OCCURRENCE AND DETECTION OF AMPC B LACTAMASE AMONG CLINICAL ISOLATES OF ENTEROBACTERICIAE AT A TERTIARY CARE CENTRE

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CERTIFICATE- I

This is to certify that the dissertation work entitled **"Occurrence and detection of AmpC β lactamases among clinical isolates of Enterobacteriaceae at a tertiary care hospital"** submitted by **Dr. M. Parimalam,** is work done by her during the period of study in this department from January 2016 to July 2017. This work was done under the guidance of **Dr. J. Jayalakshmi**, Professor, Department of Microbiology, PSG IMS&R.

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CERTIFICATE- II

This is to certify that this dissertation work titled **"Occurrence and detection of AmpC β lactamases among clinical isolates of Enterobacteriaceae at a tertiary care hospital"** of the candidate **Dr. M. Parimalam** with registration number **201514404** is for the award of the degree **M.D. Microbiology, Branch IV.** I personally verified the urkund.com website for the purpose of plagiarism check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows 5% percentage of plagiarism in the dissertation.

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DECLARATION

I **Dr. M. Parimalam**, do hereby declare that the thesis entitled, **"Occurrence and detection of AmpC β lactamases among clinical isolates of Enterobacteriaceae at a tertiary care hospital"** is a bonafide work done by me under the guidance of **Dr. J. Jayalakshmi**, Professor, Department of Microbiology, PSG Institute of Medical Sciences & Research. This study was performed at the PSG Institute of Medical Sciences & Research, Coimbatore, under the aegis of the The Tamilnadu Dr MGR Medical University, Chennai, as part of the requirement for the award of the MD degree in Microbiology

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Introduction

INTRODUCTION

Discovery of antibiotics to treat infections caused by bacteria has been one of the most important developments of modern medicine. However, widespread antibiotic usage has led to the rise of drug resistance among bacteria. Now, antibiotic resistance is a serious global problem, resulting in increased health care costs, morbidity and mortality. (1)

Mechanism of bacterial resistance to antimicrobial agents is complex and dynamic; the important ones being, production of ß – lactamases, AmpC Class ß – lactamases and Metallo- ß – lactamases.

AmpC class ß – lactamases are cephalosporinases that are poorly inhibited by clavulanic acid. They can be differentiated from other extended spectrum ß – lactamases (ESBLs) by their ability to hydrolyse cephamycins like cefoxitin as well as other extended – spectrum cephalosporins. (1) Organisms producing plasmid mediated Amp-C ß – lactamases were first reported in the 1980's. The genes are encoded on large plasmids containing additional resistance genes leaving few therapeutic options.(1) Amp- C ß – lactam resistance (AmpC-R) in Enterobacteriaceae, their spread among other members and treatment failure with broad spectrum cephalosporins have been documented. (2) Detecting Amp C isolates is clinically important, not only because of their broader cephalosporin resistance, but also because carbapenem resistance can arise in such strains by further mutations, resulting in reduced

porin expression. [3,4] However, most clinical laboratories and physicians remain unaware of their clinical importance.

Current detection methods of AmpC – R is challenging and technically demanding on a routine basis. There are no CLSI guidelines for its detection. Multiplex PCR for AmpC-R detection is available as a research tool, but is expensive and is not yet available for routine use. As a result, organisms producing these types of ß – lactamases often go undetected and therefore have been responsible for several nosocomial outbreaks [5]

We wished to address this issue by evaluating various phenotypic methods to detect AmpC ß – lactamases and compare against the genotypic methods .

Aim and Objectives

AIM AND OBJECTIVES

AIM:

To evaluate the presence clinical of Amp C beta- lactamase among and isolates of Enterobacteriaceae Amp C to detect the genetic basis for production of these strains.

OBJECTIVES:

- To screen for Amp C β – lactamase producing clinical isolates of Enterobacteriaceae.
- To compare different AmpC phenotypic detection methods.
- To detect the presence of Amp C genes among these clinical isolates and compare against the phenotypic methods.
- To know the antibiotic sensitivity pattern of these clinical isolates.

Review of Literature

REVIEW OF LITERATURE

HISTORY OF AmpC BETA-LACTAMASES

The frequency of Gram negative bacterial infections producing disease has varied widely over the past century. Before the use of antibiotic drugs, Gram negative infections were uncommon in the 1920 and 1930's. [6] In the 1960's to 1980's, percentage of infections caused by Gram negative pathogens increased. Prior exposure to antibiotic drugs was, and remains today, a principal risk for developing Gram negative infections .

In later years, Gram positive pathogens became more prominent. because of introduction of 3rd generation in 1985. Since, 1990's gram negative pathogen became prominent. Gram positive pathogens are treated with penicillin's, 1st, 2nd, 3rd generation of cephalosporins of beta-lactamase,

Beta-lactamases are heterogenous bacterial enzymes that cleave the beta-lactam ring of penicillins and cephalosporins to inactivate the antibiotic. [7] ESBL are enzymes that mediate resistance to 4th generation cephalosporins and monobactam, but, do not affect cefamycins, or carbapenems. They are inhibited by beta-lactamase inhibitor combinations such as clavulanic acid, sulbactam & tazobactam. Therefore, organisms

resistant to 3rd generation cephalosporins but sensitive to beta-lactam- beta-lactam inhibitor combination are likely to contain ESBL. (8) they; also named, as penicillinase, are enzymes produced by bacteria that provide multiresistance to beta- lactam antibiotics as penicillin, cephalosporin, cephamycin, carbapenems., they are relatively resistant to beta-lactamase.

These drugs have a common compound in the molecular structure, a four - atom ring called as Beta-lactam. (8) The lactamase enzyme breaks the beta-lactam ring open, deactivating the molecules of antibacterial properties. These are encoded by chromosomal genes, and by transferable genes on plasmids, and transposons.

They frequently reside on integrons which often carry multiple resistance determinants. Among Gram negative organisms, the rise in ampicillin resistance is due to the TEM-1 a plasmid encoded beta-lactamase named after a Greek patient Temoniera, in whom the first isolate was recovered. (9) These antibiotics are mostly used to treat a broad spectrum of Gram positive and Gram negative bacteria.

PENICILLINASE:

Penicillinase is a particular type of beta-lactamase, appearing to have specificity for penicillins, hydrolyzing the beta-lactam ring. Weight of penicillinases tend to cluster near 50 kD. Penicillinase was the first beta-lactamase to be identified. It was first isolated by Abraham and Chain in 1940 from Gram negative *E.coli* . Beta-lactam antibiotics are a broad class of antibiotics, that contain a beta lactam ring in their molecular structure. It includes penicillin derivatives, cephalosporins, monobactams and carbapenams.

AmpC CLASS BETA-LACTAMASES:

Among the beta-lactamases, the most common is production of ESBLs & AmpCs. Ambler class C and Bush group 1 beta-lactamase enzyme, AmpC Beta-Lactamases are being reported worldwide. These enzymes confer resistance to penicillin, 1^{st}, 2^{nd} and 3^{rd} gen cephalosporins cephamycin,and monobactams. These are poorly inhibited by clavulanic acid , sulbactam etc. but are inhibited by cloxacillin, and phenyl boronic acid. These are cephalosporinases which are not inhibited by Clavulanic acid, tazobactam & sulbactam. Amp C Beta-lactamase production is frequently associated with production of multi drug resistance. (10) Gram negative bacilli resistant to

beta- lactams are increasingly being isolated from ill and hospitalized patients.

Selected AmpC Beta lactamases of Gram negative bacteria:

ACC-1, ACT-1, CEF-1, CMY FAMILY, DHA-1, DHA-2, FOX FAMILY, LAT FAMILY, MIR-1, MOX-1 AND MOX-2. [11]

Amp C Gene:

Transmissible plasmids have acquired genes for Amp C enzymes, which consequently can now appear in bacteria lacking or poorly expressing a chromosomal bla Amp C gene such as *E.coli, Klebsiella,* and *Proteus mirabilis.*

Amp C INFECTION:

Carbapenems can usually be used to treat infection due to Amp C producing bacteria, but, carbapenem resistance can arise in some organisms by mutation that reduces influx- outer membrane porin loss / enhanced efflux or efflux pump activation.

The first bacterial enzyme reported to destroy penicillin was the Amp C beta-lactamase of *E.coli.* In 1965, Swedish investigators started a study of genetics of penicillin resistance in *E.coli.* (12) Mutations with stepwise increased resistance were termed ampA & ampB.

Amp A strains overproduced beta lactamase, was regulatory role for the ampA gene. Amp B gene were found to have an altered cell envelope, AmpC was the structural gene for the enzyme In 1981, ampC gene from *E. coli* was reported, it differed from TEM-1 penicillinase.

