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Newer β -Lactamases and *E.coli* – A Cause of Concern

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Additional information is available at the end of the chapter

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1. Introduction

In 1941, the Magic Bullet Penicillin was first used clinically in an Oxfordshire constable, Albert Alexander, but within one year Rammelkamp reported the identification of isolates of *Staphylococcus aureus*, resistant to this miracle drug [1]. Within a short span of 70 years, from discovery of Penicillin to Tigecycline, some hospital strains have developed which are resistant to almost all available antimicrobials and the mankind is busy in writing obituary for antimicrobials [2].

Infact, the rising trend of developing resistance to multiple antibiotics in microbes, leads to therapeutic failure. Presently, antimicrobial resistance (AMR) is a major threat to patient care and disease control throughout the world.

The World Health Day theme on 7th April 2011 was 'Antimicrobial resistance and its global spread'. World Health Organisation has raised the issue in 2011 that "Combat drug Resistance - No action today, No cure tomorrow" [3]. Not only the Gram positive bacteria like Methicillin Resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant Enterococci (VRE) etc. even the several Gram negative bacteria of Enterobacteriaceae family e.g. *E.coli*, *Klebsiella pneumoniae*, *Enterobacter sp. etc.* and *Pseudomonas aeruginosa*, *Acinetobacter baumani*, *Burkholderia species* etc. also develop resistance to most of the antibiotics now a days.

Actually, the pace in which bacteria develop resistance is much higher than the rate of development of newer antimicrobials. The antibiotic resistance is mainly developed due to inappropriate and irrational use of antibiotics. In 2011, two new terms were coined i.e. multidrug resistant (MDR) and Extreme drug resistant (XDR) organisms. MDR is defined as non-susceptibility to one or more antibiotics in three or more antibiotic classes and XDR is defined as non-susceptibility to all potentially effective antimicrobials respectively [4]. β – lactamases are the important mechanism of drug resistance among the Gram negative bacteria.

Though *E.coli* is a commensal of human or animal intestine, *E.coli* is the most commonly isolated bacterial species in clinical laboratories and is incriminated in infectious diseases involving every human tissue and organ system [5]. Theodor Escherich, a German pediatrician identified *Escherichia coli* in 1885 and named it '*Bacterium coli commune*' [6]. He also established the pathogenic role of *E.coli* through his studies. The nomenclature '*Bacterium coli*' was widely used until 1919 when Castellani and Chalmers described the genus *Escherichia* [7]. The type species is *Escherichia coli*. Some species like *E.fergusonii*, *E.hermanii* and *E.vulnerris* are considered opportunistic pathogens and are associated with wound infection in humans [8]. *Escherichia albertii*, the sixth species associated with cases of diarrhoea in children of Bangladesh, has been proposed by Huys et al in 2003 [9].

About 80% cases of urinary tract infection are due to *E.coli* [8]. It is commonly involved in Gram negative sepsis and endotoxic shock. *E.coli* can cause diarrhoea, wound infections, pneumonia in hospitalized patients and meningitis in neonates.

E.coli is the organism that is most widely studied in its various aspects. *E.coli* is the model organism for studies on cell structure, growth and metabolism. It is considered to be good vehicle for the cloning of genes from prokaryotic and eukaryotic cells and for expression of gene products [8].

E.coli is susceptible to 97% ethylene trioxide (ETO) and 95% Hydrochlorofluorocarbon-ETO (HCFC-ETO) respectively [10]. Chlorine 1 ppm is capable of eliminating approximately 4 log₁₀ of *E.coli* O157:H7 within a minute [11]. *E.coli* strains causing intestinal and extra intestinal infections, exhibits many virulence factors such as: Adhesins, O antigens, Hemolysin, Siderophore production / Iron sequestration / Aerobactin production, Cell surface hydrophobicity, Outer membrane proteins e.g. Porins etc. Haemagglutinin, Verotoxin, Cytotoxic necrotizing factor (CNF), Enterotoxin, Colicins, Gelatinase production, Serum resistance are other major virulence factors of *E.coli*. Complete serotyping of *E.coli* is based on three antigen detection as O, K & H i.e. O:K:H. If fimbrial virulence factor is present then serotype should be expressed in terms of O:K:H:F.

A. *E.coli* – Role as a pathogen

They are classified into two types [12] – (a) Enterovirulent *E.coli*, (b) Uropathogenic *E.coli*

- a. **ENTEROVIRULENTE***E.coli*: Adherence is one of the most important virulence mechanisms of enterovirulent *E.coli*.

Enterovirulent *E.coli* strains are classified into

Enteropathogenic *E.coli* (EPEC), Enterotoxigenic *E.coli* (ETEC), Enteroinvasive *E.coli* (EIEC), Verocytotoxin – producing *E.coli* (VTEC), Enteroaggregative *E.coli* (EAEC), Diffusely adherent *E.coli* (DAEC) etc.

- b. **UROPATHOGENICE***.Coli* (UPEC)

E.coli is the predominant uropathogen isolated from acute community acquired urinary tract infections and is responsible for 85% of asymptomatic bacteriuria and more than 60%

of recurrent cystitis [13]. *E.coli* strains involved in urinary tract infections includes O groups 1, 2, 4, 6, 7 etc.

B. Laboratory diagnosis

E.coli can be detected in the laboratory by conventional tests [5]. *E.coli* are Gram negative rod, motile and on Mac Conkey's agar form lactose fermenting nonmucoid colonies. Routine biochemical tests done for *E.coli* are : Catalase test: Positive, Oxidase test: Negative, Triple sugar iron agar test: Acid slant/Acid butt with gas, Indole test: Positive, Methyl red test: Positive, Voges-Proskauer (VP) test: Negative, Citrate utilization test: Negative, Nitrate reduction test: Positive, Oxidative / Fermentative test (Hugh & Leifson): Fermentative metabolism, Lysine Decarboxylase test: Positive.

1.1. Antibiotic sensitivity pattern

Intrinsic resistance is rarely seen in genus *Escherichia*. Susceptibility of individual strain to different antimicrobials varies greatly, thus antibiogram is used as an epidemiological marker. This resistance pattern is plasmid mediated. *E.coli* shows transferable resistance to one or multiple drugs [14]. Recently *E.coli* isolated from intestinal as well as extraintestinal specimens has become resistant to most of the routinely used antibiotics.

A. Beta - lactamases

Beta lactamases are enzymes produced by wide range of Gram negative and Gram positive bacteria. This enzyme is responsible for resistance to β -lactam antibiotics like Penicillins, Cephalosporins, Cephamycins and Carbapenems. The β -lactamase enzymes break the beta-lactam ring and thus inactivate the antibacterial properties. Abraham and Chain (1940) discovered penicillinase, first β -lactamase to be identified in *E.coli* before Penicillin was introduced for clinical use [15]. The name β -lactamase was given by Pollock in 1960 [16]. First plasmid mediated β -lactamase was "TEM" named after the name of first patient Temoniera in whom the enzyme was first detected [17].

