostic of carbapenem resistance and positive by modified Hodge test, 60 isolates (*E coli, K pneumoniae* and

Enterobacter spp.) were subjected to PCR for the detection of carbapenemase genes (bla_{NDM}, bla_{VIM}, bla_{IMP} and bla_{KPC})..

Twenty faeces samples were collected from patients identified to have been infected with carbapenemase producing organisms to look for colonization. The samples were plated onto selective MacConkey agar (0.5mg/L meropenem).[80] Following identification of the isolates by standard methods and the carbapenem susceptibility noted they were checked for carbapenemase production by Modified Hodge test.

The details of the methods are described as follows:

Screening for carbapenem resistance by Antibiotic susceptibility testing (AST)

All the isolates of Enterobacteriaceae were subjected for antibiotic susceptibility testing by Kirby Bauer disc diffusion technique and looked for possible carbapenem resistance. Interpretation was done according to CLSI guidelines, 2012. In the present study susceptibility was tested against antibiotics mentioned below in table 6.

Table 6: List of antibiotics used for testing the susceptibility along with the zone diameter for isolates of Enterobacteriaceae

Sl.No	Antimicrobial agent	Disc content	Sensitive (in mm)	Resistant (in mm)
1	Ampicillin	10µg	≥17	≤13
2	Piperacillin	100 µg	≥21	≤17
3	Amoxyclav	20/10µg	≥18	≤13
4	Piperacillin-tazobactam	100/10µg	≥21	≤17
5	Ceftazidime	30µg	≥21	≤17
6	Ceftriaxzone	30µg	≥23	≤19
7	Cefotaxime	30µg	≥26	≤22
8	Cefoxitin	30µg	≥18	≤14
9	Imipenem	10µg	≥23	≤19
10	Meropenem	10µg	≥23	≤19
11	Ertapenem	10µg	≥23	≤19
12	Gentamicin	10µg	≥15	≤12
13	Tobramycin	10µg	≥15	≤12
14	Amikacin	30µg	≥17	≤14
15	Ciprofloxacin	5µg	≥21	≤15
16	Levofloxacin	5µg	≥17	≤13
17	Tetracycline	30 µg	≥15	≤11
18	Cotrimoxazole	1.25/23.75µg	≥16	≤10
19	Chloramphenicol	30 µg	≥18	≤12
	For urine isolates			
20	Nalidixic acid	30 µg	≥19	≤13
21	Nitrofurantoin	300 µg	≥17	≤14
22	Norfloxacin	10 µg	≥17	≤12
23	Ofloxacin	5 µg	≥16	≤12

Determination of Minimal inhibitory concentration (MIC) for Ertapenem using E-strip

Single colony of test strain was suspended in 2.5ml of sterile peptone water and incubated at 37^{o}C for four hours. The turbidity was adjusted to 0.5 Mac Farland units, following which it was inoculated onto Mueller – Hinton Agar. The plate was allowed to dry for 3-5 mins. An Ertapenem E-strip (BioMe´rieux India Ltd., bio-Me´rieux, Marcy l'Etoile, France) was placed at the centre of the plate. The Plate was incubated at 37^{0}C overnight and interpretation made as per CLSI guidelines, 2012. The MIC breakpoints for ertapenem have been represented in the table 7.

Table 7: MIC breakpoints of Ertapenem as per CLSI, 2012[68]

	RESISTANT	INTERMEDIATE	SENSITIVE
ERTAPENEM	≥1	0.5	≤0.25

Detection of carbapenemase production by Modified Hodge Test

ATCC *E.coli* 25922 adjusted to 0.5 Mcfarland turbidity standard was inoculated on a sterility checked Muller Hinton agar plate. An Ertapenem disc 10µg (Beckton Dickenson) was placed at the centre of the plate. The test strain along with the positive control- *Klebsiella pneumoniae* BAA-1705(Carbapenem resistant) and negative control -*Klebsiella pneumoniae* BAA-1706(Carbapenem sensitive) were streaked from the edge of the disc to the periphery of the plate. The plate was incubated at 37^{0}C overnight and interpreted as follows:

a) Distortion of the zone of inhibition at the test strain streak was interpreted as POSITIVE for carbapenemase production.

b) No distortion of the zone of inhibition at the test strain streak was interpreted as NEGATIVE for carbapenemase production.

PCR amplification of carbapenemase producing genes

A total of 60 isolates positive by Modified Hodge test were analyzed for the presence of carbapenemase producing genes i.e Molecular class A (KPC) and molecular class B (NDM, IMP, VIM) enzymes.

a) DNA extraction from bacterial strains [90]

1.A single well isolated colony was taken from fresh over night growth on Mac Conkey plate and inoculated into a tube containing 2 ml Luria Bertani (LB) broth*

2. The tubes were incubated at 37^0 C in a shaker incubator overnight (18 hrs)

3. Following incubation, 1.5 ml of turbid broth from the tube was transferred to a sterile eppendorf vial of 2 ml volume.

4. The eppendorf vial containing the broth was centrifuged at 16000g for 4 mins

5. Following centrifugation, the supernatant and pellet were obtained. The supernatant was discarded retaining the pellet

6. The pellet was washed thoroughly with 1ml molecular grade water

7. The vial containing the mixture was vortexed until the pellet completely dissolved

8. The steps 4,5,6,7 were repeated to obtain the mixture

9. The mixture was again centrifuged at 16000g for 4 mins and the supernatant discarded retaining the pellet

10. Lysis buffer of 50µl volume was added to the pellet and vortexed till the pellet completely dissolved

11. The vial containing the mixture was kept at room temperature for 5 mins

12. Following this the tubes were kept in a water bath maintaining temperature of 95^0 C for 15 mins

13. The tubes were taken out from the waterbath and allowed to cool

14. The vials were centrifuged at 16000g for 4 mins

The clear supernatant (containing the DNA) obtained was transferred into a sterile, labeled eppendorf vial and were stored at -20^0 C. The supernatant was the template DNA used for the detection of carbapenemase genes.

*** Luria Bertnani broth**

Luria Bertnani broth is used for the cultivation of organisms. It is a commercially available media in premixed form.

Composition:

Tryptone- 10 g

Yeast extract- 5g

Sodium chloride- 10g

Final pH 7±0.2

Preparation of LB broth: for 100 ml

About 2.5 g of LB broth powder was weighed on an electronic weighing machine. The measured amount was transferred to a sterile flask containing 100 ml of sterile distilled water. The above mixture in the conical flask was sterilized at 121^0C for 15

min in an autoclave. The broth was dispensed into sterile test tubes and was kept in the incubator at 37^0C for 24 hrs for sterility check. The test tubes were stored in refrigerator till use.

b) Multiplex PCR for detection of carbapenemase genes

Simultaneous detection of bla_{NDM}, bla_{IMP}, bla_{VIM} and bla_{KPC} carbapenemase genes was carried out on a 96-well thermal cycler instrument. Sequence primers and amplicon sizes are shown in table 8. Known positive controls (NDM, VIM) were run together to know the specificity of PCR. Five microliters of extracted total DNA was subjected to multiplex PCR in a 30 µl reaction mixture. The PCR mixture for the detection of genes contained 1XPCR buffer (10mmol/L Tris-HCL[pH 8.3], 50mmol/L KCl), 1.5mmol/L of $MgCl_2$, 1.5 mM of dNTPs, 10 µM of each primer and 1.5 U of Taq DNA polymerase (GeNei, Bangalore). The PCR program consisted of an initial denaturation at 94^0 C for 5min, followed by 35 cycles of DNA denaturation at 94^0 C for 30s, primer annealing at 55^0C for 45s, and primer extension at 72^0 C for 1 min, followed by a final extension at 72^0C for 5 min. PCR products were analyzed by electrophoresis in a 1.5% agarose gel at 100 V in 1X TAE(40nmol/L Tris-HCL[pH 8.3], 2 mmol/L acetate, 1mmol/L EDTA) containing 0.05mg/L ethidium bromide.

Table 8: Sequence primers used for detection of carbapenemase producing genes.[91]

Gene	Primer	Sequence (5'-3')[c]	Product size (bp)[b]
bla_{IMP}	IMP- F IMP- R	GGAATAGAGTGGCTTAAYTCTC GGTTTAAYAAAACAACCACC	232
bla_{VIM}	VIM- F VIM- R	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	390
bla_{NDM}	NDM- F NDM- R	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	621
bla_{KPC}	KPC- Fm KPC- Rm	CGTCTAGTTCTGCTGTCTTG CTTGTCATCCTTGTTAGGCG	798

[a] F- forward primer; R- reverse primer

[b] Nucleotide numbering begins at the initiation codon of genes

[c] Y= C or T

Meropenem Selective MacConkey agar[80]

This was prepared by adding the meropenem antibiotic at a concentration of 0.5mg/L of MacConkey agar. To achieve this concentration, meropenem stock solution was prepared and then the required amount was added to MacConkey agar.