Amp C enzyme has molecular wt of 40,000 and pH was alkaline. (13) It has hydrolytic activity, and rapid action in 1^{st} gen of cephalosporins, slow but kinetically effective in 2_{nd} & 3_{rd} gen , slow kinetically ineffective in 4_{th} gen. and poorly inhibited by Sulphones, but, Carbapenams are nearly stable & not inhibited by Clavulanate.

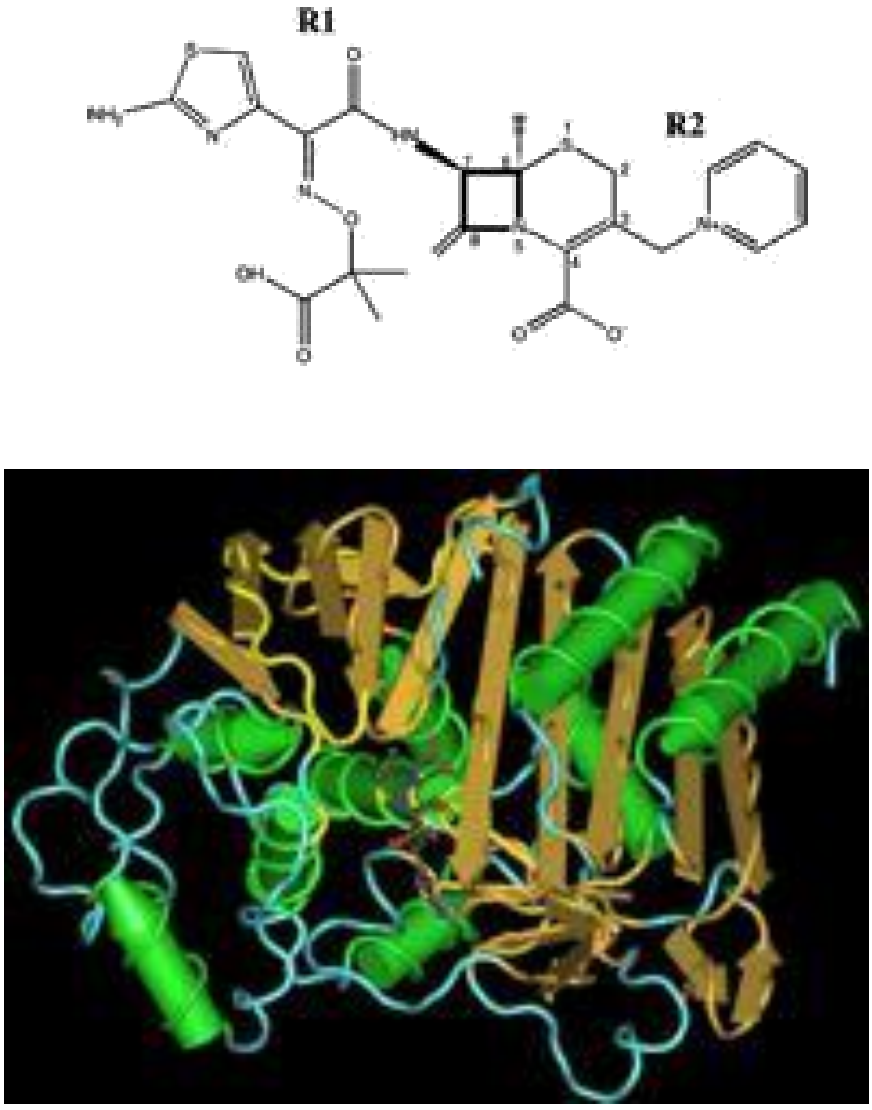


Fig -1 Molecular structure of AmpC beta lactamases from *E coli*

CLASSIFICATION.

Beta-Lactamases Can be classified according to their aminoacid structure in to four molecular classes A through D as first suggested by Ambler. [14] The Bush- Jacoby- Medeiros system classifies the enzymes according to their substrate profile and susceptibility to beta- lactamase inhibitors such as clavulanic acid in to several functional groups. [3]

Class A, C & D-Hydrolyse the β-lactam ring through a serine residue at their active site.

Class B- use Zinc to break the amide bond.

CLASS C Amp C- type beta-lactamases.

Class C type beta-lactamases that hydrolysed cephems, cephamycins, cephalosporins, Broad spectrum penicillins, monobactams beta-lactamase inhibitors.

Table – 1 Classification of Betalactamases

Functional group	Major sub group	Molecular class	Functional group	Inhibition by clavulanate
1		C	Cephalosporinases often chromosomal enzymes in GNB, but, may be plasmid encoded, confer resistance to all classes of β-lactams,	--
2	2a	A	Penicillinases, confer resistance to all penicillins, primarily from Staphylococcus, Enterococci.	+
	2b	A	Broad spectrum β-lactamases primarily from GNB.	+
	2be	A	ESBLs confer resistances to Oxyimino-cephalosporines& monobactams	+
	2br	A	Inhibitor resistance TEM β-lactamases	- (+) for tazobactam
	2c	A	Carbenicillin hydrolyzing enzymes	+
2	2d	D	Cloxacillin hydrolyzing enzymes	+/_
	2e	A	Cephalosporinases confer resistance to monobactam	+
	2f	A	Carbapenem-hydrolysing enzymes with active site serine.	+
3	3a,3b,3c	B	Metallo β lactam classes,except monobactams.	_
4			Miscellaneous unsequenced enzymes that do not fit into other groups	_

Types of AmpC beta-lactamases.

Chromosomal- & plasmid mediated.

SPICE. ORGANISMS. :

Colloquial acronyms for Gram negative bacteria that have inducible, chromosomal, β – lactamase genes known as AmpC .Resistance may not be detectable initially, but, appears after a period of exposure to β – lactam antibiotics. Organisms in these groups include, *Serratia spp, Pseudomonas, indole positive Proteae, Providencia, Morganella morganii,Citrobacterspp, Enterobacter* spp. [15] These organisms are more virulent than susceptible gram negative bacteria.

Plasmid-mediated; transmissible.. Mostly detected organisms without intrinsic amp c gene are *Ecoli ,Klebsiella ,Proteus mirabilis & Salmonella spp.,*

Amp C Beta- lactamases are typically encoded on the chromosome of many gram –ve bacteria including *Citrobacter, Serratia, and Enterobacter spp* where its expression is usually inducible. AmpC type beta-lactamases may also be carried on plasmids. [16]

Amp C beta-lactamases, in contrast to ESBLs, hydrolyse broad and extended spectrum cephalosporins, but are not inhibited by beta-lactamase inhibitors such as clavulanic acid

Differentiating ESBL & beta-lactamases.

ESBL& AmpC β-lactamases are in a variant of de-repressed state, cause hydrolysis of β-lactams, it can be difficult to identify the cause of resistance. But,both these are share the hydrolytic activity to the penicillins and the 1^{st},3^{rd} generation of cephalosporins. And they are inherently resistance to the inhibitors. such as Amoxy-clavulanate, Ampicillin- sulbactam, and in some cases of Piperacillin-Tazobactam. [17] Conversely, ESBL- producing organisms may or may not be resistance to these same agents. AmpC producing organisms are susceptible to ATM, where ESBL are fundamentally resistance to ATM.

Even in a wild type strain, Ampc β lactamases will hydrolyse against the cephamycins ,but ESBLs are susceptible to cephamycins. Fourth generation cephalosporins are very stable opposite to AmpC beta-lactamases, and relatively stable against ESBLs.

Different type of resistances are played. One of the resistance type is Acquired resistance to cephalosporins is due to

1. Affinity of antibiotics was reduced due to altered target proteins of PBS

2. Impermiability of antibiotic, so it does not reach the active site of action

3. Elaboration of beta-lactamases which destroy cephalosporinases.

AmpC Beta Lactamases can be differentiated by their ability to hydrolyse cephamycins like cefoxitin as well as other ESBL. (18)

Organism producing plasmid mediated AmpC Beta- lactamases were first reported in 1980's, after the introduction of 3rd generation cephalosporins they acquire Amp C beta-lactamases on plasmids and hyper produce chromosomal AmpC , which is normally produce at lower level

Genes for AmpC also found on plasmids that transfer non inducible cephalosporin resistance.

Amp C β-Lactamase resistance.

AmpC producing organisms causes reistance to penicillins & cephalosporins through hydrolyasis and opening of the beta-lactam ring, it will also producing inherently resistant to the activity of cephamycins.