Classification of β -Lactamases can be done by -

- a. Functional or Bush Jacoby Mederios classification [18]
- b. Molecular or Ambler classification [19]

Ambler in 1980s classified β - lactamases into various groups and according to him, there are three main classes as A, C and D [19]. Metallobetalactamases belong to class B which exhibits potent hydrolyzing activity not only against Carbapenems but also to other β - lactam antibiotics. *E.coli* is one of those Gram negative bacteria that has potential to develop all the major classes of β - lactamases including Metallobetalactamases.

Determination of β -lactamase production could be done by acidometric method, iodometric method and Chromogenic cephalosporin method using nitrocephin [20].

B. Extended spectrum beta – lactamase (ESBL)

Extended spectrum beta – lactamase (ESBL) producing strains are emerging pathogens causing Health care associated infections (HAI) and pose great therapeutic challenge in recent years.

ESBLs were first detected in *Klebsiella pneumoniae* in 1983 [21, 22]. ESBL are classified under molecular AMBLer class A penicillinase having serine residue at their active site. ESBLs are responsible for resistance to one or more β -lactam antibiotics including third generation cephalosporins. Typically ESBLs are derived from genes for TEM-1, TEM-2 or SHV-1 by mutations that alter the amino acid sequence around the active site of β -lactamases [23,24]. ESBLs are inhibited by β -lactam inhibitors like Clavulanic acid, Sulbactam and Tazobactam. ESBLs are produced by Enterobacteriaceae family, *Pseudomonas aeruginosa*, *Acinetobacter species* etc.

Detection of Extended Spectrum β -lactamase (ESBL) production:

The ESBL in *E.coli* is detected by two approaches: 1. Screening tests and 2. Confirmatory tests. Screening test detect reduced susceptibility to indicator drugs. According to Clinical Laboratory Standard Institute (CLSI) guidelines indicator drugs used for *E.coli* & *Klebsiella* spp. are Cefotaxime, Ceftazidime, Ceftriaxone or Aztreonam [25]. Screening tests are not specific because mechanism other than ESBLs may also give positive results. Therefore, positive screening test should be followed by confirmatory tests.

Different confirmatory tests include Double disk approximation test, [26,27]. Comparison of Minimum inhibitory concentration (MIC) or inhibitory zone around disk in presence or absence of β -lactamase inhibitor [28], Vitek ESBL test [29], Etest [30], three-dimensional test [31] etc. For identification of specific ESBL different molecular detection methods can be applied like DNA probes, PCR with oligonucleotide primers oligotyping, PCR followed by restriction fragment length polymorphism analysis, ligase chain reaction and nucleotide sequencing etc [32]. Commonly primers used for detecting bla genes are [33]:

CTX Forward – CGCTTTGCGATGTGCAG

Reverse - ACCGCGATATCGTTGGT

These techniques are available only in research centers and are beyond the scope of routine Clinical Microbiology Laboratories in India considering the presence of too many different types of ESBLs and the high cost.

C. Amp C beta-lactamase [34]

Amp C β -lactamases were first reported in 1988. Amp C β -lactamases are found either on chromosome (inducible) or on plasmid (non-inducible). Amp C β -lactamase producing bacteria show resistance to most of Cephalosporins including Cephamycin (Cefoxitin, Cefotetan) except Carbapenems. But they also hydrolyze Penicillins and Aztreonem. These are not inhibited by beta-lactam inhibitors such as Clavulanic acid, Tazobactam and Sulbactam.

Detection of AMPC β -Lactamase production: Presently, all plasmid mediated Amp C β -lactamases have similar substrate profile to chromosomal Amp C β -lactamases. But the only difference is chromosomal Amp C β -lactamases are inducible where as plasmid mediated Amp C β -lactamases are uninducible [35]. Amp C β -lactamase producing strains could be detected by disc antagonism test [36], modified three dimensional test (MTDT) [37], Amp C disc test [38], double disk synergy test and disc potentiation test using 3-amino-phenyl-boronic acid (APB) etc [39].

D. Carbapenemases

These include β – lactamases which cause Carbapenem hydrolysis, with elevated Carbapenem MICs and they belonged to molecular classes A, B and D. Molecular classes A, C and D include the β – lactamases with serine at their active site, whereas class B β – lactamases are all metalloenzymes which require Zn^{++} for their activation [40].

Molecular class A carbapenemase – Class A serine carbapenemases belong to functional group 2f include chromosomally encoded NMC(Not Metalloenzyme Carbapenemase), IMI (Imipenem hydrolyzing β -lactamase) and SME(*Serratia marcescens* enzyme) and plasmid mediated KPC (*Klebsiella pneumoniae* carbapenemase) and GES / IBC(integron borne cephalosporinase), etc [40]. All have the ability to hydrolyse Carbapenems, Cephalosporins, Penicillins and Aztreonem and all are inhibited by Clavulanic acid and Tazobactam. The chromosomal class A carbapenemase are infrequently found and can be induced by Imipenem and Cefoxitin. The KPC (*Klebsiella pneumoniae* carbapenemase) producing strains are found in *Klebsiella pneumoniae*, *Enterobacter* species, *Salmonella* species and other Enterobacteriaceae [41,42].

Class D Serine carbapenemases - The OXA (Oxacillin hydrolysing) β -lactamase with carbapenemase activity was detected by Patow et al in 1993 and the enzyme was purified from *Acinetobacter baumannii* [40]. They have been also found in Enterobacteriaceae and *P.aeruginosa* and were described as penicillinase capable of hydrolyzing Oxacillin and Cloxacillin [43,44]. They are not inhibited by Clavulanic acid and EDTA and were designated as ARI-1 (Acinetobacter Resistant to Imipenem) and reside on large plasmid.

Metallobetalactamases (MBL)

They belong to molecular class B β – lactamases, requiring one or more divalent cations (Zn^{++}) for their activation [45] and have 3 characteristics [2] –

1. Hydrolyze carbapenems
2. Resistant to clinically used β – lactamase inhibitors and
3. Inhibited by EDTA, a metal ion chelator.

The first MBL detected was chromosomally encoded and was detected in *Bacillus cereus* [46]. They possess a high level of resistance to all Carbapenems, Penicillins, Cephamycins, Cephalosporins and beta-lactamase inhibitor combinations.

Classification of MBLs

On the basis of Imipenem and other β -lactam hydrolysis, MBLs are classified into different subgroups as [47] –

Subgroup3a- possess broad spectrum activity; Subgroup3b – preferential avidity for carbapenem; Subgroup 3c – hydrolyze carbapenems poorly compared to other β -lactam substrate.

At molecular level MBLs are classified into [48] –

Class B1 – possess key Zn co-ordinating residues of three histidines and one cysteine eg. IMP, VIM, GIM and SPM – 1,

Class B2 – possess asparagine instead of histidine at first position of principal Zn binding motif, NXHXD. e.g. *Aeromonas* species and *Serratia fonticola* enzyme SFH – 1.