Ingredients: for 1 L

Anhydrous MacConkey agar- 51.53g

Meropenem stock solution- 1 ml (0.5mg/L)

Preparation of meropenem stock solution

The requirement was 0.5mg/L of MacConkey agar. Meropenem antibiotic was obtained from Biocon Company as PenmerTM 500, which contained 500 mg of

anhydrous meropenem. According to the instruction, it was reconstituted in 10 ml of distilled water. Once it was reconstituted, each ml solution contains 50 mg of meropenem (50 mg/ml). To achieve our requirement of 0.5mg/L, 1ml of the reconstituted mixture was in turn diluted in 99 ml of distilled water which accounts to a meropenem concentration of 0.5mg in 1 ml.

Preparation of selective MacConkey agar

Using an electronic weighing machine, 51.53g of anhydrous MacConkey agar was weighed. It was transferred into a sterile conical flask containing 1 litre of distilled water. To the above mixture 1 ml of meropenem stock solution was added. The above mixture in the conical flask was sterilized at 121^0 C for 15 min in an autoclave. The prepared media was then poured into petri plates of 110mm size. It was allowed to solidify. The plates were kept in an incubator at 37^0 C for sterility check. Following this the plates were stored in refrigerator until use.

RESULTS

Between January 2012- May 2013 (1year 6 months) 1,245 organisms belonging to family Enterobacteriaceae were isolated from different clinical samples. Among them 175(14.05%) of the isolates were found to be resistant to any one of the carbapenems and 1070(86%) were sensitive to carbapenems. Resistance to ertapenem, imipenem and meropenem was 64%, 68%, and 89%, respectively (Fig 5). The MIC values for ertapenem varied widely among the isolates of Enterobacteriaceae. The resistant range of MIC values for ertapenem was from 1.5->32µg/ml (Table 9, Fig 6).

Table 9: MIC levels against Ertapenem of different organisms of Enterobacteriaceae which were found to be positive in screening test.

Ertapenem MIC range						
	Sensitive	**Intermediate**	**Resistant**			
Organisms	≤0.25µg/ml	0.5-0.95µg/ml	1-1.5 µg/ml	2-6 µg/ml	8-24 µg/ml	≥32 µg/ml
K. pneumoniae	11	5	2	21	13	5
E.coli	26	9	3	27	9	2
Enterobacter spp.	8	5	1	12	2	3
K.oxytoca	-	1	-	3	-	-
Proteae group	-	-	1	1	2	-
Citrobacter spp	-	-	-	1	1	-
TOTAL	46	20	7	65	27	10

All those found to be resistant to any one of the carbapenems were subjected to Modified Hodge test (Fig 7). Modified Hodge test could confirm 108 (61.71%)

isolates to be positive for carbapenemase production and 67(38.28%) were negative. (Graph I) The ratio of MHT positive to MHT negative is 1.7:1.4. Thus, taking positivity in Modified Hodge test as the criteria the prevalence of carbapenemase producing Enterobacteriaceae was found to be 8.67% among the clinical isolates. This is represented in table 10.

Graph I: Results of Modified Hodge test done on carbapenem resistant organisms detected by screening

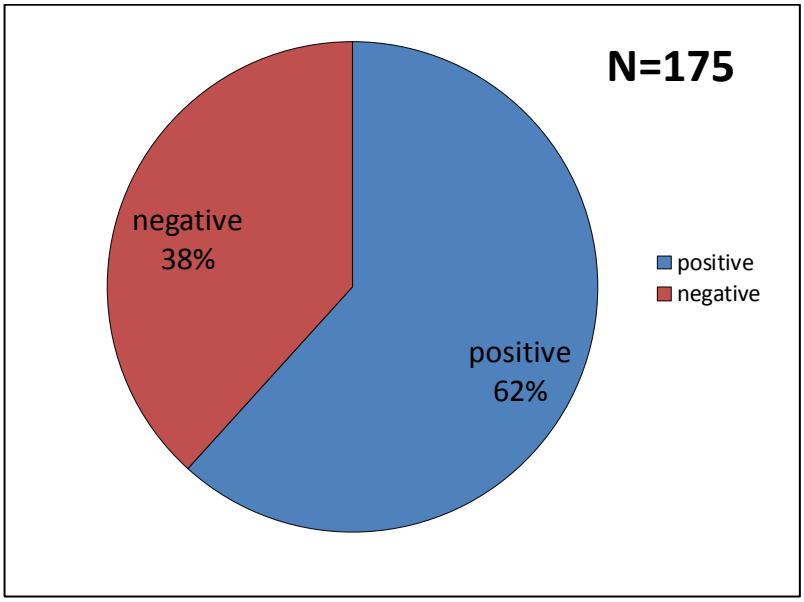


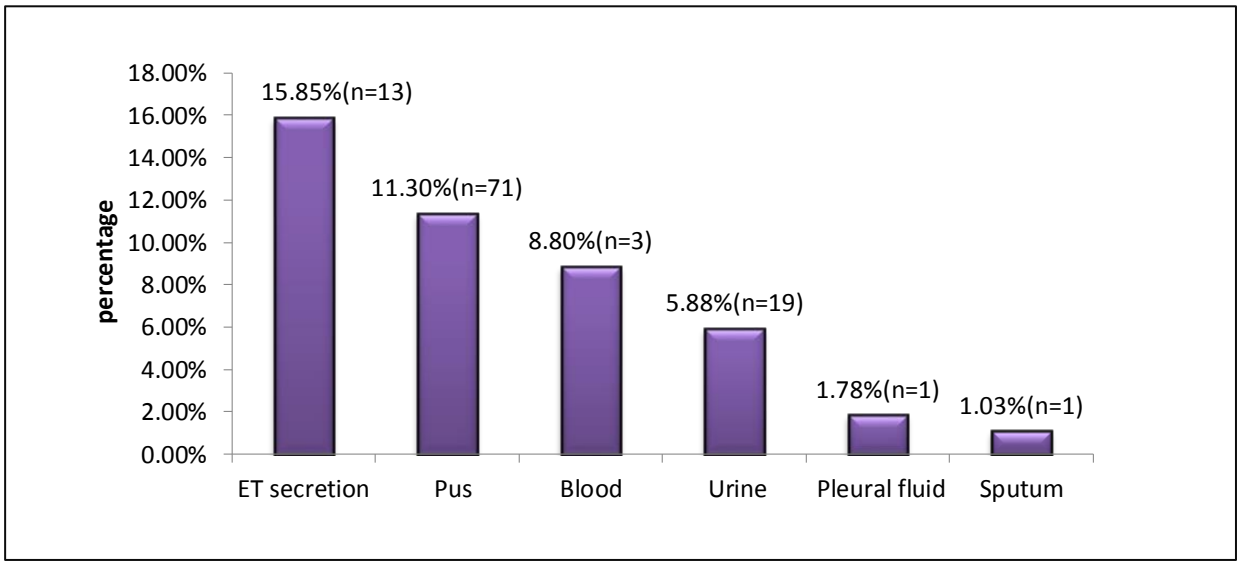
Table 10: Results of screening & confirmatory tests for carbapenemase production

No. of isolates belonging to Enterobacteriaceae screened	No. positive in Screening test n (%)	Bacteria confirmed as carbapenemase producing among screening test positives	Bacteria confirmed as carbapenemase producing among all those tested
1,245	175 (14.05%)	108/175 (61.71%)	108/1,245 (8.67%)

Table 11: Isolates from the various clinical samples screened and those confirmed as producing carbapenemase

Clinical samples	No of isolates screened	No. of isolates positive on screening	No of isolates confirmed n (% positive)
1) Pus	628	109	71(11.30%)
2) Urine	323	40	19(5.88%)
3) Sputum	97	5	1(1.03%)
4) ET secretion	82	13	13 (15.85%)
5) Pleural fluid	56	1	1 (1.78%)
6) Blood	34	4	3 (8.8%)
7) Miscellaneous	25	3	0
TOTAL	**1245**	**175**	**108**

Graph II: Sample wise distribution of carbapenemase producing isolates of Enterobacteriaceae



The isolation of carbapenemase producing organisms from different specimens is presented in table 10. The highest percentage of carbapenemase producing isolates were obtained from endotracheal secretions (15.85%). Carbapenemase producing organisms were also isolated from pus and urine samples: 11.46% and 5.88%