Genes encoding for AmpC β-lactamases are plasmid mediated or chromosomally mediated. (19) In general chromosomal AmpC will undergo hyperproduction in the suitable environment. Some gram negative organisms of 'SPACE' ORGANISMS are in a state of chromosomally mediated Amp C hyperproductive state. Here,

Ampicillin, Amoxycillin, and Cefazolin are regarded as strong inducers, excellent substrate of Amp C β-lactamases.Beta-lactamase inhibitors are also inducers of AmpC and can gives to treatment failure with drugs appeared susceptible. AmpC β-lactamase commonly with in the

periplasm porin entry of these agents is the rate limiting step in hydrolysis by AmpC β –lactamases, due to the zwitterionic structure , does not require porin for entry to the periplasmic space can overcome the inactivation rate. (20) AmpC genes located on plasmids are constitutively produced, where as chromosomally mediated Amp C acting in a hyper production state. Baseline state represses AmpC production.

The antimicrobials that hydrolyse bacterial cell wall, a series of 1,6,anhydro-N-acetylmuramic acid peptides are produced and they buildup they compete with uridine di phosphate- N –acetylmuramic acid peptides for binding with Amp R, a transcriptional regulatory enzyme thyat at a base line

Another regulatory protein, AmpD, It was responsible for cleavage of stem peptides from 1,6,anhydro-N-acetyl muramic acid which decreases their affinity to bind to Amp R

Induction of AmpC transcription occurs as increased concentration of 1,6, anhydro N acetyl muramic acid, so not able to cleave all of the stem peptides leads to increased Amp C transcription. So induction only explains the wild type resistance profile of the AmpC producing organisms. Stable de-repression is felt to be the reason for AmpC over expression leading to resistance but, susceptible in vitro. [21] This occurs secondary to Amp D mutation., which ultimately inhibit cleave of peptide bond. These resistance mechanism, identify the most clinically prudent empiric antimicrobial therapy difficult .

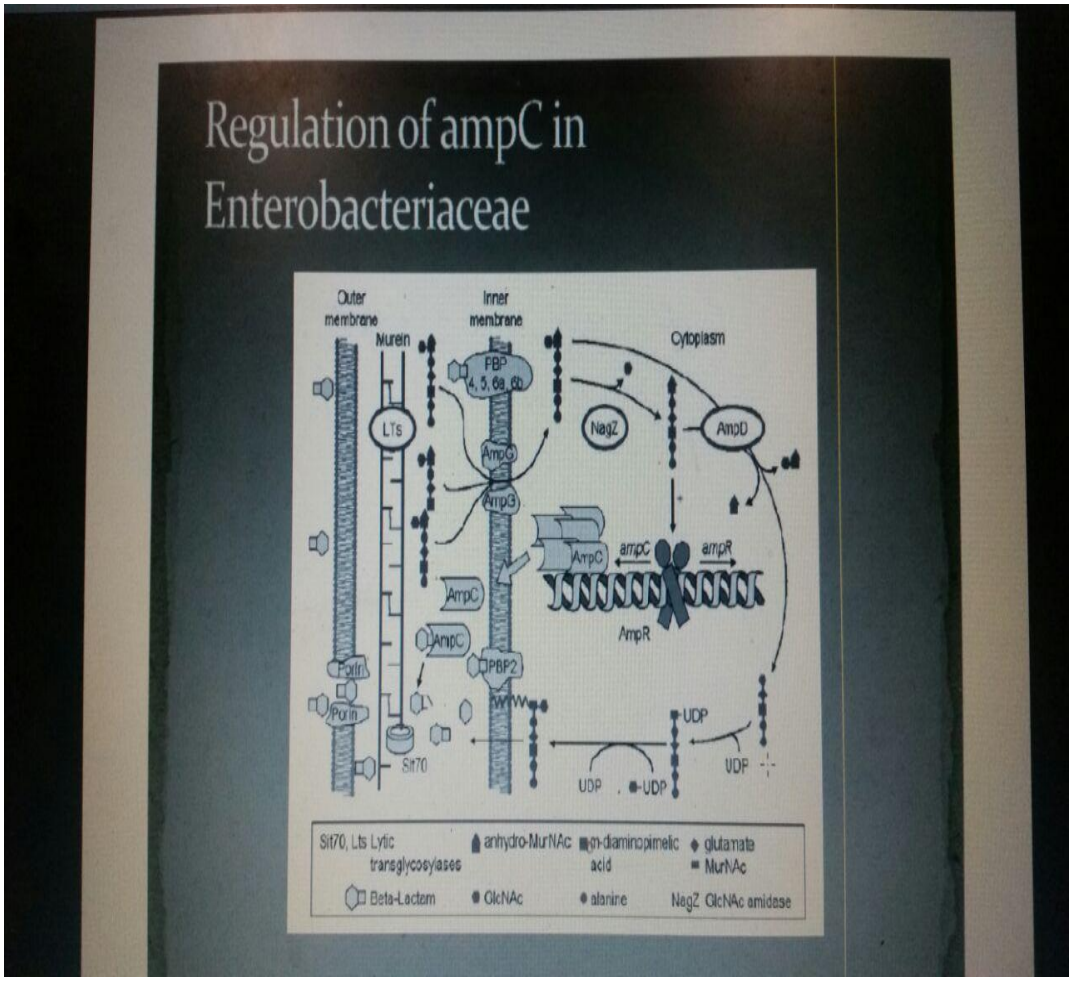


Fig -2 : Regulation of AmpC in Enterobacteriaceae

Beta lactam differ in their inducing abilities strong inducers, good substrates for AmpC betalactamase are ampicillin, amoxicillin, benzyl penicillin& Ist gen of cephalosporins. Cefoxitin, imipenam are also strong inducers but are much more stable for hydrolysis.

Weak inducers and weak substrates but can be hydrolysed if enzymes are supplemented are 3 rd, 4 th gen cephalosporins, piperacillin & aztreonams. MIC of weakly inducing oxyimino beta lactam are increased with AmpC hyperproduction. [22] But, MIC of strong inducers shows little change with regulatory mutations.

Beta lactam inhibitors are also inducers, especially clavulanate, has little inhibitory effect on AmpC, but can paradoxically appear to increase AmpC mediated resistance.this effect of clavulanate is important for P.aeruginosa. and this antagonize the antibacterial activity of Ticarcillin. [23]

PUMPS & PORINS:

Important determinants of the resistance spectrum is the rate at which the substrate is delivered to the enzymes. The concentration of β-lactam substrate in the periplasm is a permeability function of the cell's outer membrane, and through the porin channel β-lactam penetrate and of efflux pump, which transport them out of the cell. Vu & Nikaido noted out, concentration of β-lactam in the periplasm necessary to inhibit target penicillin binding protein, decreasing the number of porin entry channel or increasing efflux pump expression can lower influx and further augments enzyme deficiency.[24] But, carbapenem resistance in clinical isolates involves various combination of overproduction of

AmpC, decreased production of porin channel for drug entry, and activation of other efflux systems. Zwitterionic molecules such as Cefepime,& Cefpirome have the advantage of penetrating the outer bacterial cell membrane rapidly than Cefotaxime, Ceftrioxone.[25] .

Global distribution of strains producing plasmid determined cephalosporinases. They have been found in

Africa, - Algeria, Tunisia.

Asia; India, Japan, Pakistan, South Korea.

Europe; France, Germany, Greece, Italy, Sweden, UK.

Middle east ; Saudi Arabia,

North America; US.

South ; central America

Plasmid mediated AmpC β lactamase have been discovered world wide, according to resistance produced by

CMY ; Cephamycin ‘ 60.00.00,000- 60 Crore

FOX ; Cefoxitin. ; 1.55,00,000

MOX ; Moxalatem ; 1.04,00,000

LAT ; Latamoxif ; 3,00,000

To the type of β lactamase such as AmpC type ; ACT ; 20,00,000

ACC ; (AMBLER CLASS C)

To the site of discovery MIR; (MIRIAM HOSPITAL IN PROVIDENCE)

-69,00,000

DHA ; Dharan hospital in Saudi arabia. ; 1.07,00,000

In Greece - Plasmid mediated LAT-2, CMY-2, have been found in clinical isolates of *Enterobacter aerogenes* simultaneously with its appearance in clinical strains of *E.coli, & Klebsiella.* In France; plasmid mediated ACC -1 found in *E.coli, Proteus spp,* from urine samples.

In UNITED STATES ; Ceftrioxone resistance *Salmonella* was isolated from symptomatic patients.

CMY-2; Spread from Pakistan to UK , India TO UK, Algeria to France.

CMY-4; From India to Sweden.

Mox-2;p Greece to France.

ACC; Tunisia to France, FOX - from Guatimala to Germany.