Class B3 – MBL L₁ unique among all β -lactamases in being functionally represented as a tetramer.

The numbering scheme has been recently updated to accommodate newly discovered MBLs.

MBLs are inhibited by EDTA (Ethylene diamino tetraacetic acid), 2 Mercaptoethanol, 5-Mercaptoacetic acid, 2 Mercapto propionic acid, Copper Chloride and Ferric Chloride. MBLs are classified mainly into two types – Chromosomally encoded and Plasmid encoded or Acquired or Transmissible type. Usually metallo β lactamase producing strains are susceptible to Colistin or Polymyxin B. MBLs do not hydrolyze aztreonam very well, which characteristic is different than ESBLs or Class A β -lactamases [48].

The acquired MBLs are further classified into different types depending on their place of origin as VIM (Italy or Greece), SPM (Brazil), GIM (Germany), SIM (Korea), DIM (Dutch), NDM/PCM (New Delhi metallo β lactamases/Plasmid coded metallo β lactamases).

NDM – 1: NDM – 1 was named after New Delhi, capital of India as NDM – 1 and was first described by Young et al in December 2009 in an individual who acquired infection in a Hospital in India due to Carbapenam resistant *Klebsiella pneumoniae* strain [49].

In March 2010, researchers from Mumbai found that most of Carbapenam resistant bacteria carried bla_{NDM-1} gene. The gene is carried on plasmids and is readily transferred between different strains of bacteria by horizontal gene transfer. All these strains were resistant to most of routinely used antibiotics like Aminoglycosides, β -lactams, Quinolones but sensitive to Tigecycline and Colistin [50]. Recently, Espinal et al identified a new variant of NDM-1 in *Acinetobacter baumannii* and designated it as NDM-2. They reported that, the clonal dissemination of a NDM-2 producing *A. baumannii* was isolated in an Israeli rehabilitation ward [51]. Recently, a new variant of the New Delhi metallo-enzyme (NDM) carbapenemase, NDM-4 and NDM-5, was identified in *E.coli* from two patients both of them had a history of hospitalization in India [52,53].

Detection of Metallo β -lactamase production

Carbapenems often used as an antibiotic of last resort for treating serious infections caused by multi-drug resistant (MDR) organism. Reduced susceptibility to any Carbapenem can be used as a screen for carbapenemases. Positive screening tests are to be followed by a confirmatory test for MBL production.

Although a variety of phenotypic methods have been proposed for the detection of carbapenemases, none have been recommended by CLSI. The classical Hodge [54], Modified Hodge test (MHT) [55] are economical approach for detection and confirmation of carbapenemase activity and Re – Modified Hodge test [56] for detection of MBL. However, the first two tests cannot differentiate between a class A carbapenemase and MBL, making a further confirmatory test necessary. Imipenem is more sensitive but less specific Carbapenem for this test allowing detection of even OXA carbapenemases.

MBL detection tests involving inhibitors such as ethylene diamine tetraacetic acids (EDTA) and 2-Mercaptopropionic acids (2-MPA) have been recommended by various workers [57]. Tris/EDTA disks can also be used in combination with a Carbapenem disk to detect Carbapenem - hydrolyzing enzymes and to differentiate between class A enzymes and MBLs. MBLs are inhibited by the Tris/EDTA disk. The inhibition of MBL can be enhanced by the addition of chelators. Double disk synergy test (DDST) [55] and Disk potentiation tests [58] are based on this principle. For detection of MBL many other methods used are MBL E-test using imipenem/imipenem-EDTA [59], reduction of MIC in presence of EDTA and polymerase chain reaction (PCR) [60].

Commonly primers used for detecting Class B metalloenzyme genes are [40]:

VIM-1 Forward – TTATGGAGCAGCAACCGATGT

Reverse - CAAAAGTCCCGCTCCAACGA

PCR is specific for gene family IMP, VIM, etc. and hence, many other specific primers can be used for different MBL genes. The main disadvantage of PCR is that it requires tailor-made DNA primers and cannot differentiate between variants and may not detect new variants.

E. Detection of *Klebsiella pneumoniae* carbapenamases (KPCs) [61]

KPCs can be mainly detected by Combined disk method using Imipenem and Imipenem with Phenyl boronic acid, Molecular methods like PCR etc.

Recently, Carbapenem Resistant Enterobacteriaceae (CRE) pose a real threat to Medical fraternity as the increased frequency with which Enterobacteriaceae cause infection and the mortality associated with infection caused by CRE. Most of the studies reported newer β -lactamases including MBL production in nonfermenters like *Pseudomonas aeruginosa*, *Acinetobacter species* etc. There are very few studies that report MBL production in Enterobacteriaceae [19]. Hence, we have conducted the study to detect newer β - lactamases producing *E.coli* strains by phenotypic methods, isolated from different clinical specimens.

2. Material & methods

A total Number of 450 *E.coli* strains isolated from different clinical specimens like urine, stool, blood, pus etc. were studied. The strains were characterized as *E.coli* according to conventional identification tests [5]. *E.coli* ATCC 25922 was used as positive control for all the conventional tests. Few recent tests were also included to identify *E.coli* which could reduce the number of biochemical tests and there by cost also e.g. Motility- Indole- Lysine (MIL) medium [62], Methylumbelliferyl- β -D- Glucuronide(MUG) MacConkey's medium [63]. All the *E.coli* strains isolated from urine samples were subcultured on Hi chrome UTI agar for direct detection of *E.coli*.



Photo 1 : Motility – Indole –
Lysine medium
Motility +VE,
Indole production test +ve,
Lysine decarboxylase test +ve.



Photo 2: MUG Mac Conkey's agar
Typical bluish fluorescence



Photo 3 : Hi Chrome UTI agar : *E.coli*

2.1. Antibiotic susceptibility test

Antibiotic susceptibility test for all 450 strains of *E.coli* were done using Mueller Hinton (MH) agar plate with commercially available antibiotic discs (Himedia Pvt Ltd, India) by Kirby Bauer disc diffusion method [64] according to CLSI guidelines [65]. *E.coli* ATCC 25922 was used as control.

Lawn culture of test strain (turbidity adjusted to 0.5 McFarland standard) was put on MH Agar plate. The antibiotic disks were put on inoculated plate with all aseptic precaution. Antibiotic susceptibility test was done for Aminoglycosides like Amikacin (AK-30 μ g), Gentamicin (GEN-10 μ g), Cephalosporins like Ceftazidime (CAZ-30 μ g), Cefotaxime (CTX-30 μ g), Fluoroquinolones like Ciprofloxacin (CIP-5 μ g), Monobactams like Aztreonam (AT-30 μ g), Carbapenems such as Imipenem (IPM-10 μ g), Etrapanem (ETP-10 μ g) etc. For urine sample an additional disk of Nitrofurantoin (NIT-300 μ g) and only for MBL producing strains, Colistin (CL-10 μ g) disk were used

All 450 *E.coli* strains were tested for newer β -lactamases e.g. Extended Spectrum β -lactamases (ESBLs), AMPC β -lactamases Metallobetalactamases (MBLs) and Klebsiella pneumoniae producing Carbapenemases (KPCs) [28, 30, 39, 58, 59, 61]. As Metallobetalactamases are also found in carbapenem susceptible organisms, we have screened carbapenem sensitive strains also for MBL production.