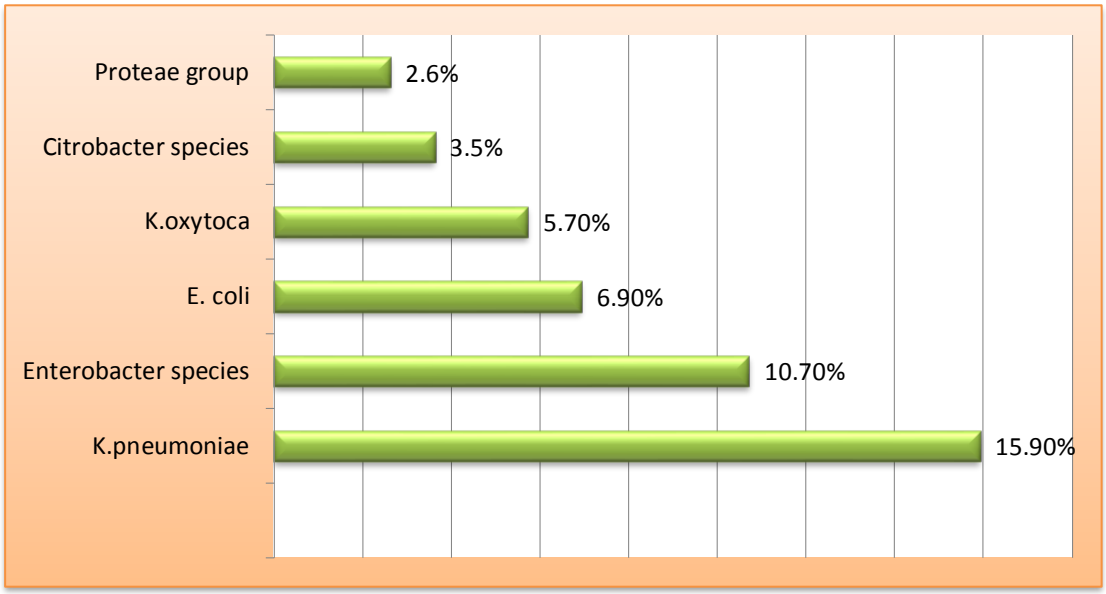
respectively. There were 3(8.8%) carbapenemase producing strains of the 34 isolates from blood. Among 71 pus samples, in 11 of the samples more than one organism was isolated.

The various species of Enterobacteriaceae isolates producing carbapenemase are represented in table 12 and Graph III.

Table 12: Carbapenemase producing strains among carbapenem resistant isolates of Enterobacteriaceae

Isolates	**Total no screened**	**screened positives**	**Confirmed positives among screening test positives n (%)**	**Confirmed positives among all those tested**
E. coli	596	76	41/76 (53.94%)	41/596 (6.87%)
K.pneumoniae	258	57	41/57 (71.92%)	41/258 (15.89%)
Enterobacter species	168	31	18/31 (58.06%)	18/168 (10.7%)
Proteae group	115	4	3/4 (75%)	3/115 (2.6%)
Citrobacter species	56	3	2/3 (66%)	2/56 (3.5%)
K.oxytoca	52	4	3/4 (75%)	3/52 (5.7%)
TOTAL	**1,245**	**175**	**108/175 (61.71%)**	**108/1,245 (8.67%)**

Graph III: Carbapenemase producing strains among carbapenem resistant isolates of Enterobacteriaceae



K pneumoniae ranked highest (15.89%) among the carbapenemase producing isolates and the distribution was highly significant ($p < 0.001$). It was followed by *Enterobacter spp. (10.7%)* and *E.coli (6.87%),* among the species of Enterobacteriaceae producing carbapenemase. The percentage was comparatively lower in *Proteae group* & Citrobacter spp. Among the *Proteae group*, one each of *Proteus mirabilis*, *Providencia rettigeri* and *Morganella morganii* were positive for carbapenemase production. Though, number are fewer in the latter group data shows that carbapenemase production is encountered and consequent resistnace to the drug are also manifested by these species of *Proteus* and *Citrobacter*.

Table 13: Ward wise distribution of carbapenemase producing isolates of Enterobacteriaceae

Isolate/ Ward	ICU	Surgery	Ortho	OBG	NS**	US***	Paed	Med*	Burns ward
K pneumoniae	14	13	3	4	3	2	1	-	1
E. coli	10	10	7	5	1	3	1	3	1
***Enterobacter* spp**	6	4	3	3	2	-	-	-	-
***Proteae* group**	1	1	-	-	-	-	-	-	1
***Citrobacter* spp**	1	-	-	-	1	-	-	-	-
K oxytoca	1	2	-	-	-	-	-	-	-
TOTAL	33 (31%)	30 (28%)	13 (12%)	12 (11%)	7 (6.5%)	5 (4.6%)	2 (1.85%)	3 (2.7%)	3 (2.7%)

*Med-medicine ward, **NS-neurosurgery ward, ***US-urosurgery ward

Majority of the carbapenemase producing organisms were recovered from cases admitted at ICU (31%) followed by general surgery wards (28%). The most commonly isolated species were *K pneumoniae* and *E coli* from all the wards. All the carbapenemase producing organisms were detected from ICU and general surgical wards, whereas in other wards only *E.coli, K.pneumoniae* and *Enterobacter spp* were found. In the medical wards it was comparatively lower. (Graph IV)

Graph IV: Ward wise distribution of carbapenemase producing isolates of Enterobacteriaceae

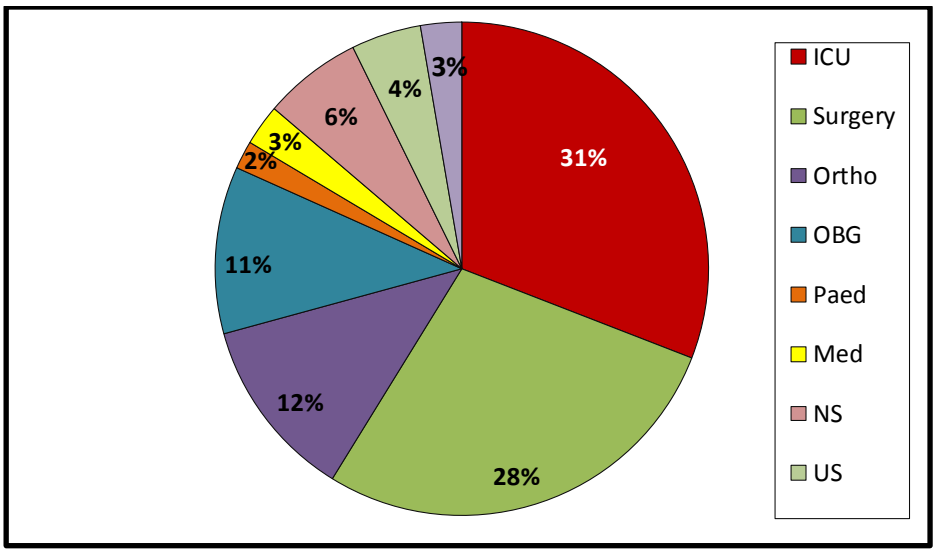
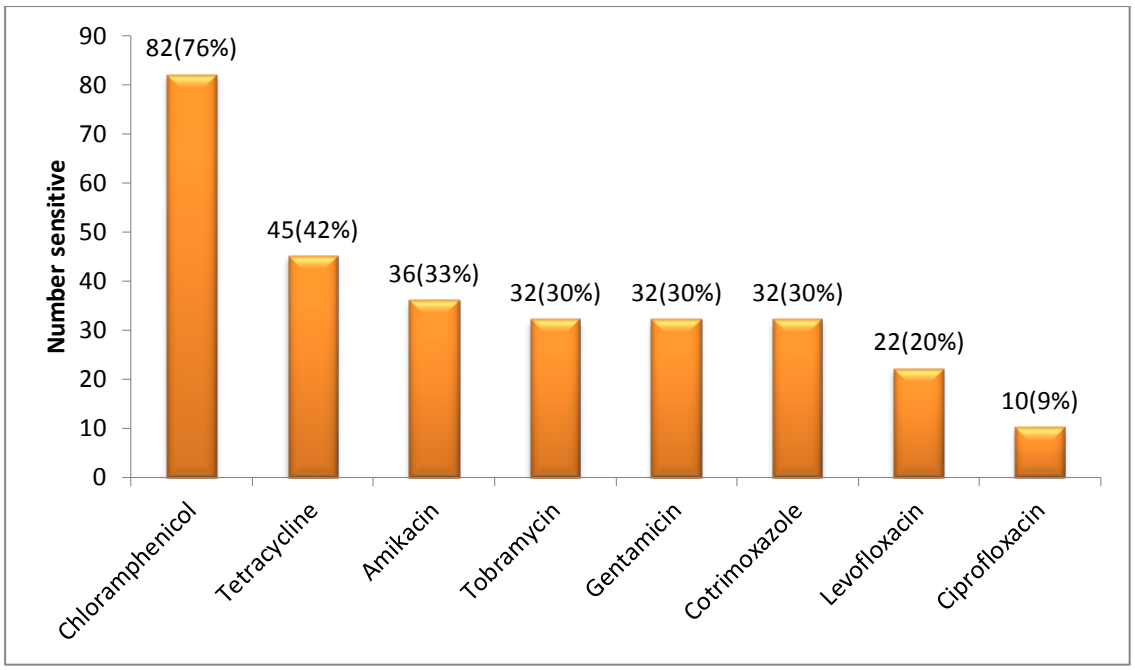


Table 14: Antibiotic sensitivity pattern of carbapenemase producing isolates (N=108)

Antibiotics	Number sensitive
Chloramphenicol	82 (76%)
Tetracycline	45 (42%)
Tobramycin	32 (30%)
Gentamicin	32 (30%)
Amikacin	36 (33%)
Cotrimoxazole	32 (30%)
Levofloxacin	22 (20%)
Ciprofloxacin	10 (9%)

Graph V: Antibiotic sensitivity pattern of carbapenemase producing strains



All the 175 isolates were found to be resistant to ampicillin, piperacillin-tazobactam, and 3rd generation cephalosporins as expected. Among 108 carbapenemase producing Enterobacteriaceae majority of them were found to be sensitive to Chloramphenicol(76%) followed by Tetracycline(42%). The sensitivity to Cotrimoxazole and Aminoglycosides varied from 30%-32%. Resistance to Fluoroquinolones was comparatively higher.