Most of the plasmid mediated Amp C enzymes are isolated from ICU,or post operative, post organ transplant cases, malignancy with

antimicrobials users Most Amp C producing nosocomial infections are the causes of outbreaks.

PHYLOGENY:

The ancient enzymes of serine beta lactamases were originated more than 2 billion yrs ago. AmpC enzymes are divided from a common ancestor in to class A & class D.AmpC enzymes from organism of same genus cluster together, but, AmpC beta lactamases of *Pseudomonas*, Enterobacteriaceae, *Acinetobacter* are distinctly related. [26]

PLASMID –MEDIATED AmpC BETA LACTAMASES.

Plasmid mediated Amp C beta-lactamase.

Since, 1989 plasmid –encoded AmpC genes found around the world in nosocomial isolates and easily detected in Enterobacteriaceae family. Minor differences in amino acid sequence given rise to families,

43 CMY alleles, 7 varietes of FOX, 4 varieties of ACC, LAT, & MIR, 3 varieties of ACT, MOX, 2 variety of DHA. These are determined by chromosomal genes, and represent progenitor for the plasmid - determined enzymes.

This plasmid enzyme- related, and very closely to chromosomally determined AmpC enzymes. (27)

Ex- CMY has 6 varieties;CMY-1, 8, 9, 10,11,& 19 related to chromosomally determined AmpC enzymes in *Aeromonas spp*. Other enzymes are related to *Citrobacter freundii*.

LAT-2 was identical to CMY-2, LAT-3 was identical to CMY-6, and LAT4 was to LAT-1

Plasmid – mediated enzymes confer resistance to broad spectrum of beta-lactams including penicillin, oxy imino- beta –cephalosporins, cephamycins, Astreonam. but, ACC-1 resistance to cephamycins, and cefoxitin inhibited. The genes for ACT-1,DHA-1,DHA-2,& CMY-13 are linked to ampR genes, and are inducible. while others are not. AmpC plasmids lack ampD genes, but the expression of ACT-1 increased with the loss of chromosomal Amp D function. (28)

AmpD – deficient *E. coli* producing ACT-1 sensitive to imipenam, but, the same in *K.pneumoniae* carrying ACT-1 plasmid associated with a loss of outer membrane porins, they provide, resistance to carbopenams, oxy-imino-beta- cephalosporins. But, remain sensitive to cefepime.

Plasmids carrying genes for AmpC beta- lactamases carry other gene resistance including aminoglycosides, chloramphenicol, quinolones,

sulphonamides, tetracyclines, trimethoprims, & TEM-1, CTX-M3,SHV varieties. The AmpC gene is usually a part of integran and the same gene can be incorporated in to different backbone on different plasmids. A variety of genetic elements are implicated in the mobilization of AmpC genes onto plasmids. [29]

The insertion sequence associated with many CMY alleles- CMY-2,4,5,7, 12, 14 15,16,21,31,& 36, as well as ACC-1, 4. This sequence plays a dual role, and involved in the transposition of adjacent genes,has ability to mobilize a chromosomal bla gene on to a plasmid. It can supply an promoter for the high level expression of neighbouring gene other. Bla-AmpC genes are situated adjacent to an insertion sequence common region, involved in gene mobilization in to class1 integrons. [30]

Genes for several CMY varieties , DHA-1, MOX-1, are so linked. But, the gene for CMY-13, and its ampR gene are bounded by directly repeated IS26 elements made up of a transposase gene with inverted terminal repeat segments. Other elements are involved in capturing the genes for FOX-5, MIR-1, &MOX-2.

Chromosomal AmpC Enzymes.

High level AmpC beta-lactamase production by mutation, the development of resistance upon therapy is concern. Ex - biliary tract infection with malignant bile duct obstruction was identified as risk factor for resistance development. Combination therapy did not prevent resistance emergence.low level expression of AmpC beta-lactamase in *E.coli*, high level producers identified in clinical specimens,as cefoxitin - resistance isolates with stronger AmpC promoters or mutations that destabilize the normal AmpC attenuater. (31)

PCR, and sequencing in different promoter or attenuator varients. In a few strains, the integration of an insertion element created a new & stronger ampC promoter. (32) These strains are not only resistant to cefoxitin but also typically resistant to ampicillin,, ticarcillin,cephalothin, and beta- lactam combination with clavulanic acid and have reduced susceptibility or resistant to ESBL .

Few *E. coli* strains with up promoter mutation have some alteration in bla AmpC , expanding its resistance spectrum, with loss of outer membrane porins can augment the resistance phenotype further. These strain remain susceptible to cefepime and imipenem, but, may become ertapenem resistance upon therapy is concern. .

Clinical relevance:

Plasmid- mediated AmpC enzymes.

Infection caused by gram – ve organisms expressing plasma mediated AmpC beta-lactamases. But, its action limited, because they are usually resistant to all beta- lactam antibiotics. Except for cefepime, cefpirome, and the carbapenams constitutive over expression . of AmpC beta-lactamases in gram –ve organisms occur either by deregulation of the amp C chromosomal gene or by transferable ampC gene on a plasmid.the transferable ampC gene products are commonly called plasmid mediated AmpC beta- lactamases. [33] Mobilization from the genome of species carrying inducible/ de- repressed bla/ampC such as *Citrobacter frreundii* and *Morganella morgagnii* by plasmids in to *E. coli,* and *K. pneumonia*.

The recognition of plasmid mediated AmpC beta-lactamases in *E.coli* and *Klebsiella spp*, the world wide distribuition AmpC resistance is important. So, very difficult to detect clinically, and no CLSI guideline also. The detection of AmpC production is challenging, since the hyperproduction of enzyme in association with OMP F porin loss in *E.coli* or porin deficiency in *K.pneumoniae* can produce phenotypic resistance. Detection of plasmid-mediated AmpC producing isolates is critical for epidemiological studies, hospital infection control. Because the gene can be spread to other organisms.

Genes for Amp C β- Lactamases are found on chromosomes of several members of the Enterobacteriaceae family including E. coli, Enterobacter, shigella, providencia, Citrobacter freundii, Morganella morganii, Serratia marcesens, (34)

Amp C β-LACTAMASE- associated Diseases

In recent years, various studies, have been conducted on the occurrence of AmpC producing bacteria in humans. It is being observed more and more AmpC Producing bacteria play a major role in health care facilities as the pathogen that cause so called Nosocomial infection, Hospital acquired infection, & risk of infection via food with the different type of pathogens , especially *Salmonella*, *EHEC*, *Klebsiella*., and also produced wound infection, urinary tract infection, VAP,Meningitis, septicemia, CAUTI, mortality and morbidity increased in immunocompromised patients, than immune competent. (35)

To Enable bacteria to produce AmpC, they must carry the necessary genetic information- Resistance genes. As they are passed from one bacteria generation to the next during cell division called "VERTICAL TRANSFER', the propagation and distribuition of these bacterium also contribute towards the spread of the resistance genes. Poor hygiene, in hospitals, animal shed, & home play a major role in the carryover of the bacteria.

Because the resistance gene vary often lie on transmissible gene section, they can also be e xchanged between bacteria of same type or different types called .HORIZONTAL GENE TRANSFER' The big problem here is the harmless intestinal bacteria can pass on the genes for Amp C to pathogenic bacteria, such as ' Salmonella' Infections are transmitted by food stock home handlers, pet animal handlers, health care workers. So, risk of infections occurred by in between animals or animals to human by pet handlers. (36)

In India Amp C enzyme producers were found among Gram Negative bacteria in Guru Tegh Bahadur Hospital Delhi in 2003. In the same year Subha et al., in Chennai, Shahid et al in Aligarh and Ratna et al., in Karnataka reported Amp C β lactamase producing Enterobacteriaceae in the clinical isolates. (38)

Detection of Amp C

Detection of the resistance medicated by class C β Lactamases remains a challenging issue considering that transferable plasmid mediated class C β – lactamases are of world wide concern. Several methods that use the Kirby- Bauer disk potentiation method with some β Lactamases inhibitors or the three dimensional methods have been developed and a cefoxitin agar medium based assay that uses preparations of bacterial cell extracts also developed. However these methods are technically intricate and interpretation

of their results in not sufficiently simple for routine use in clinical microbiology laboratories. PCR or multiplex PCR analysis are able to provide satisfactory results in the identification and classification of genes for β Lactamases, but equipment availability is limited to medical institutions such as university hospital. They are costly and require time consuming techniques. An enzyme-linked immunosorbent assay has also been developed and has known sensitivity and specificity for the detection of certain class C β - Lactamases. This technique is less costly than genetic methods, but it is not sensitive for the detection of class C β Lactamases that possess less than 70% homology to CMY – 2. [39] Thus practical and simple methods for detection of the resistance medicated by plasmid- mediated class C β- Lactamases are urgently needed for enhanced infection control.