2.2. Detection of newer β -lactamases

2.2.1. Detection of ESBL production [28]

Combined disk method as per CLSI guideline and ESBL E-test were used for ESBL detection.

In Combined disk method, lawn cultures of test strains (turbidity adjusted to McFarland 0.5 standard) were put on MH agar plates. Ceftazidime (CAZ) 30 μ g disc and Ceftazidime plus Clavulanate (CAC) 30 μ g plus 10 μ g discs were put widely apart on that MH plate. After overnight incubation at 37 $^{\circ}$ C increase in zone diameter of ≥ 5 mm with CAC disk as compared to CAZ disk alone was considered positive for ESBL detection.

In ESBL E-test, lawn culture of test strain (turbidity adjusted to McFarland 0.5 standard) was done on a MH agar plate & ESBL E-test strip (AB Biomeriux) was placed. After overnight incubation at 37°C, MIC ratio of ceftazidime/Ceftazidime Clavulanic acid (TZ/TZL) ≥ 8 or deformation of ellipse or phantom zone present was considered positive for ESBL production.

2.2.2. Detection of AmpC β -lactamases [39]

For, detection of Amp C β -lactamase producing strains substrate inducer combination of Imipenem (10 μ g) / Ceftazidime(30 μ g) disks and for confirmation disk potentiation test using 3 aminophenyl boronic acid (100 mg/ml) was used.

In Disk potentiation test, lawn culture of test strain (turbidity adjusted to McFarland 0.5 standard) was done on MH agar plate. Two ceftazidime(30 μ g) disks with centre to centre distance of 30mm were placed on that MH plate. 3-aminophenylboronic acid (APB) was dissolved in DMSO at a concentration of 100mg/ml. 10 μ l of this APB solution was added to one of the ceftazidime disk. After overnight incubation at 37°C, an increase in zone size of ≥ 5 mm around the Ceftazidime - APB disc compared to Ceftazidime disc only was recorded as a positive result for Amp C β -lactamase production.

2.2.3. Detection of both ESBL & AmpC β -lactamase producing strains [66]

As ESBL and AMPC β -lactamase can be produced by a single strain and ESBL production is suppressed if the same strain also produces Amp C β -lactamases, we followed the following methods.

Lawn culture of test strain (turbidity adjusted to McFarland 0.5 standard) was done on MH agar plate. To detect the strains producing both ESBL and AMPC β -lactamases, we used one disk containing Ceftazidime and Clavulanic acid (CAC) and the other 02 disks containing Ceftazidime (CAZ) only, placed widely apart. On CAC disk 10 μ l of 3-aminophenyl boronic acid (3-APB) (100mg/ml) solution was put. 3-APB inhibit the growth of AmpC β -lactamases and ESBL genes can be expressed whereas 10 μ l of 3-APB solution was also put on one of the CAZ disk. The plates were incubated 37°C overnight. The zone diameter of ≥ 5 mm around CAC disk with 3-APB compared to CAZ only was recorded as ESBL positive and increase in zone diameter of ≥ 5 mm around CAZ and 3-APB disc compared to zone diameter of CAZ only was considered positive for AmpC β -lactamase production.

2.2.4. Detection of metallo β lactamases (MBL)

All 450 *E.coli* strains were screened for Carbapenemase activity by Classical Hodge test [54] and for MBL production by Re-Modified Hodge test [56], DDST [55], DP test [58] and MBL ETest.

Re-Modified Hodge Test (Re-MHT) [56]: All 450 *E.coli* strains were subjected to Re-modified Hodge test for detection of carbapenemase activity. The broth culture of *Escherichia Coli* ATCC 25922 was adjusted to a turbidity of 0.5 McFarland standards and was used to put lawn culture on MH agar plates with sterile swab. After drying, a 10 μ g Imipenem disc (HiMedia) was placed

at the centre and 10 µl of 50mM zinc sulfate solution was added to Imipenem disk. Then, a test strain of *E.coli* was streaked from the edge of the disk to the periphery in four different directions. The plate was incubated overnight at 37°C. The presence of a cloverleaf shaped zone of inhibition due to MBL production by the test strain was considered as positive Re - Modified Hodge test (Re - MHT).

Imipenem-EDTA double disk synergy test (DDST) [55]: The IMP-EDTA double disk synergy test was performed for detection of Metallobetalactamases. Test strains i.e. *E.coli* (turbidity adjusted to 0.5 McFarland standard) were inoculated on to Mueller Hinton agar plate. After drying, a 10µg Imipenem disk and a blank sterile filter paper disk (6mm in diameter, Whatman filter paper no.2) were placed 10mm apart from edge to edge. 10 µl of 50mM zinc sulfate solution was added to the 10 µg Imipenem disk. Then, 10µl of 0.5 M EDTA (Sigma, USA) solution was applied to the blank filter paper disk. As disodium-EDTA is difficult to be solubilised in sterile water, we had used dipotassium-EDTA which is easily soluble in sterile water. Enhancement of the zone of inhibition towards the EDTA disk was interpreted as a positive result.

Disk Potentiation Test (DP) [58]: The IMP-EDTA combined disk test was performed for detection of metallobetalactamases. Test strains (turbidity adjusted to 0.5 McFarland standard) were inoculated on to MH agar plate. Two imipenem disk (10 µg) were placed on the plate wide apart and 10 µl of 50mM zinc sulphate solution was added to each of the imipenem disks. Then 10µl of 0.5M EDTA solution was added to one of the disk and the plates were incubated at 35° C for 16-18 hrs. If the increase in inhibition zone with the Imipenem and EDTA disk was ≥ 7 mm than the imipenem disk alone, it was considered as MBL positive.

MBL E-Test – Confirmatory test

The MBL E-test was done and interpreted using test strains and Quality control strains according to manufacturer's instructions. Overnight broth culture of test strain (turbidity adjusted to 0.5 McFarland standard) was used to inoculate MH agar plate. The MBL E-test strip was put on that inoculated MH plate with a sterile forceps and plates were incubated at 37°C for 18-20 hrs. After incubation, MIC ratio of Imipenem /Imipenem-EDTA (IP/IPI) of ≥ 8 or deformations of ellipse or phantom zone indicate MBL production.

Colistin E test: All MBL producing *E.coli* strains were tested with Colistin E test (AB bioMerieux, Solana, Sweden). The Colistin E-test was done and interpreted using test strains and Quality control strains according to manufacturer's instructions.