Among the 40 urine samples which yielded carbapenemase producing organisms, 48% of the isolates were found to be sensitive to Nitrofurantoin, 38% sensitive to Nalidixic acid and 35% sensitive each to Norfloxacin and Ofloxacin.

Table 15: Carbapenemase producing genes carried by the isolates of Enterobacteriaceae found to be positive by MHT

Organisms	Only NDM	Only IMP	NDM+IMP	NDM+VIM	NDM+IMP+VIM
K.pneumoniae(N=30)	26	-	1	1	-
E.coli (N=23)	7	2	9	2	2
Enterobacter spp.(N=7)	7	-	-	-	-
TOTAL	**40**	**2**	**10**	**3**	**2**

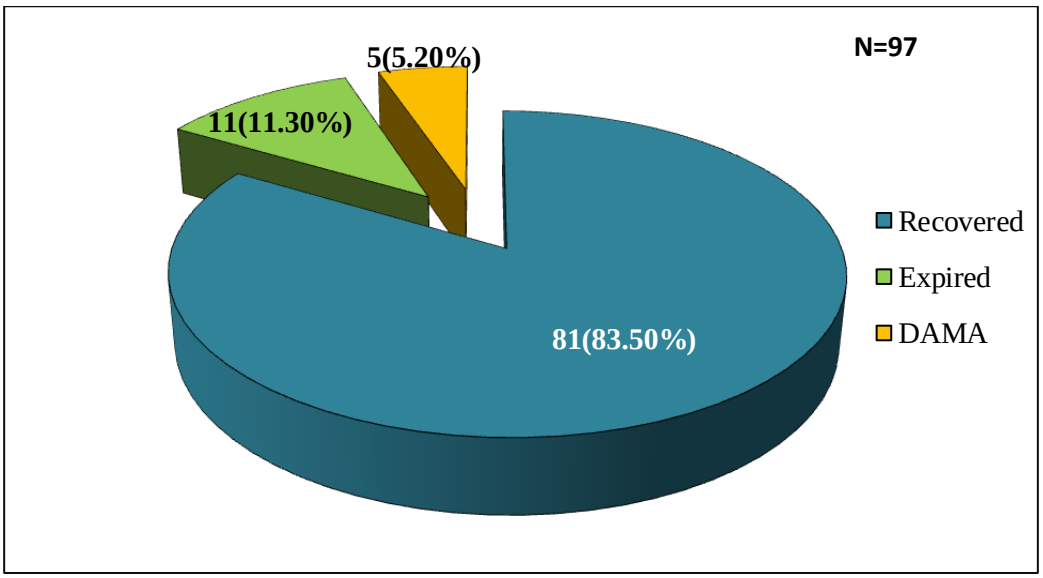
Sixty of 108 isolates were subjected to multiplex PCR. The isolates subjected to this analysis belonged to the major carbapenemase producing species: *K.pneumoniae*, *E.coli* and *Enterobacter* spp. Among them 30 were *K.pneumoniae*, 23 were *E.coli* and 7 were *Enterobacter* spp. The molecular analysis was done for these isolates by PCR technique for the detection of carbapenemase producing genes (blaNDM, blaIMP, blaVIM, blaKPC). The carbapenemase producing genes carried by the isolates is presented in table VII. Among the 60 isolates, 57(95%) were found to be harbouring one or more of carbapenemase producing genes. Fifty five of carbapenemase producing strains carried blaNDM, among which 10 isolates also harboured blaIMP, 3 harboured blaVIM and 2 isolates were positive for NDM, IMP and VIM (Fig 8). Additionally, blaVIM gene was detected in two strains of *E.coli*. There were 3 isolates that tested positive by Modified Hodge test and were negative by PCR. All of them were *E.coli*. BlaKPC was not found in any of the isolates. We could not look for blaOXA.

Table 16: The outcome in patients infected with carbapenemase producing isolates of Enterobacteriaceae

Outcome	Number
Recovered	81(83.5%)
Expired	11(11.3%)
DAMA*	5(5.2%)
TOTAL	**97**

*DAMA- Discharge against medical advice

Graph VI: The outcome in patients infected with carbapenemase producing organisms



Ninety seven patients were infected with carbapenemase producing strains. Among them 81(83.5%) patients recovered following treatment with antibiotics according to the sensitivity pattern or local dressing in cases of wound infections (Fig 9), 11(11.3%) patients expired and 5(5.2%) patients went against medical advice.

Table 17: Clinical characteristics of 11 patients infected with carbapenemase producing Enterobacteriaceae who expired during the study period

Patient/Age (Yrs)/ Sex	Ward	sample	Diagnosis	Comorbidities	organism	MIC (µg/ml)	Carbapenemase gene present
S/25/M	Surg	ET secretion	SDH*	Increased intracranial pressure	*K pneu*	24	NDM, IMP
S/30/M	ICU	sputum	Head injury	Increased intracranial pressure	*K pneu*	>32	NDM
V/37/M	Surg	pus	Diabetic foot	Diabetes mellitus	*K pneu*	8	NDM
N/50/M	ICU	ET secretion	Head injury	Increased intracranial pressure	*K pneu*	24	Not done
L/50/F	ICU	ET secretion	Asthma	Asthma	*Enterobacter*	24	Not done
C/50/M	ICU	pus	Psoas abscess	-	*E coli*	32	NDM, IMP
S/50/M	ICU	ET secretion	SAH**	Increased intraventricular pressure	*Enterobacter*	>32	Not done
S/53/M	ICU	ET secretion	B/l pneumonia	-	*K pneu*	32	NDM
B/62/M	ICU	ET secretion	Head injury	Intracranial haemorrhage	*K pneu*	24	NDM
K/70/M	ICU	pus	sepsis	Advanced age	*E coli*	32	NDM, IMP
N/70/M	ICU	pus	SAH	Increased intraventricular pressure	*E coli*	18	NDM

*SDH-sub dural haemorrhage, SAH**-sub arachnoid haemorrhage

Majority of the patients who died were males and above 50 years of age (73%). *K.pneumoniae* was the most common isolate obtained from endotracheal secretions of patients admitted in ICU. Among the 8 tested for the presence of carbapenemase genes, all of them possessed blaNDM and in 3 of the isolates additional blaIMP was also detected. These patients had associated comorbidities

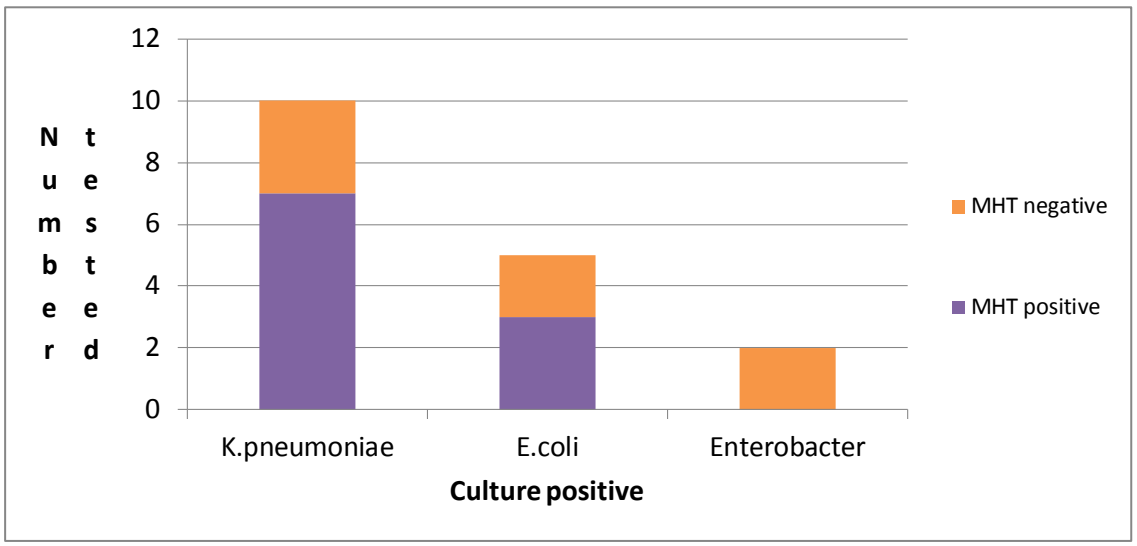
such as raised intracranial pressure, intracranial haemorrhage, advanced age and diabetes mellitus, which could have been the cause of the death.