There are no CLSI or other approved criteria for Amp C detection.organism producing Amp C beta lactamase gives positive ESBL screening test, but fail the confirmatory test and increased sensitivity to clavulanic acid so, confirmatory test are needed.

Phenotypic method;

- Amp C disc test
- Disc antagonism test
- Ceftazidime- imepenem disc antagonism test.
- Boronic acid inhibitor based test
- Cloxacillin combined disc diffusion test
- Double disc synergy test
- Modified three dimension test

MOLECULAR METHOD BY MULTIPLEX PCR METHOD.

Phenotypic test cannot distinguish various families of plasmid mediated Amp C enzymes but, also identified chromosomally determined Amp C enzymes with an ESBL. For this, the current method of Gold standard for plasmid mediated Amp C detection, multiplex PCR has been improved by utilizing Six primer pairs,to which a seventh pair for CEF-1 beta lactamase could be added. [40] Compared to ESBL producers, isolates producing AmpC beta-lactamase are resistant to additional betalactams and betalactam inhibitors and developing resistance to Carbapenems.

PREVENTION AND CONTROL OF INFECTION CAUSED BY AmpC PRODUCING ORGANISMS.

Amp C Producing bacteria are carried in the feces, which may spread via food chain, thereby producing reservoir of multiple resistant bacteria in the gut. It can be spread from person to person by contaminated hand, untrained person handling with urinary catheter in hospitalised patient, so, urinary infection commonly occur in patient after admission, also associated chest infection, wound infection causing septicemia, so, increasing prevalence of mortality and morbidity .

Common principle of control measures are Hand washlng standard precaution and scrupulous hand hygiene are used. Area of concern, particularly transmission of infection occur in neonatal ward surgical ward, ophthalmic ward, post operative ward, & important of Burns patient care are needs intervension therapy

There are different type of MDR –GNB are greate or lesser concern, flexibly judged by Microbiologist,& Infection prevention, control team. So, continuous screening, review, diagnosis are done. Drug resistant pattern are continuously monitored.

Decontamination procedures are followed, excessive usage of antimicrobials are avoided or controlled by Health board team. Avoid reusable drugs infection surveillance is very important.

Materials and Methods

MATERIALS AND METHODS

This study was conducted after obtaining IHEC approval at the Diagnostic microbiology department, PSG Hospitals.

STUDY PERIOD: Jan 2016 – July 2017.

GEOGRAPHIC AREA: PSG Hospitals, Coimbatore

SAMPLE SIZE:

Out of the 71067 various specimens received for culture and sensitivity at the microbiology department during the study period, 16552 microorganisms were isolated, of which 7123 (43.3%) were enterobacteriaceae identified after processing by standard microbiological techniques, colony morphology Gram's staining, motility and biochemical reactions. (Fig-3a & 3b).

Sample size determination:

Formula used : n= t 2 x p (1 – p) / m^2

Where,

n = required sample size.

t= confidence level at 95% (standard value of 1. 96)

p= estimated prevalence of Amp C producers .

m = margin of error at 5% (standard value of 0.05)

Es timated prevalence from hospital statistics (p) = 0. 02

n = 1.96 x 1. 96 x 0. 2 (1 – 0. 2) / 0. 05 x 0.05

n= 245 .8 (250)

Sampling: Consecutive sampling

Inclusion Criteria: All Gram negative clinical isolated identified as Enterobacteriaceae

Exclusion Criteria:

1. Gram positive Cocci / Bacilli
2. Gram neagative bacilli other than Enterobacteriaceae

FLOW CHART:

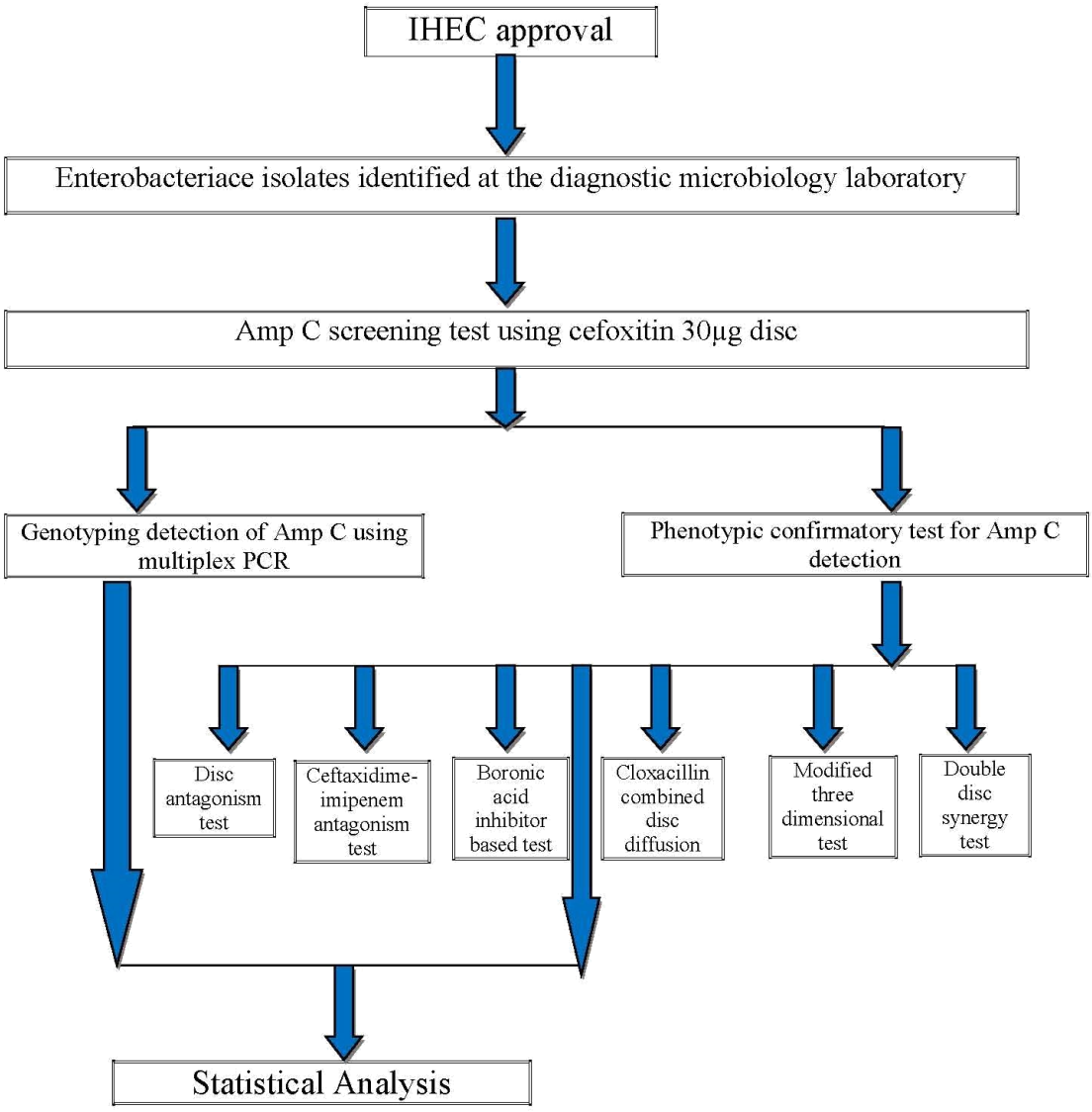

IHEC approval
Enterobacteriace isolates identified at the diagnostic microbiology laboratory
Amp C screening test using cefoxitin 30µg disc
Genotyping detection of Amp C using multiplex PCR
Phenotypic confirmatory test for Amp C detection
Disc antagonism test
Ceftaxidime-imipenem antagonism test
Boronic acid inhibitor based test
Cloxacillin combined disc diffusion
Modified three dimensional test
Double disc synergy test
Statistical Analysis

Fig – 3a: Culture and biochemical characteristics of *E coli*

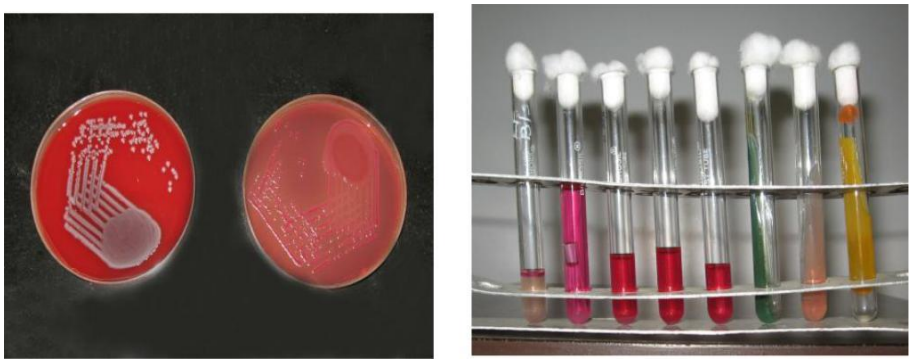


Fig – 3b: Culture and biochemical characteristics of *Klebsiella pneumoniae*

The enterobacteriacae identified were subjected to antibiotic susceptibility testing by Kirby Bauer's disc diffusion method.