2.2.5. Detection of Klebsiella pneumoniae carbapenemases (KPCS)

It was done by Combined disk method [61]. Lawn culture of test strain (turbidity adjusted to 0.5 Mc Farland) was put on MH agar plate and 2 Imipenem (10 µg) disks were put widely apart. To one Imipenem disk 10 µl Phenyl boronic acid solution (400µg/disk) was put. Then the MH agar plates were incubated at 37°C overnight. After incubation, the test should be considered positive when growth inhibitory zone around the disk containing Imipenem and Phenyl boronic acid was ≥ 5 mm compared to zone diameter of Imipenem alone.

2.2.6. Detection of class D enzymes

Several workers have reported that Class D enzymes i.e. OXA – 48 type are the most difficult carbapenemase producers to be identified phenotypically [42,43]. Hence, we did not include detection of Class D carbapenemase in our study.

3. Observation

Figure 1: Incidence of MBL, ESBL & Amp C β –lactamase producing *E.coli* strains (n = 450)

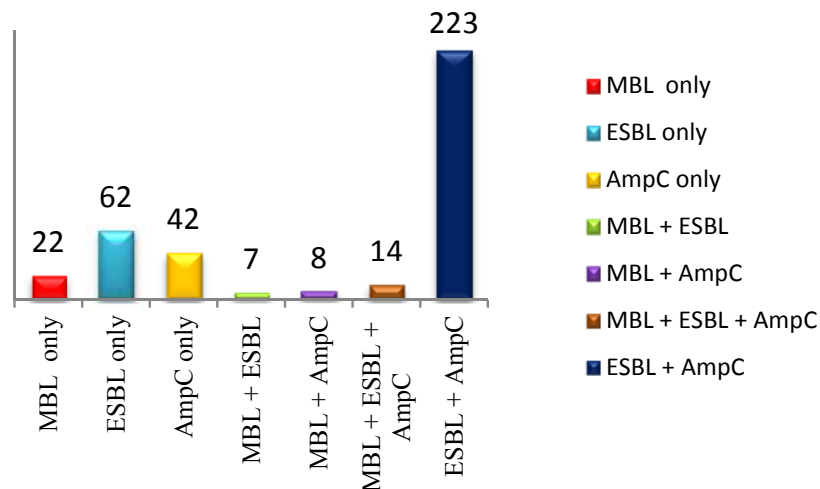


Figure 1. Shows incidence of different β – lactamases e.g. MBL, ESBL, AMPC β – lactamases producing *E.coli* strains. Out of 450 *E.coli* strains studied, 378 (84%) strains produced any of the 3 types of β – lactamases i.e. MBL, ESBL and Amp C β –lactamases, either alone or in combinations. Photographs of different methods used to detect newer β -lactamases phenotypically is given below (photo 4,5,6,7,8,9,10,11,12)



Photo 4 : ESBL Combined disc test+ve



Photo 5 : ESBL Etest :positive



Photo 6 : Both ESBL & AmpC β- lactamase +ve

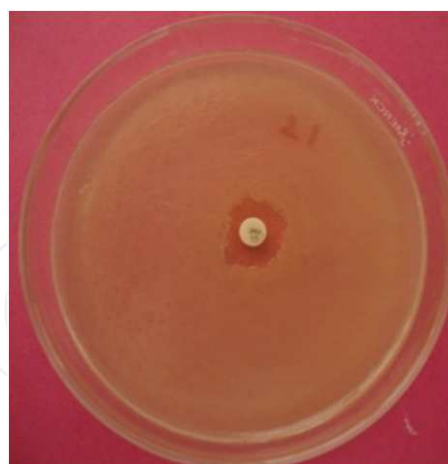


Photo 7 : Classical Hodge test +ve

3.1. Detection of MBL

In photo 5 ESBL E Test positive shows MIC of Ceftazidime (TZ) 6 µg/ml and Ceftazidime Clavulanic acid (TZL) 0.25µg/ml respectively i.e. MIC ratio of TZ/TZL is 24. Out of 378 β - lactamase producing *E.coli* strains 223(59%) produced both ESBL and Amp C β –lactamases. Out of total 51 MBL producing *E.coli* strains 14 (27.5%) strains produced all the three types of β – lactamases i.e. MBL, ESBL and AMPC β – lactamases. In Photo 11 MBL E Test positive shows MIC of Imipenem (IP) 24 µg/ml and Imipenem-EDTA (IPI) < 1µg/ml respectively i.e. MIC ratio of IP/IPI is > 24 and also presence of Phantom zone.

All 51MBL positive *E.coli* strains were sensitive to Colistin with MIC range from 0.032 to 0.25µg/ml and were detected by E test for Colistin (bioMe’rieux) (Photo 12).

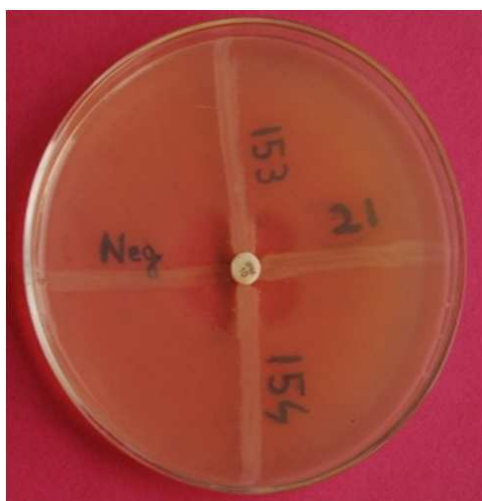


Photo 8 : Remodified Hodge test +ve



Photo 9 : DDST test +ve



Photo 10 : Disc potentiation test +ve



Photo 11 : MBL E test +ve

Photo 12: Colistin E test : MIC 0.125 µg/ml

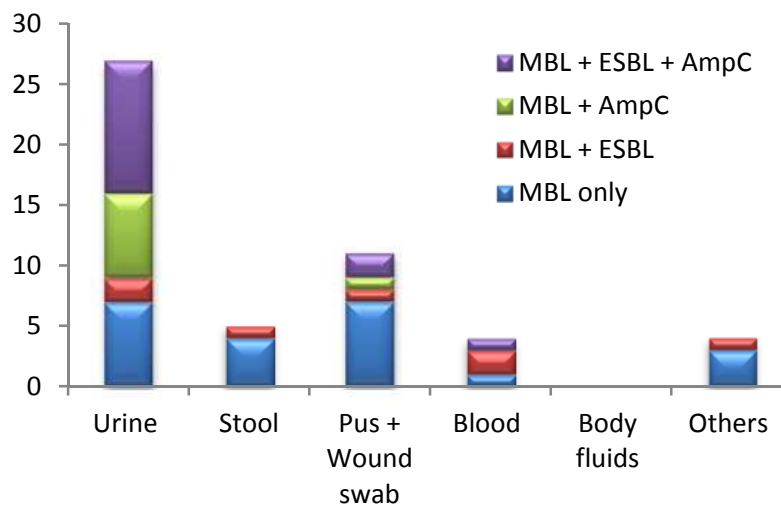


Figure 2. Isolation of MBL producing *E.coli* strains from different clinical specimens (n = 51)

Out of total 450 *E.coli* strains 218 (48.4%) were isolated from urine, 92 (20.4%) from stool, 61 from pus and wound swab, 30 from blood, 10 from body fluids and 39 from other specimens e.g. Endotracheal (ET) tube secretions, broncho-alveolar lavage etc.