Isolation of carbapenem resistant strains from faeces samples of 20 patients infected with carbapenemase producing Enterobacteriaceae is presented in table 17.

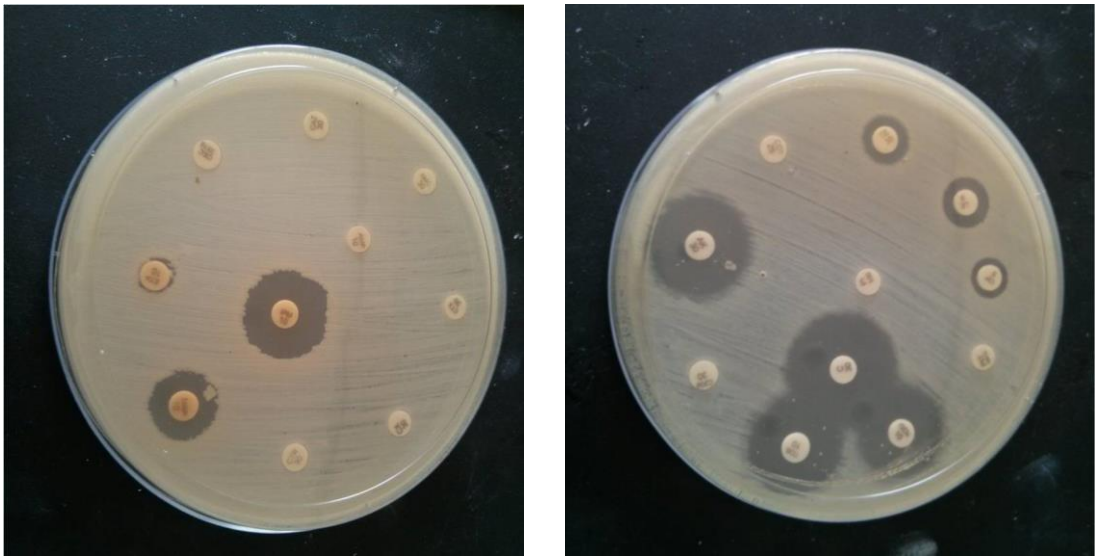
Table 18: Isolation of carbapenem resistant organisms from faeces samples of patients infected with carbapenemase producing strains.

Organisms	**No. which showed growth and was MHT positive**	**No. which showed growth but MHT negative**
K.pneumoniae (n=10)	7	3
E.coli (n=5)	3	2
Enterobacter (n=2)	-	2
TOTAL (n=17)	**10 (59%)**	**7 (41%)**

Graph VII: The MHT results of the carbapenem resistant isolates from faeces samples (N=20)



Of the 20 faeces samples collected, 17(85%) yielded growth of carbapenem resistant organisms and remaining 3 showed no growth on selective MacConkey agar. Among them 10 were *K.pneumoniae*, 5 were *E.coli* and 2 were *Enterobacter* spp. Among these 17 isolates, 10 (59%) were positive by modified Hodge test and 7 (41%) of them were found to be negative by modified Hodge test.



A **B**

Fig 5: Antibiotic susceptibility testing for various antibiotics

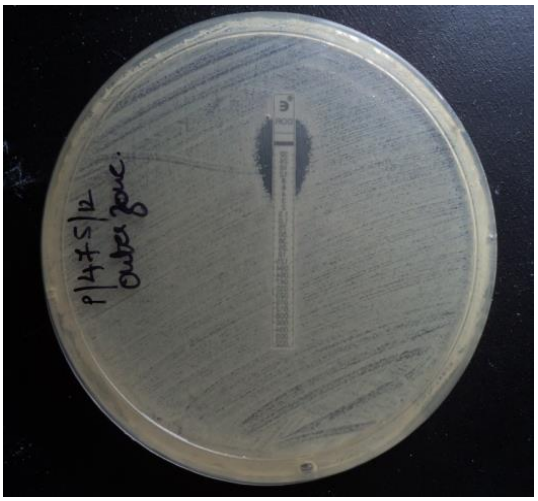


Fig 6: MIC determination using Ertapenem E test strip

Fig 7: Modified Hodge Test

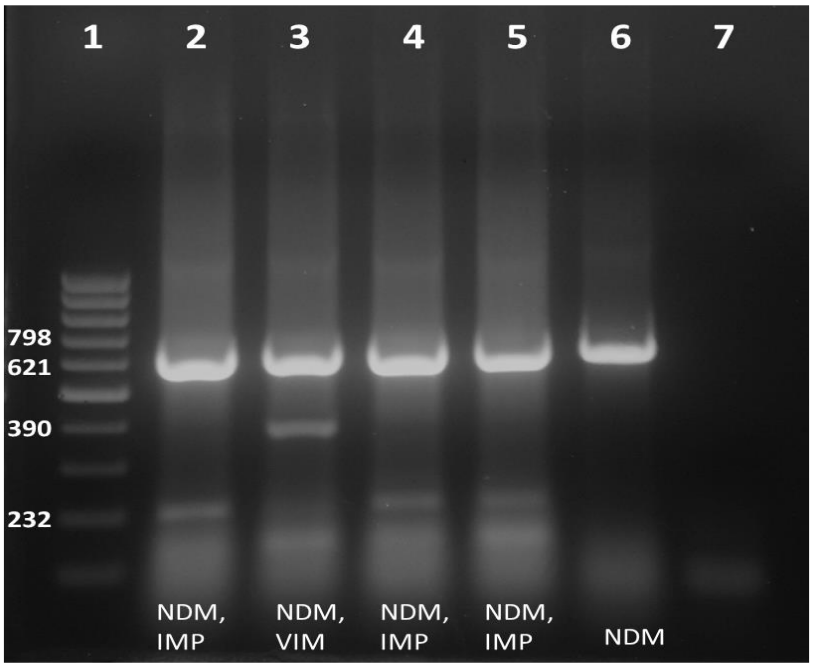


Fig 8 a: Results of multiplex PCR detecting blaNDM, blaIMP, blaVIM and blaKPC. The size of each amplicon is indicated on the left.Lane 1: Generuler 1kb plus DNA ladder. Lane 2,4,5 showing bands for blaNDM and blaIMP. Lane 3 showing bands for blaNDM and blaVIM