Antibiotic susceptibility testing by Kirby Bauer's Disc Diffusion method:

The Kirby-Bauer method is used for antimicrobial susceptibility testing which is recommended by the CLSI 2015. The accuracy and reproducibility of this test are dependent on maintaining a standard set of procedures as described here.

At least three to five well-isolated colonies of the same morphological type were selected from an agar plate culture. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 4 to 5 ml of a suitable broth medium such as peptone water culture was incubated at 35°C until it achieved or exceeded the turbidity of the 0.5 McFarland's standard(usually 2 to 6 hours). The turbidity of the actively growing broth culture was adjusted with sterile saline or broth to obtain turbidity optically comparable to that of the 0.5 McFarland's standard by visually comparing the inoculums tube and the 0.5 McFarland's standard against a card with a white back ground and contrasting black lines. That resulted in a suspension containing approximately 1 to 2×10^8 CFU/ml for *E.coli* ATCC 25922.

Inoculation of Test Plates:

Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab. The dried surface of a Muller –Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step the rim of the agar was swabbed. The lid was left for 3 to 5 minutes, but not more than 15 minutes ,to allow for any excess surface moisture to be absorbed before applying the antibiotic discs.

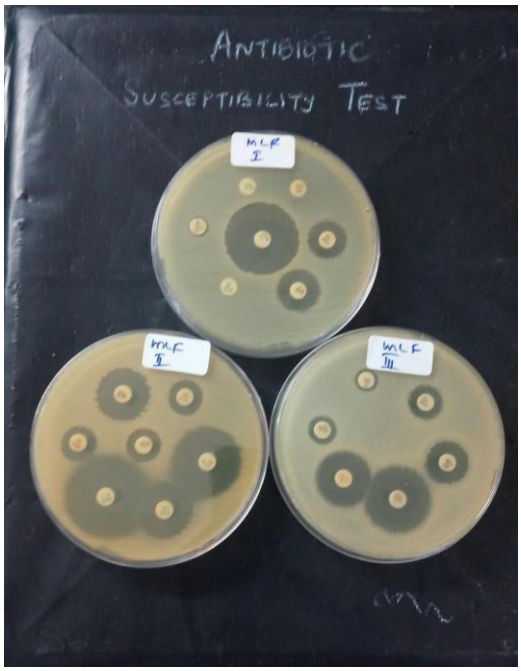
Application of Discs to Inoculated Agar Plates:

Antimicrobial discs were dispensed onto the surface of the inoculated agar plate and were pressed down to ensure complete contact with the agar surface distributed evenly so that they were no closer than 24 mm from center to center. The plates were inverted and placed in an incubator set to 35°C within 15 minutes after the discs were applied.

Reading Plates and Interpreting Results:

After 16 to 18 hours incubation, each plate was examined. If the plate was satisfactorily, streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth.. The diameter of the zones of complete inhibition were measured, including the diameter of the disc, zones were measured to the nearest whole millimeter, using sliding calipers or a ruler, which was held on the back of the inverted petriplate. (Fig –4).

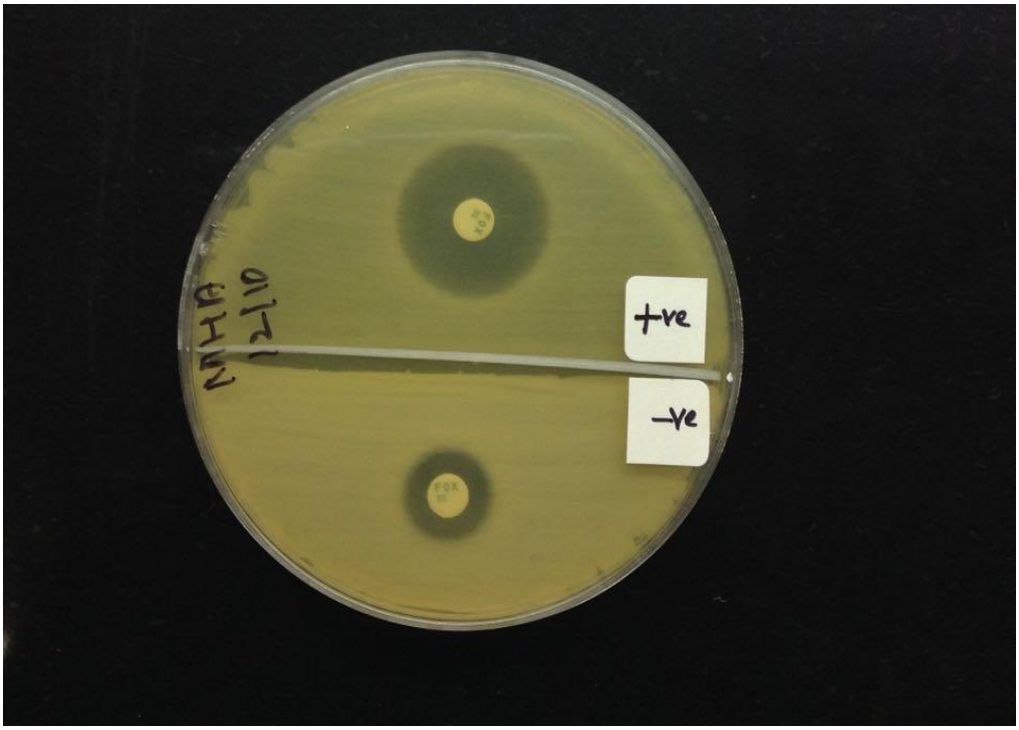
Fig –4: Antibiotic susceptibility testing



Cefoxitin Screening test:[42]

All the organisms speciated as Enterobacteriaceae were subjected to Cefoxitin screening by the standard Kirby bauer's disc diffusion method ***CLSI 2015*** [43]. A 30 µg Cefoxitin disc was placed on the inoculated medium and incubated at 37 degree C overnight. The zones of clearing around the discs were measured .For all the isolates cefoxitin resistance was identified when the zone diameter was ≤ 18 mm (Fig - 5) Cefoxitin resistance isolates were subjected to the following AmpC ß – lactamases detection methods.

Fig – 5 : AmpC betalactamases screening assay using 30µg Cefoxitin disc



About **256 non repeat** randomly selected cefoxitin resistant isolates were tested for AmpC β lactamase production by each of the following tests.

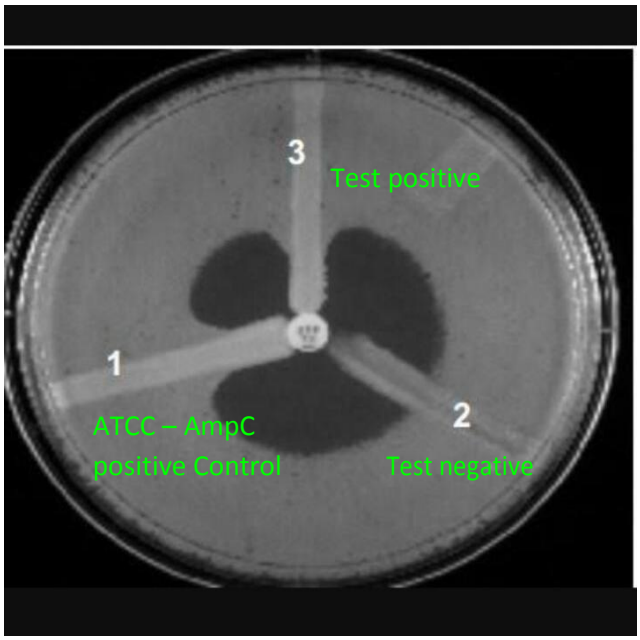
1. Modified three dimensional test ; CONFIRMATORY TEST.
2. CIAT; Ceftazidime-Imipenem antagonism test.
3. Boronic acid inhibitors based test.
4. Cloxacillin- combined disc diffusion test.
5. Disc Antagonism test
6. Double disc Synergy test.