Figure 2 shows out of total 51 MBL positive *E.coli* strains maximum 27(53%) strains were isolated from urine followed by 11(21.6%) strains from pus and wound swab. Out of 27 MBL positive *E.coli* strains isolated from urine 12(44.4%) had history of catheterization and 2(7.4%) had history of instrumentation in urethra (e.g.dilatation, etc). Only 01 urine sample received from High Dependency Unit and that *E.coli* strain produced all 3 types of β – lactamases i.e. MBL, ESBL and Amp C β – lactamases In our study, total 14 *E.coli* strains were positive for all 3 types of β – lactamases i.e. MBL, ESBL and AMPC β – lactamases, and out of which 11 (78.6%) strains were isolated from urine samples which was quite alarming. No MBL producing *E.coli* strain was isolated from body fluids. Out of 92 stool samples, 5 (5.4%) were MBL producers, 15 (16.3%) were only ESBL producer and 13 (14.1%) were only AMPC β – lactamase producer.

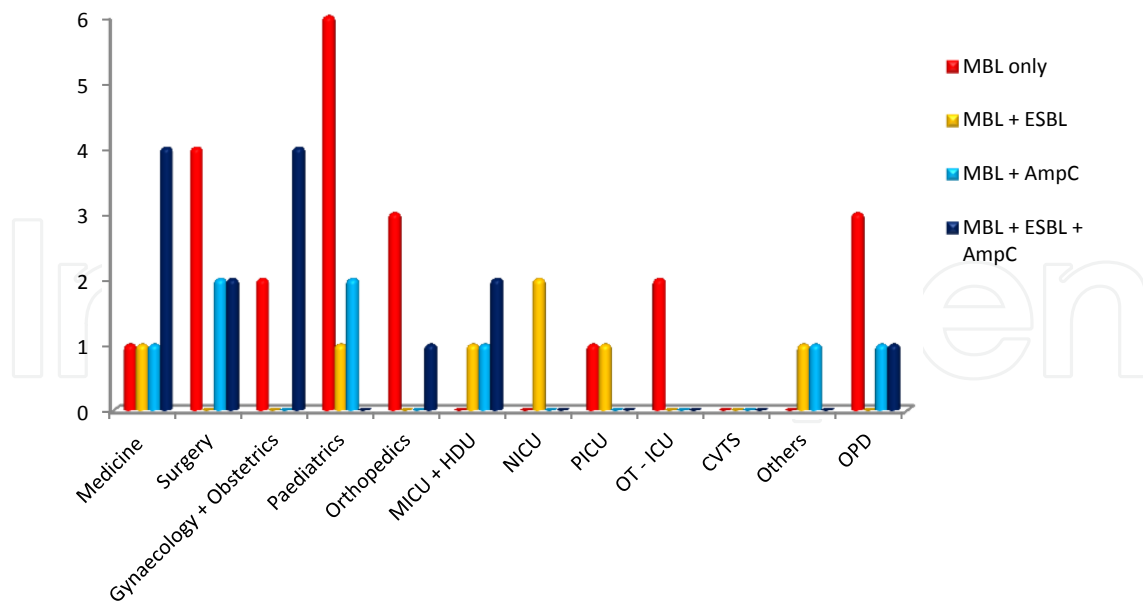


Figure 3. Isolation of only MBL and MBL with other β - lactamase producing *E.coli* strains from different clinical specialties (n = 51)

Figure 3 shows maximum 9/51 (17.7%) MBL producing *E.coli* strains were isolated from Pediatrics ward. No MBL producing strain was isolated from Cardiovascular & Thoracic Surgery (CVTS) ward. From Medicine ward, 16 *E.coli* strains were only ESBL producers and 10 were only Amp C β – lactamase producers.

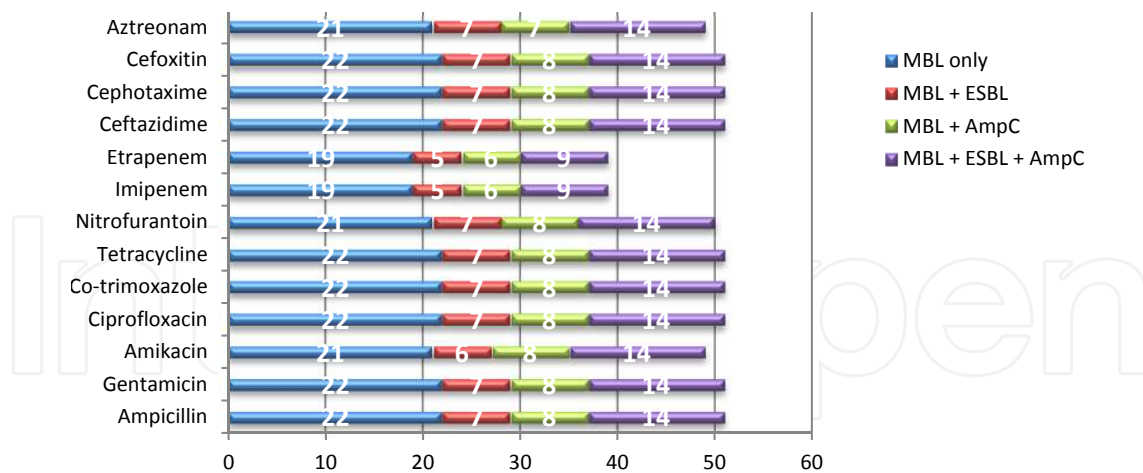


Figure 4. Antibiotic resistance pattern of MBL producing *E.coli* strains (n = 51)

Figure 4 shows out of total 51 MBL producing *E.coli* strains, 39 (76.5%) strains were resistant to Imipenem and Etrapanem by disc diffusion method. The MBL producing strains of *E.coli* showed total resistance to Ampicillin, Gentamicin, Ciprofloxacin, Co – trimoxazole, Tetracycline, Ceftazidime, Cephalexime and Cefoxitin. But all MBL positive *E.coli* strains (100%) were sensitive to Colistin. Out of total 450 *E.coli* strains, only 58.9% strains were sensitive to

Amikacin and only 28.2% strains were sensitive to Ciprofloxacin. Nitrofurantoin was used for urine specimen only (n = 218) and 67.9% strains were sensitive to Nitrofurantoin. Amongst the 51 MBL producing *E.coli* strain, 12 (23.5%) strains were sensitive to Imipenem and Etrapanem by disk diffusion test. Out of 12 Imipenem sensitive MBL producing *E.coli* strain, 5 (41.7%) strains produced all 3 types of β – lactamases.