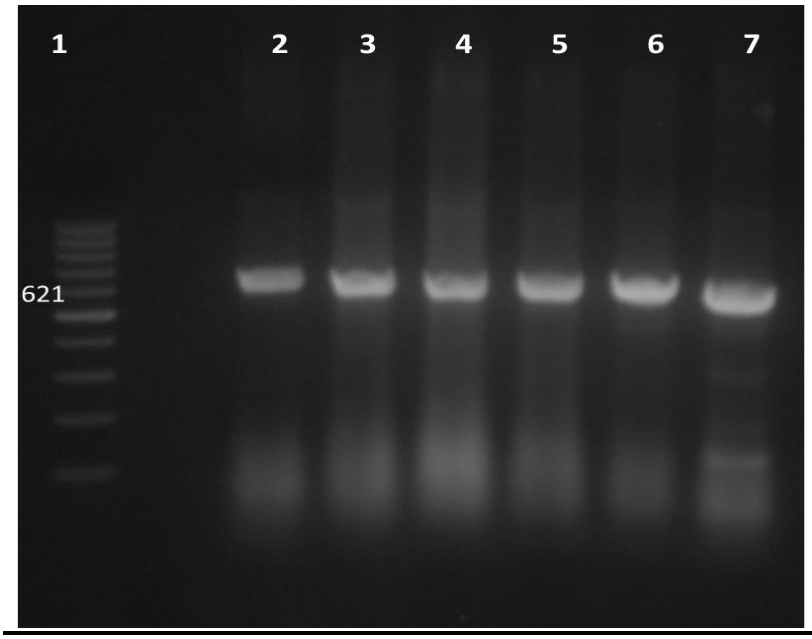
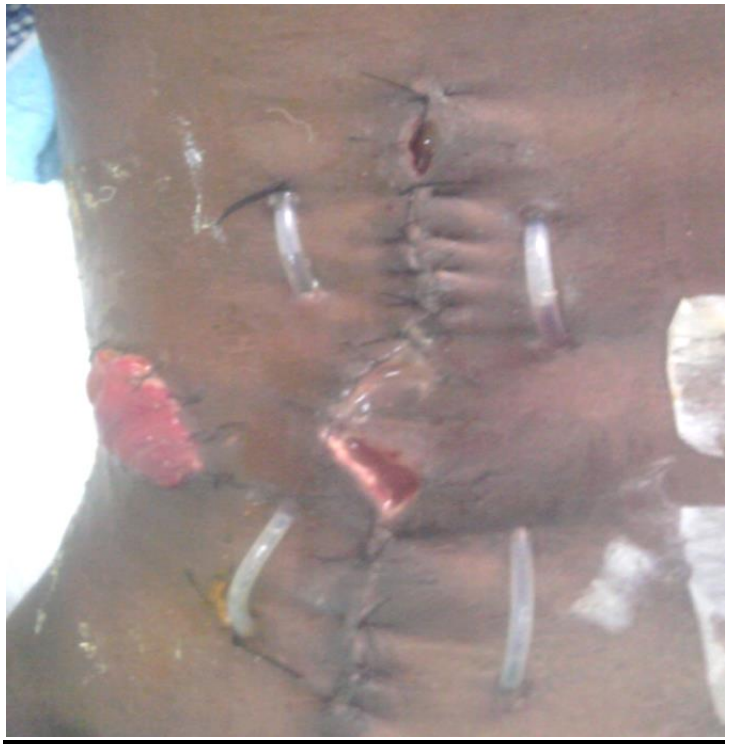


Fig 8 b: Results of multiplex PCR. Lane 1: DNA ladder, Lanes 2-7 showing bands only for blaNDM. Amplcon size indicated on the left



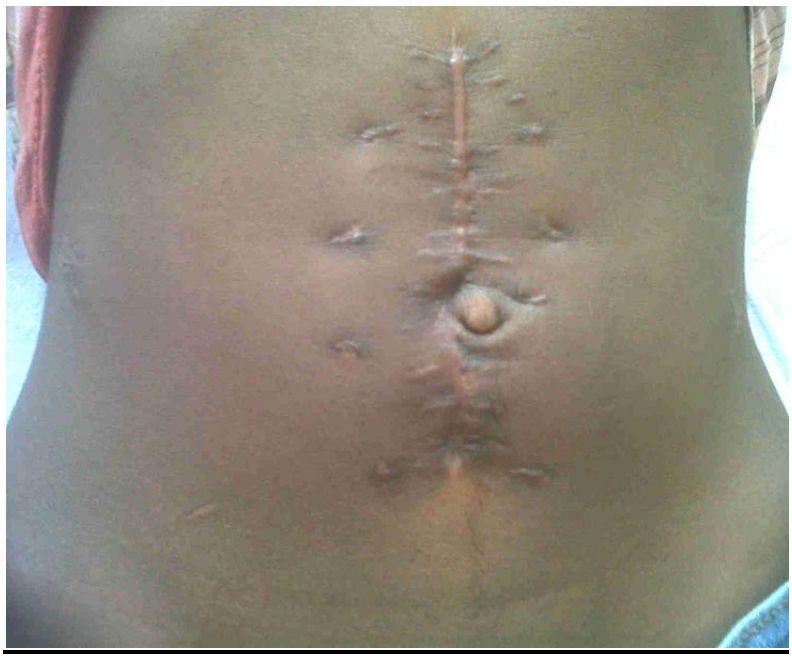


Fig 9: A post operative wound infection caused by carbapenemase producing *E.coli* before (above) and after treatment

DISCUSSION

The most common cause of bacterial resistance to betalactam antibiotics is the production of beta lactamases. Bacteria produce a variety of beta lactamases, because of this protective repertoire of genes which code for these enzymes there is a constant addition to the list of available cell wall acting antibiotics. The bacteria respond to antibiotics by developing resistance against them. The most recent drugs discovered in the series of antibiotics are carbapenems.[2] Bacteria have manifested resistance to them by multiple mechanisms. The most common mechanism being production of hydrolytic enzymes: carbapenemases. Carbapenemases represent a major group of beta lactamases currently being identified among resistant bacteria worldwide in large numbers.[5] In recent years, genes that code for carbapenemase enzymes have spread from *P.aeruginosa* to members of Enterobacteriaceae.[8] These enzymes are plasmid mediated and multidrug resistance is a characteristic feature of strains producing these enzymes.[6]

The overall prevalence of carbapenemase producing Enterobacteriaceae is found to vary from one geographical area to another and from one institute to another. We found that the prevalence of carbapenem resistant Enterobacteriaceae in patients with enterobacterial infections was 14.5%. This observed resistance to carbapenems is low when we compare our data with that reported from Delhi published in 2010, wherein the prevalence of carbapenem resistant Enterobacteriaceae among cases admitted to ICU was 13% and 51% in *E.coli* and *K.pneumoniae*, respectively.[73] Another study from Delhi, similarly reported high prevalence of carbapenem resistance among members of Enterobacteriaceae which varied from 17% to 22%.[92] Our data contrasts to the report from a multicentric study conducted in India in which the carbapenem resistance among members of Enterobacteriaceae was 2.7%.[72] The

moderately high carbapenem resistance in our institute may be partly due to the increased scrutiny of carbapenem resistant strains for carbapenemase production by Modified Hodge Test.

In our study the screening test was done using ertapenem, imipenem and meropenem and the resistance observed was 64%, 68%, and 89% respectively. The resistance rate of meropenem was much higher indicating that meropenem was better suited for detection of carbapenem resistance. This observation was in contrast to a study conducted in china which reported ertapenem to be a better indicator for screening of Carbapenem resistant Enterobacteriaceae species.[93] There was 100% correlation between those that were resistant to ertapenem by disc diffusion and MIC levels. The range of MIC values of ertapenem for 108 isolates of Enterobacteriaceae was from 1.5->32µg/ml, which is similar to a multicentric study who referred to about the same MIC.[72] This wide variation observed in MIC of ertapenem could be due to the type and expression of carbapenemase enzyme, the presence of other resistance mechanisms such as ESBL's and AmpC beta lactamases, reduced permeability and/or efflux pumps.[82]

Among the 175 found to be resistant to carbapenems, the Modified Hodge test was able to detect 61.7% (108/175) of screened positive isolates of Enterobacteriaceae as producing carbapenemase. Although, 38.28% were found to be carbapenem resistant they were negative for Modified Hodge test. The reason for this discrepancy could be due to multiple mechanisms such as mutation in the porin proteins, loss of porin expression, hyperproduction of ESBL or AmpC [61] and increased efflux systems.[42] Thus, it becomes important to study these resistance mechanisms in addition to detection of carbapenemase production. In concordance with our study SENTRY Antimicrobial Surveillance Program conducted in India, showed that

Modified Hodge test was able to detect 66.7% of carbapenemase producing isolates among the carbapenem resistant organisms.[72] Modified Hodge test is the only phenotypic method that has been recommended by CLSI guidelines for detection of carbapenemase production, its sensitivity varies from as high as 90%[rai]to as low as 50%.[94] The modified Hodge test, a simple method for detection of carbapenemase production, however acts as an useful indicator in monitoring the hospital infection control activities to limit the spread of carbapenemase producing organisms. The MIC levels of ertapenem correlated well with the results of MHT, in contrast to a report by Chande C et al who observed that 3 of 50 carbapenem resistant strains which showed MIC levels diagnostic of resistance were found to be MHT negative. This could be attributed to the other mechanisms of carbapenem resistance.[95]

The prevalence of carbapenemase producing Enterobacteriaceae in our health care set up is 8.67% as detected by Modified Hodge test in contrast to a report from East Delhi, India wherein the prevalence was 6.2% in members of Enterobacteriaceae.

In the present study, among 108 carbapenemase producing isolates we observed that the most common carbapenemase producing isolate was *Klebsiella pneumoniae* (71.92%). This observation has been similarly reported in other studies from different countries as shown in table 19.

Table 19: Percentage of *K.pneumoniae* producing carbapenemase reported from various studies from different countries

Sr. No.	Study series	Percentage	Country
1.	Kumaraswamy et al[10]	61	India, Pakistan & UK
2.	Deshpande et al [81]	45.5	India
3.	Castanheira et al [72]	61	India
4.	Vaux S et al [96]	62	France
5.	Xia Y et al [93]	22	China

High incidence of resistance in *K. pneumoniae* is probably because its mechanism of resistance is plasmid mediated. *Klebsiella pneumoniae* is a common nosocomial pathogen. The various exogenous sources of *K. pneumoniae* are hands of hospital staff, contaminated medical equipment while endogenous source is the gastrointestinal tract of patients. It had been reported earlier to have been transmitted from patient to patient through health care givers. There are also reports on isolation of *K. pneumoniae* from endotracheal tube connectors, blood pressure cuffs.[97] In 2009, Walsh et al conducted a study in India which showed that *K. pneumoniae* was isolated from various environmental sources such as sewer, soil and drinking water.