Modified three dimensional (M3D) AmpC assay[44]:

The M3D assay was performed as described by Coudron et al and was used as the ***Gold Standard*** for detecting AmpC-R.It detects both inducible and non inducible ampc β lactamases producing isolates. Crude enzyme preparations was made by freezing and thawing five times the cell pellets from centrifuged tryptic soy broth culture. A lawn culture of E coli ATCC 25922 was made on the Muller Hinton agar. A Cefoxitin disc (30µg) was placed in the centre of the plate. With a sterile Surgical blade, a slit (3 cm) beginning 3mm away from the edge of the disc was cut in the agar in an outward radial direction. A small circular well is made, 5mm inside the outer edge of the slit. By using a pipette 30µl of enzyme preparation was dispended into the slit, avoiding slit overfill. AmpC positive and negative controls were included in the study. The plates were incubated overnight at 37º C.

The isolates showing clear distortion of zone of inhibition of Cefoxitin is taken as AmpC producers. (Fig - 6).

Fig – 6: AmpC Detection using Modified 3 dimensional (M3D) assay



Ceftazidime- Imipenem Antagonism Test[45]:

This method is also used for the detection of wild type inducible Amp C producers.

A 0.5 Mc Farland of test isolate was taken with the help of a sterile non toxic cotton swab and spread over Muller Hinton agar plate. Ceftazidime (30µg) and imipenem (10µg) disc were placed 20 mm apart from centre to centre.It was incubated at 35 º C for 16 to 20 hrs.

Interpretation; isolates showing blunting of Ceftazidime zone of inhibition adjacent to imipenem disc are confirmed as positive for inducible AmpC β Lactamase production. (Fig –7).

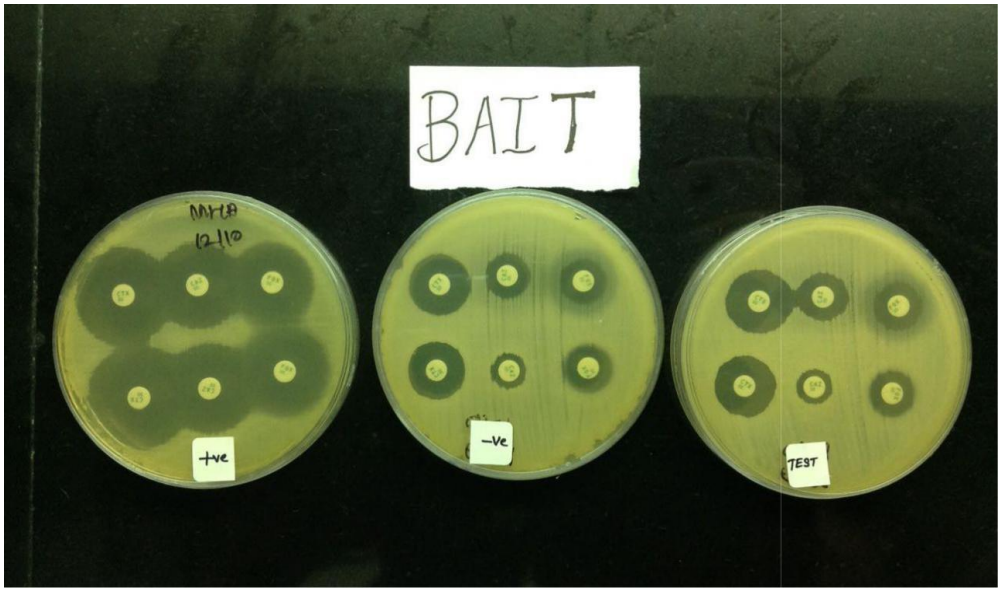
Fig – 7: AmpC Detection using Ceftazidium – imipenem antagonism (CIAT) assay

Boronic acid inhibition based test[44] ;

This test was carried out for the detection of both inducible as well as non inducible AmpC β- Lactamase producing isolates against Cefoxitin (30 µg) , Cefotaxime (30 µg) and Ceftazidime (30 µg) antibiotic discs with and without boronic acid(400µg). Preparation of boronic acid stock solution; Phenylboronic acid 120 mg was dissolved in 3ml of dimethyl sulfoxide (DMSO) , 3 ml Of sterile distilled water , added to this solution to get a final concentration of 20 mg /ml .now from this stock solution 20 µ l were added to each of the cephalosporin/ boronic acid (30µ g /400µg.). A 0. 5 Mc Farland of test isolate was spread over Muller Hinton agar plate and inoculums was allowed to dry for 5 to 10 min with lid in place. The above mentioned antibiotic discs- Cefoxitin (30 µg) , Cefotaxime (30 µg) , Ceftazidime (30 µg) with and with out boronic acid (400 µg) were applied using aseptic technique at a distance of 20 mm from each other The plates were incubated in inverted position at 35 ^{0} C for 16 to 20 hrs.

Interpretation: A ≥ 5mm increase in zone diameter for either anti microbial tested in combination with boronic acid versus its zone when tested alone confirms Amp C .β- lactamases production. (Fig –8).

Fig – 8: AmpC Detection using Boronic Acid inhibition test (BAIT) assay



Cloxacillin combined disc diffusion test [46].

This test was used for inducible and non inducible Amp C detection using Cefoxitin (30 µg) and Ceftazidime (30 µg) antibiotic discs with and with out Cloxacillin (400 µg) .Antibiotic discs : Cefoxitin (30 µg) and Ceftazidime (30µg). were obtained commercially while

Cefoxitin/ cloxacillin disc was prepared in laboratory by adding 10 µl of cloxacillin stock solution to each cephalosporin (30 µg) disc.

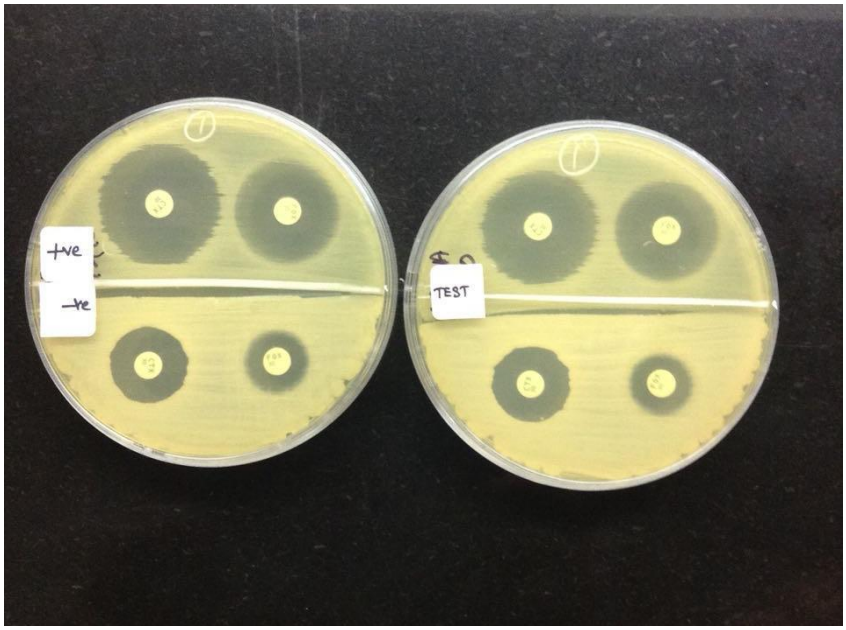
Preparation of cloxacillin stock solution:

20 mg of cloxacillin powder was dissolved in one ml of sterile distilled water to get a final concentration of 20 mg/ ml. the solution was vortexed for one min to make it homogenous. Now from this stock solution 10 µl were added to each of the cephalosporin / cloxacillin disc (30 µg/ 200µg).

A 0.5 M c Farland of test isolate was spread over Muller- Hinton agar plate and inoculums was allowed to dry for 5 to 10 min. The above mentioned antibiotic disc are applied using aseptic technique at a distance of 20 mm from each other.The plates were incubated in inverted position at 37 ^{0} C for 16 to 20 hrs

Interpretation: A ≥ 5mm increase in zone diameter for either antimicrobial tested in combination with cloxacillin versus its zone when tested alone confirm Amp C β- Lactamase Production. (Fig –9)

Fig – 9: AmpC Detection using Disc Cloxacillin combined disc diffusion test assay



Disc antagonism test[47]: For inducible AmpC detection

A 0.5 Mc Farland of test isolate was taken with the help of a sterile cotton swab and spread over Muller- Hinton agar plate. Cefotaxime (30 µg) and Cefoxitin (30 µg) disc were placed 20 mm apart from centre to centre. The plates were incubated at 37^0 C for 16 to 20 hrs .