Strains	Phenotypic methods		
	Re - MHT	DDST	DP
MBL + ve (n = 51)			
By MBL – E test	45	46	51
MBL – ve (n = 399)			
By MBL – E test	401	402	399
False negative	6	5	0
False positive	4	2	0
Sensitivity %	88.2	90.2	100
Specificity %	99	99.5	100
Positive predictive value	91.8	95.8	100
Negative predictive value	98.5	98.8	100
Efficiency	97.8	98.5	100

Table 1. Performance of different phenotypic methods compared to MBL – E test in identifying MBL + ve *E.coli*

Table 1 shows Sensitivity, Specificity, Positive predictive value, Negative predictive value and Efficiency calculated for Re – Modified Hodge test (Re – MHT), Double disk synergy test (DDST) and Disk potentiation (DP) test, compared to MBL – E test in identifying MBL positive *E.coli* strains. MBL – E test is considered as standard phenotypic reference method for detection of MBL positive strains. The sensitivity of Re - MHT was 88.2% and specificity was 99% whereas sensitivity of DDST was 90.2% and specificity was 99.5%. DP test was having sensitivity and specificity of 100%. The efficiency of Re – MHT was 97.8%, DDST was 98.5% and DP was 100%, when compared to MBL - E test as standard reference method.

4. Discussion

The emergence of antibiotic resistance occurs by a) spontaneous mutation and vertical gene transfer and b) horizontal gene transfer through transformation, transduction, conjugation, transposons (jumping genes) etc. The rapidity of development of antimicrobial resistance in organisms, leads to selection pressure of antibiotics like 3rd generation of cephalosporin- ESBL inhibitor combination, Monobactams and Carbapenems. Recently, Carbapenem resistant Enterobacteriaceae (CRE) pose a real threat to Medical fraternity as the increased frequency

with which Enterobacteriaceae cause infection and the mortality associated with infection caused by CRE and ESBL producing bacteria. In mid1990, CTX-M 15 was first reported as ESBL in India. Now, CTX-M 15 is established as globally dominant ESBL and primary cause of acquired resistance to 3rd generation Cephalosporins in Enterobacteriaceae. Walsh TR et al in year 2005 noted that MBL genes have spread from *Pseudomonas aeruginosa* to Enterobacteriaceae and a clinical scenario for MBL appears to simulate the global spread of ESBL in recent future. bla_{NDM-1} gene on plasmid can be readily transferred between different strains of bacteria by horizontal gene transfer [47].

In the present study, 52(11.6%), 56(12.4%) and 342 (76%) *E.coli* strains were isolated from Outpatient Departments(OPDs), Intensive Care units (ICUs) and Inpatient Departments(IPDs) respectively. Maximum 25 (44.6%) *E.coli* strains were isolated from Medicine ICU (MICU) and High dependency unit (HDU). In a previous study conducted in our laboratory in 2008, Basak et al have already reported the incidence of ESBL producing *E.coli* in our hospital as 41.3% [67] whereas 5 years after, in the present study, the incidence of ESBL producing *E.coli* were 68%, out of which only 13.8% strains produced ESBL alone and other strains produced ESBL, Amp C β -lactamases and MBL in combination. Pakzad I et al in 2011 have reported 28% of their *E.coli* strains as ESBL producers [68]. Sinha et al in 2008 had reported that 40.8% of *E.coli* strains were ESBL producers and 24% were AMPC β -lactamase producers [69]. 37.5% and 47.8% of *E.coli* strains were reported to be Amp C β -lactamases producers in the study conducted in Chennai, India and Kolkata,India respectively, whereas in our study 65.3% *E.coli* were Amp C β -lactamase producers.

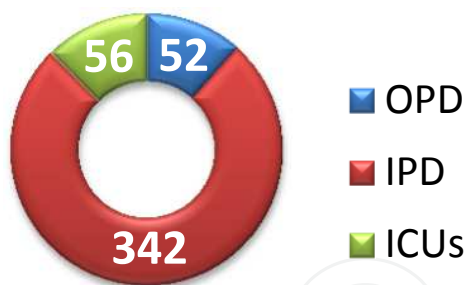
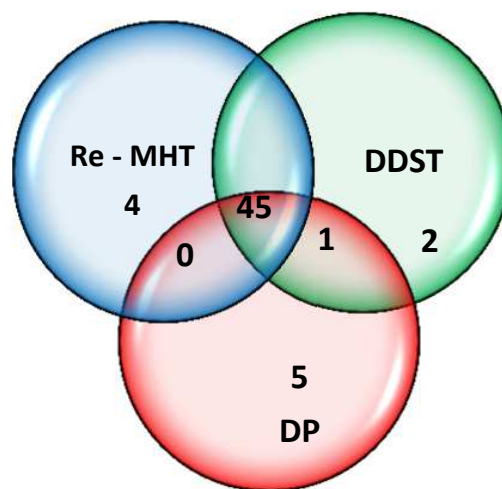


Figure 5. Isolation of *E.coli* strains from OPD, IPD and ICUs



Figure 6. Isolation of *E.coli* strains from different ICUs (n = 56)

Various authors have reported MBL producing *E.coli* strains from 2.9% (Pandya et al from Gujrat, India) [70], to 6.8% (Tsakris et al from Greece) [61] to 25% (Enwuru NV et al from Nigeria) [13]. In the present study 51(11.3%) MBL producing *E.coli* strains were isolated. MBL production was detected in both Imipenem resistant (39/41 i.e. 95.1%) and Imipenem sensitive 12/409 i.e. 2.9% strains also. It indicates that if only Imipenem resistant strains would have been screened, 2.9% MBL producing strains would have been missed. But no *Klebsiella pneumoniae* producing carbapenemases were detected in our present study. In 2011, Tsakris et al reported 15.9% KPC producing *E.coli* in their study. Tsakris et al have also reported that 19(43.2%) *E.coli* strains produced Amp C β –lactamases and ESBL and 15(34%) *E.coli* strains produced ESBL [61]. In another study in 2012, Gupta V et al have reported 17(68%) *E.coli* strains to be ESBL positive [71].



Disk Potentiation test (DP)
 True + ve : 51
 Re – modified Hodge test (Re – MHT) Double Disk Synergy test(DDST)
 True + ve : 45 True + ve : 46
 False + ve : 4 False + ve : 2

Figure 7. Phenotypic detection of metallo-beta-lactamase (MBL) producing *E.coli* strains by various methods. (n = 450)

Walsh et al in 2002 have reported that the MBL – E test results were in 100% agreement with the results from the genotypic Polymerase chain reaction (PCR) and biochemical methods [59]. They have also reported that the E test MBL strip IP/IPI has the ability to detect MBLs both chromosomally and plasmid mediated, in aerobic and anaerobic bacteria. This novel method could be used by Clinical Laboratories to monitor the emergence of the MBL [59].