In our study, majority of the carbapenemase producing isolates of family Enterobacteriaceae were isolated from endotracheal secretion 13(15.85%) followed by pus 71(11.30%) and urine 19(5.88%). A similar observation was made in a study conducted at Bangalore in St Johns medical college, which reported an isolation rate of 16% from respiratory secretions but the highest isolation being from urine samples (42%)[86] in our study urine isolation was low. Similarly in a study from Mumbai reported highest isolation of carbapenemase producing organisms from urine samples (45%).[81] The carbapenemase producing organisms have been found to cause infections in the ICU, this is also true with data from our institution. This may account for the highest isolation from ET secretion. The CDC criteria to diagnose ventilator associated pneumonia (VAP), however requires 100,000 colony forming units (CFU/ml) along with a demonstrable radiological evidence in the lung.[98] In many of the patients from whose ET secretion carbapenemase producing organisms were isolated they could be colonizers rather than pathogens. However, they have to be confirmed by quantitative estimations and other supportive criteria. The existence of

the organisms in the ICU settings though could not be done in our study is worth noting.

In the present study 31% of carbapenemase producing isolates of Enterobacteriaceae were recovered from cases admitted in ICU, followed by 28% from surgical wards. This observation was in accordance with Gaibani P et al which also reported 31% isolation from patients admitted in ICU.[8] However, a study by Deshpande et al reported a comparatively higher isolation rate of 63% of carbapenemase producing Enterobacteriaceae from cases in ICU.[81] Isolation of more number of carbapenemase producing isolates from ICU could be due to the different use of invasive devices (intubation tubes, surgical drains, intravascular devices) and also patients with comorbidities are admitted in ICU worsening the situation.[8] The aggressive use of antibiotics in ICU also could be a selective factor for colonization of carbapenemase producing organisms. The equipments also are prone to get colonized. Prevention of hospital associated infections is the key.[97] In our study more than half the infections were from ICU and surgical wards, therefore prevention has to be concentrated in these areas.

The isolates were tested for susceptibility to various other drugs. It was observed that there was total resistance (100%) to Ampicillin, Piperacillin-tazobactam, and 3rd generation Cephalosporins which is similar to the observations made in other studies.[82,83,99,100]In our study 76% of isolates were found to be sensitive to Chloramphenicol. This could be because it is seldom used as a first line antibiotic due to its serious side effects. Chloramphenicol can be used as an alternative drug if found to be sensitive on susceptibility testing. A quote by Wareman and Wilson which states "Therapy with chloramphenicol must be limited to infections for which the benefits of the drug outweigh the risks of the potential toxicities. When other

antimicrobial drugs that are equally effective and potentially less toxic are available, they should be used instead of chloramphenicol."[101]However, in situations such as in the present study it was considered as an alternative antibiotic used to treat post-operative wound infections in orthopaedics caused by carbapenemase producing Enterobacteriaceae and it was found to be effective. Similarly, 42% of the carbapenemase producing isolates were found to be sensitive to tetracycline as this is also a rarely used drug, It was used in a patient with LSCS wound infection and was found to be effective.

The carbapenemase producing organisms showed a sensitivity that varied from 30-32% for Cotrimoxazole and Aminoglycosides. The isolates exhibited a higher resistance for Fluoroquinolones (90%). A study done by Huang S et al showed the co-existence of QRD and ARD genes were highly prevalent among the carbapenem resistant *Enterobacter cloacae* accounting for their resistance to Fluoroquinolones and Aminoglycosides, respectively.[99]

However, looking at the susceptibility pattern still these (Chloramphenicol, Tetracycline) drugs seem to have a place in treating infections caused by carbapenemase producing isolates of Enterobacteriaceae. None of the patients were treated with tigecycline. Colistin was used to treat two head injury cases who responded well to the therapy. Local and regular dressing was effective in majority of the post-op wound infection cases and did not require administration of antibiotics.

Sixty isolates positive by modified Hodge test were subjected to molecular analysis. Among them 57 isolates were found to harbor one or more of carbapenemase genes. We could classify into 3 categories: those that possessed only NDM- 40 isolates, those that possessed NDM along with any other genes- 15 isolates and those organisms which possessed only other genes apart from NDM- 2 isolates.

According to our findings, NDM was the most predominant type carbapenemase that was produced by isolates of Enterobacteriaceae. Similar observations have been seen in other studies which are shown in the table 20.

Table 20: Detection rate of NDM type carbapenemase produced by isolates of Enterobacteriaceae reported from other studies

Year	Study	Percentage of NDM detected	Country
2007	Castanhiera et al	61%	Multicentric
2009	Kumaraswamy et al	1%-44%	India
2009	Bora et al	5.2%	India
2009	Sultan BA et al[102]	93%	Pakistan
2009	Deshpande et al	91%	India
2010	Nordmann et al	96%	UK
2011	Nagaraj S et al	75% in *K.pneumoniae* and 66% in *E.coli*	India

The increasing reports on NDM producing organisms among members of Enterobacteriaceae are of major concern and cannot be ignored. Given the fact that NDM producing isolates are resistant pathogens every attempt should be made to document all relevant clinical details. In this study we also found that the organisms were harbouring multiple carbapenemase genes. KPC was tested for but none of the isolates were found to harbor this gene. Similarly as reported in a study from Bangalore.[86] We could not test for the genes of OXA-48 type carbapenemase.

Of the 60 isolates only 3(5%) were found to be negative by PCR thus indicating false positive result on MHT. This indicates that modified Hodge test is an adequate screening method for detecting carbapenemase producing Enterobacteriaceae. This is in accordance with a study done by Deshpande et al which

reported 22/24 (91%) isolates detected as carbapenemase producers by MHT.[81] However, Girlich D et al reported a low sensitivity of 50% for NDM producers by modified Hodge test, but the sensitivity of the test increased to 85% on addition of $ZnSO_4$.[94] False positivity by modified Hodge test depends on type of carbapenemase being produced, the inoculum size and also the existence of other mechanisms of resistance (CTX-M type of ESBL).

In the present study, of the 97 patients found to be infected with carbapenemase producing organisms, 81 (83.5%) recovered, 11 (11.3%) expired, while 5 (5.2%) were discharged against medical advice. Thus in our study a mortality rate of 11.3% was encountered in patients infected with carbapenemase producing organisms; the mortality rate observed was comparatively lower. In contrast to our experience, a study from Taiwan, in 2010 reported a mortality rate of 37.3% in patients infected with carbapenem resistant organisms.[103] Similarly, a study from Israel, reported mortality rate among patients infected with carbapenem resistant *K.pneumoniae* to be 43.7%. This study compared the mortality rate with those patients infected with carbapenem sensitive *K.pneumoniae* which was 29%.[63]

In our study, 9 of 11(82%) deaths were among patients admitted to the ICU. In most of the studies, ICU acquired infections due to carbapenem resistant organisms are associated with more severe clinical outcomes and higher morbidity and mortality.[104] We found that patients with associated co-morbidities such as increased intracranial pressure, intraventricular bleeding, diabetes mellitus and advanced age were seen among our patients admitted to the ICU. Eight of the patients were above 50 years and 6 of them were put on mechanical ventilation. These observations are in line with studies from Israel, Taiwan, Greece and Brazil.[103,105,106] Mortality among patients with carbapenemase producing organisms is associated with severity of

illness and with well known, serious comorbidities, but not *per se* with carbapenem resistance.[63] There are several other parameters which serve as risk factors for mortality such as previous exposure to antibiotics, central venous catheterization, urinary catheterization and nasogastric tube insertion.[64]

Active surveillance of patients who are thought to be at high risk for infection with carbapenem resistant Enterobacteriaceae (CRE) is strongly advised. Sites of colonization with CRE include lower gastrointestinal tract, oropharynx, skin and urinary tract. CDC recommends collection of stool samples or rectal swabs for screening of CRE.[107] In our study, when 20 stool samples from patients who had yielded carbapenemase producing organisms from their lesions, were plated on selective MacConkey medium, we could recover 17(85%) carbapenem resistant organisms. This observation was high when compared to a study from Israel, which reported an isolation rate of 24% on screening for CRE from rectal swabs.[103] Of these only 10(59%) were found to produce carbapenemase; rest of them, 7 (41%) were not carbapenemase producing though they manifested resistance to carbapenems as evidenced by high MIC levels for ertapenem. Thus, based on the results of screening, we found that patients fell into three categories- the first group of patients who had carbapenemase producing organisms isolated from the lesion and also from their stool sample. There were 10 patients who belonged to this group. The second group in whom carbapenem resistant organisms could be isolated from stool sample but they were not carbapenemase producing. There were 7 patients in this group. Lastly, there were 3 patients in whom no carbapenem resistant organisms could be isolated from their stool samples. It is interesting to note that patients yielding carbapenemase producing organisms from their lesions harbor carbapenem resistant organisms in

their gut which may have mechanisms other than carbapenemase production to account for resistance to carbapenems.

K.pneumoniae was the predominant carbapenemase producing isolate identified by screening (70%). This was in agreement with 2 different studies from Israel which reported *K.pneumoniae* to be the predominant isolate detected.[108] Three of the 10 carbapenemase producing isolates were *E.coli* (30%). In contrast to our findings a study from Rawalpindi, Pakistan, conducted at two military hospitals reported a prevalence of 18.5% in which the predominant isolate producing carbapenemase was *E.coli*(47%).[109]

Six samples out of 10 that were found to contain Enterobacteriaceae that produced carbapenemase were obtained from patients admitted to the ICU. A similar observation was reported from a study conducted in France, which stated that colonization with carbapenem resistant organisms increased regularly from 5.6% after 1 week to 58.6% after 6 weeks in the patients admitted to ICU. The main risk factor for colonization was prior imipenem exposure.[104]

The cultures from clinical samples of patients infected with carbapenem resistant strains will identify only a fraction of the population colonized with such strains and the asymptomatic patients who are colonized with carbapenem resistant strains are missed. Therefore, it becomes important to screen all patients irrespective of carbapenem resistant organisms being isolated from lesions or not. These patients who harbor carbapenem resistant strains in their gut serve as reservoirs for transmission of infection. The purpose of conducting surveillance studies is for the implementation of control measures thereby limiting the spread of multidrug resistant isolates. These studies also help the health care workers to take decisions as to which antibiotic to administer empirically if patients are found to be colonized by

carbapenem resistant organisms. One has to choose an antibiotic which covers these organisms also. Such a strategy may have a potentially better outcome. [110]

Thus, our study shows that carbapenem resistant organisms is not infrequent: It accounts for 14.05% among the isolates of Enterobacteriaceae that were screened. Carbapenemase production is one of the mechanisms we encountered in carbapenem resistant organisms. It accounts for about 2/3rd (61%) of the strains that are resistant to carbapenems. The remaining 1/3rd of the carbapenem resistant strains exhibit resistance to carbapenems by mechanisms other than carbapenemase production.

The most common type of carbapenemase was NDM type, though others such as VIM and IMP were also involved.

Many of the patients infected with carbapenemase producing organisms may also harbor carbapenem resistant isolates in their gut. All this should be borne in mind while treating and controlling carbapenem resistant organisms.

CONCLUSION

In our study it was found that carbapenem resistant strains accounted for 14.05% among clinical isolates of Enterobacteriaceae. The most common mechanism of resistance to carbapenems was carbapenemase production; which accounted for about 2/3rd of the strains found resistant to carbapenems. The remaining 1/3rd exhibited resistance to carbapenems by mechanisms other than carbapenemase production.

Modified Hodge Test was a useful test for detection of carbapenemase production, as among the 60 isolates positive by MHT that were subjected to multiplex PCR, 57 (95%) were found to be harbouring one or more of carbapenemase producing genes. NDM type carbapenemase was the predominant type.

The carbapenemase producing strains were found to be sensitive to Chloramphenicol and tetracycline, which are seldom used as first line antiobiotics. They were used for treating post operative wound infections and were found to be effective.

In our study we found that 83.5% of the patients recovered following treatment with antibiotics according to the sensitivity pattern or local dressing in cases of wound infections. The mortality rate of 11.3% was more commonly seen among patients admitted to the ICU. Many of the ICU patients who succumbed to the illness had life threatening comorbidities in addition to infection with carbapenemase producing organisms. Many of the patients infected with carbapenemase producing organisms were found to harbor carbapenem resistant organisms belonging to family Enterobacteriaceae in their gut.

SUMMARY

Our study included the screening of isolates of Enterobacteriaceae obtained from various clinical samples for carbapenem resistance and detection of carbapenemase production by Modified Hodge test and also to follow up the patients infected with such strains.

A total of 1,245 isolates of Enterobacteriaceae were screened for carbapenem resistance. Among them 175(14.05%) of the isolates were found to be resistant to any one of the carbapenems. Modified Hodge test could confirm 108 (61.71%) isolates to be positive for carbapenemase production and 67(38.28%) were negative. The prevalence of carbapenemase producing Enterobacteriaceae was 8.67%.

Majority of carbapenemase producing isolates were obtained from endotracheal secretions (15.85%) followed by pus (11.46%) and urine samples (5.88%).

K pneumoniae (15.89%) was the most common isolate found to be producing carbapenemase followed by *Enterobacter spp.* (10.7%) and *E.coli* (6.87%).

Majority of the carbapenemase producing organisms were recovered from cases admitted at ICU (31%) followed by general surgery wards (28%). The most commonly isolated species were K pneumoniae and E coli from all the wards.

Among 108 carbapenemase producing Enterobacteriaceae majority of them were found to be sensitive to Chloramphenicol (76%) followed by Tetracycline(42%).

Among the 60 isolates subjected to multiplex PCR, 57(95%) were found to be harbouring one or more of carbapenemase producing genes. Fifty five of carbapenemase producing strains carried blaNDM, among which 10 isolates also

harboured blaIMP, 3 harboured blaVIM and 2 isolates were positive for NDM, IMP and VIM. Additionally, blaVIM gene was detected in two strains.

Among 97 patients infected with carbapenemase producing strains 81(83.5%) patients recovered following treatment with antibiotics according to the sensitivity pattern or local dressing in cases of wound infections, 11(11.3%) patients expired. Among 11 patients who expired, majority of them were males and above 50 years of age (73%). The most common isolate was *K.pneumoniae* obtained from endotracheal secretions of patients admitted in ICU.

Twenty stool samples collected from patients found to be infected with carbapenemase producing organisms were plated onto selective MacConkey agar. Of these 17(85%) yielded growth of carbapenem resistant strains and 10(59%) among them were carbapenemase producing organisms

Thus, our study showed that resistance to carbapenems is common among members of family Enterobacteriaceae isolated from patients. The most common type of carbapenemase detected was the NDM type. The carbapenem resistant Enterobacteriaceae exhibited sensitivity to Chloramphenicol and Tetracycline, which were used in some patients successfully. The finding that patients infected with carbapenemase producing organisms also are colonized with carbapenem resistant organisms stresses that one has to keep this phenomenon in mind while instituting control measures against carbapenemase producing Enterobacteriaceae.

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KEY TO MASTER CHART

Ak	Amikacin
Amp	Ampicillin
Amc	Amoxyclav
BPH	Bening prostatic hypertrophy
C	Chloramphenicol
C.diversus	Citrobacter diversus
Cac	Ceftazidime-Clavulanic acid
Ca cx	Carcinoma cervix
Caz	Ceftazidime
COT	Co-trimoxazole
Cip	Ciprofloxacin
Ctr	Ceftriaxzone
Ctx	Cefotaxime
Cx	Cefoxitin
DAMA	Discharge against medical advice
Diabfoot	Diabetic foot
DOA	Date of admission
DOD	Date of discharge
E	Ertapenem
E coli	Escherichia coli
EDH	Extra dural hemorrhage
ET secretion	endotracheal secretion
F	Female
Frac	Fracture
Gen	Gentamicin
Hspt no	Hospital number
I	Imipenem
ICU	Intensive care unit
IMP	IMP gene
K oxy	Klebsiella oxytoca
K pneu	Klebsiella pneumoniae
KPC	KPC gene
Lab no	Laboratory number
LE	Levofloxacin
LSCS	Lower section caesarian section
M	Male
Me	Meropenem
Med	medicine
MHT	Modified Hodge test
MIC	Minimal inhibitory concentration
NA	Nalidixic acid
NDM	NDM gene
ND	Not done
Nf	Nitrofurantoin
NS	Neurosurgery
Nx	Norflox
Of	Ofloxacin
P.rett	*Providencia rettegeri*
POS	Positive
Pi	Piperacillin
PICU	Paediatric ICU
Pit	Piperacillin-tazobactum
Prot mira	*Proteus mirabilis*
R	Resistant
RTA	Road traffic accident
S	Sensitive
SAH	Sub arachnoid hemorrhage
SDH	Sub dural hemorrhage
SICU	surigal ICU
Sup cond frac	Supra condylar fracture
Te	Tetracycline
Tob	Tobramycin
US	Urosurgery
UTI	Urinary tract infection
VIM	VIM gene
Yrs	years