Omair et al in 2012, have reported that MBL – E test have been taken as a gold standard method for MBL detection [72]. Manoharan et al have reported that MBL - E test has taken as a phenotypic standard method for MBL detection though the test is expensive. Double disk synergy test (DDST) and Disk potentiation (DP) tests are economical and simple to perform but DDST is observer dependent while DP test is measurable with lesser chance of subjective error [45].

In the present study, we studied MBL positive *E.coli* strains by MBL E – test and compared the results of other phenotypic methods for MBL detection i.e. Re – Modified Hodge test (Re-MHT), Double disk synergy test (DDST) and Disk potentiation (DP) test.

In figure 7, the venndiagram showing interrelationship of Re – modified Hodge test (Re – MHT), Double disk synergy test (DDST) and Disk potentiation test (DP) for detection of MBL producing *E.coli*.

In the present study when results of all three phenotypic methods were compared with MBL – E test results, it was found that 45/51 (88.2%) MBL positive strains were positive by all three phenotypic method i.e. Re – Modified Hodge test (Re- MHT), Double disk synergy test (DDST) and Disk potentiation (DP) tests. 04 and 02 were false positive by Re – MHT and DDST methods respectively, whereas 6/51 (11.8%) and 5/51(9.8%) were false negative by Re – MHT and DDST method respectively. Amongst all three phenotypic methods, DP was best correlated with MBL – E test. By DP test 51 MBL positive *E.coli* strains were detected and no false positive and false negative result was found (Sensitivity 100% and specificity 100%).

5. Epidemiology

E.coli are responsible for various infections like urinary tract infection, diarrhoea, pneumonia, bacteremia, upper respiratory tract infections, wound infections, osteomyelitis and neonatal meningitis [73,74].

The successful outcome of clinical use of 3rd generation cephalosporines unfortunately led to the increased use and emergence of ESBL producing Enterobacteriaceae. With the emergence of ESBL and Amp C β – lactamase production in *E.coli*, *Klebsiella pneumoniae* and other Enterobacteriaceae, Carbapenems were used as last resort to treat those infections. Because of selective pressure of Carbapenems, even carbapenemases producing Enterobacteriaceae (CRE) has emerged.

Most common MBL found worldwide in Enterobacteriaceae were VIM (Verona integron encoded MBL) and IMP (active on imipenem). Multidrug resistant *E.coli* harboring New Delhi metallo β lactamase - 1 (NDM-1) isolated from a patient returned to Canada from India [75], was reported first in 2009. NDM -1 was also recognized among Enterobacteriaceae 32 from Mumbai, 13 from Varanasi and 3 from Guwahati in India and 25 isolates from eight different cities in Pakistan. These isolates were from cases of bacteraemia, ventilator associated pneumonia and community acquired urinary tract infections [76].

NDM - 1 spread largely to different countries like Australia, Japan, Brazil, Belgium, Canada, Germany etc [77]. The gene encoding NDM – 1 is called bla_{NDM-1}, located on transmissible plasmid which may include other antibiotic resistance genes also leading to extensive drug resistant phenotypes (so called ‘superbugs’). A recent report from ICU and wards of Sir Gangaram hospital Delhi, India showed 8.1% NDM – 1 positive *E.coli* [78]. In January 2011, the name of NDM–1 was changed to PCM (Plasmid encoding Carbapenem resistant metallo β lactamases) [79].

Metallobatalactamases are also found in Carbapenem susceptible organisms. This hidden MBL gene can spread unnoticed in hospitals if isolates are reported sensitive without screening for presence of MBL [48].

The prevalence of ESBL and Amp C beta lactamases in a single isolate reduces effectiveness of beta – lactam and beta - lactamase inhibitor combinations while MBLs and Amp C beta–lactamases confer resistance to carbapenems and Cephameycin. Unfortunately these enzymes usually co-exist in same isolate.

6. Prevention and control

As *E.coli* are one of the commonest cause of both health care and community acquired infections, rapid identification of beta lactamase producing *E.coli* is crucial for appropriate treatment and timely implementation of infection control measures in Health care set-up. Indeed, delayed detection of ESBLs, Amp C β – lactamase and MBL producing strains, raise the possibility of spread of these strains into the community. These issues combined with the limited therapeutic options available to treat patient infected with these organisms, have made CRE of epidemiological importance globally [80]. ESBLs and Carbapenem resistant strains may lead to outbreaks of infection in HealthCare Set-up also.

Phenotypic methods can be useful for routine detection of ESBLs and carbapenemase production, among Gram negative bacteria particularly when PCR is not available.

Screening of colonisation with multidrug-resistant organisms (MDROs) upon admission to hospitals has been advocated in patients who have already received healthcare in endemic countries. The CDC recommends, if previously unrecognized cases are identified of being infected with β -lactamase producing strains, a round of surveillance culture from high risk areas i.e. ICUs or wards from where detected, should be considered in any Health Care Setup. In addition prompt notification, must be made to infection control team members when CRE are identified in Clinical Microbiology Laboratories.

Antimicrobial stewardship has been suggested as the most important efforts to control multidrug resistant organisms (MDROs) [81]. It has been found to be most effective, if efforts are directed towards an overall decrease in antimicrobial use rather than targeting a specific antimicrobial class. Limiting use of invasive devices is another potentially important preventive mechanism for MDROs including β - lactamase producing organisms. Health care workers (HCW) should follow hand hygiene practices while giving patient care preferably using an alcohol based hand rubs or antimicrobial soap and water if hands are visibly soiled, and also follow Standard precautions and Additional precautions as per the indications.

7. Conclusion

In the present study, all MBL – E test positive *E.coli* strains (100%) were detected by Disk potentiation test also. MBL producing *E.coli* strains must be tested in both carbapenem resistant

as well as sensitive strains by Disk potentiation method using Imipenem – EDTA. Disk potentiation method is simple to perform and materials used are cheap, non-toxic, and easily accessible and allowed for objective interpretation of results. It is also quite good in detecting carbapenem sensitive MBL producing strains.

Beta-lactamase producing organisms are detected by E test, which is standard phenotypic method and also by Polymerase chain reaction (PCR) which is a gold standard, but both are costly and require expertise. Failure to detect these enzymes has contributed to their uncontrolled spread and commonly to therapeutic failures.

Hence to conclude, for detection of ESBL, combined disk method using Ceftazidime / Ceftazidime Clavulanic acid(CAZ/CAC), for detection of Amp C β -lactamases confirmatory Disk potentiation test using Ceftazidime / Ceftazidime - 3-aminophenylboronic acid and for detection of Metallo β -lactamases(MBL) producing *E.coli*, disk potentiation test using Imipenem/Imipenem - EDTA should be done in both carbapenem sensitive as well as resistant isolates by all Clinical Microbiology Laboratories to prevent its dissemination and also for a good therapeutic outcome.

Author details

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