Interpretation; isolates showing blunting of Cefotaxime zone of inhibition adjacent to Cefoxitin disc were positive for inducible AmpC β Lactamase producers. (Fig –10)

Fig – 10: AmpC Detection using Disc Antagonism test (DAT) assay

Double disc Synergy test[46] :

The Surface of the Muller – Hinton agar plates were inoculated with a 0. 5 Mc Farland suspension of test isolate. Place one Cefotaxime (30 µg) and one Ceftazidime (30 µg) disc on an inoculated MH agar plate.In between apply one Boronic Acid disc at a distance of 10

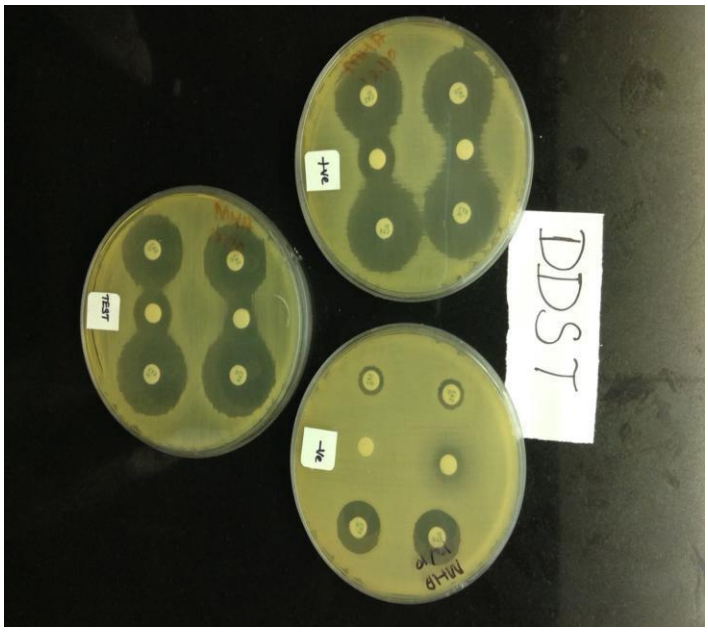
mm (edge to edge). If the strain was totally resistant to the Cephalosporins combination , the distance should be reduced to 5 mm

.Also, apply one Ceftazidime (30 µg) , and one Cefotaxime (30 µg) disc, in between the two disc at a distance of 5 to 10 mm dge to edge , place one Cloxacillin disc .

Interpretation: A keyhole or ghost zone (synergism) between Boronic Acid and any of Cefotaxime or Ceftazidime indicates the presence of an Amp C β Lactamase.

A keyhole or ghost zone between Cloxacillin and Ceftazidime and or Cefotaxime indicates the presence of an Amp C β-Lactamase. (Fig –11)

Fig – 11: AmpC Detection using Double disc synergy test (DDST) assay



DETECTION OF AMPC BY MOLECULAR METHODS:

Multiplex PCR;

Multiplex PCR was performed on randomly picked **106 cefoxitin resistant** isolates , of which **82 isolates** were confirmed phenotypically to be positive for Amp C β- lactamase production and **24 isolates** negative by modified 3 dimensional (M3D) test . For partial gene PCR amplification, primers (Table-2) specific for different beta lactamase gene are used for reaction with bacterial DNA as template.

Table – 2: Oligonucleotides used as primers for amplification of different AmpC β- lactamases gene groups (Perez and Hanson, 2002)

TARGET	PRIMER	SEQUENCE - 5' to 3'	Expected amplicon size (bp)
MOX-1,MOX-2,CMY-1, CMY-8 to CMY-11	MOXMF MOXMR	GCTGCTCAAGGAGCACAGGAT CAATTGACATAGGTGTGGTGC	520
LAT-1 to LAT-4,CMY-2 to CMY-7,BIL-1	CITMF CITMR	TGGCCAGAACTGACA GGC AAA TTTCTCCTG AACGTG GCT GGC	462
DHA-1, DHA-2	DHAMF DHAMR	AACTTTCACAGG TGT GCT GGG T CCGTACGCATACTGG CTT TGC	405
ACC	ACCMF ACCMR	AACAGCCTCAGCAGC CGG TTA TTC GCC GCA ATC ATCCCTAGC	346
MIR-1T, ACT-1	EBCMF EBCMR	TCGGTAAAGCCGATGTTGCGG CTT CCA CTG CGG CTGCCAGTT	302
FOX-1 to FOX-5b	FOXMF FOXMR	AACATGGGGTATCAGGGA GAT G CCAAGCCCG TAACCGGATTGG	190

Preparation of DNA template; DNA is extracted by boiling centrifugation method (Freshchi et al . 2005).

Preparation of reaction mixture;

Each single reaction mixture (30 µl) contained 10µl of DNA suspension, 15 pmol of each primer (Sigma) , 10 m M d NTPs, 1 U Taq DNA, polymerase, 25 Mm Mg Cl 2, and 2.5 µl of 10 x Taq buffer.

Reaction condition; PCR was performed using thermal cycler (fig – 12) with the following running conditions; initial denaturation step at 94' C for 5 min, followed by 39 cycles using following parameters;

DNA denaturation 94' C for 1 min

Primer annealing 60' C for 45 sec

Primer extension 72'C for 1 min.

After the last cycle, a final extension step at 72'C for 7 min was added.

Visualization of PCR product;

The DNA fragments of the AmpC genes amplified by PCR are identified by agarose gel electrophoresis. 5µl of amplified product from PCR is electrophoresed on 1.5% agarose gel, at constant 60 volts for 60 min with Tris Acetate EDTA (TAE) buffer. Molecular marker of 100 bp DNA ladder, is run concurrently. The gel stained with ethidium bromide is visualized under ultraviolet illumination and saving of image for the presence of bands using Multi –image Light Cabinet, is done. (Fig – 13a & 13b).

Fig – 12: PCR thermocycler

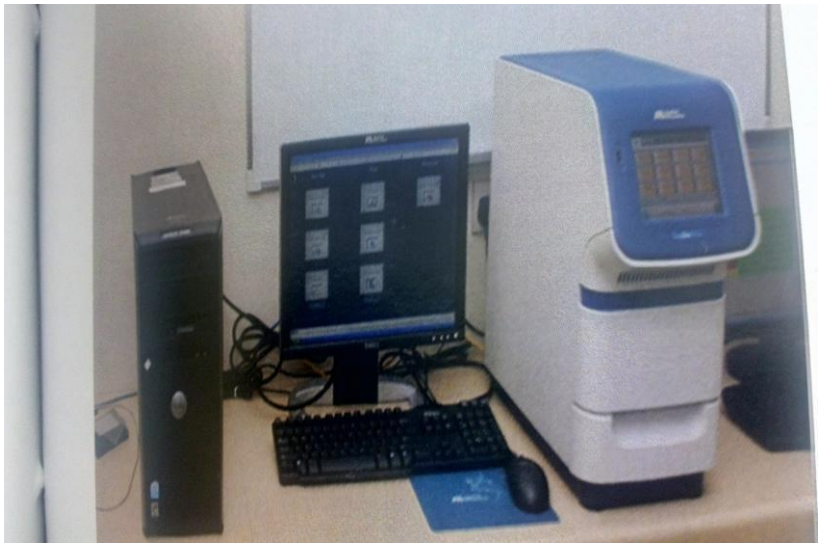


Fig – 13a: Gel electrophoresis set up

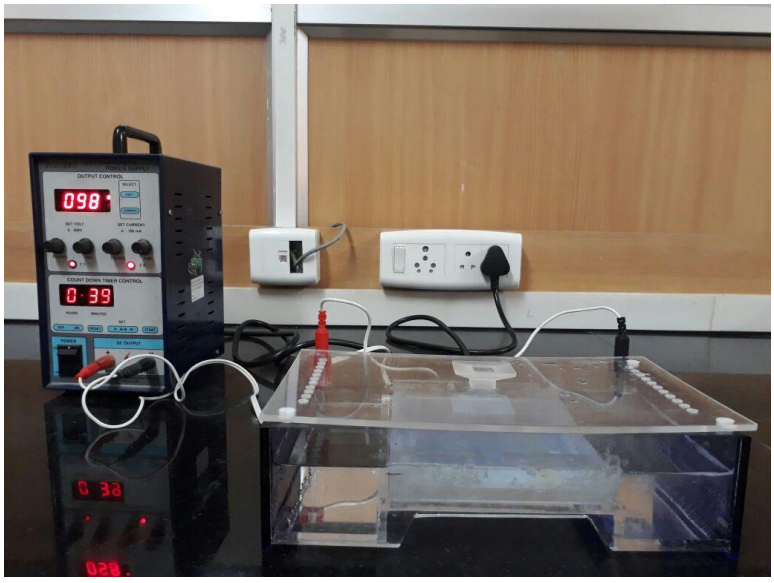
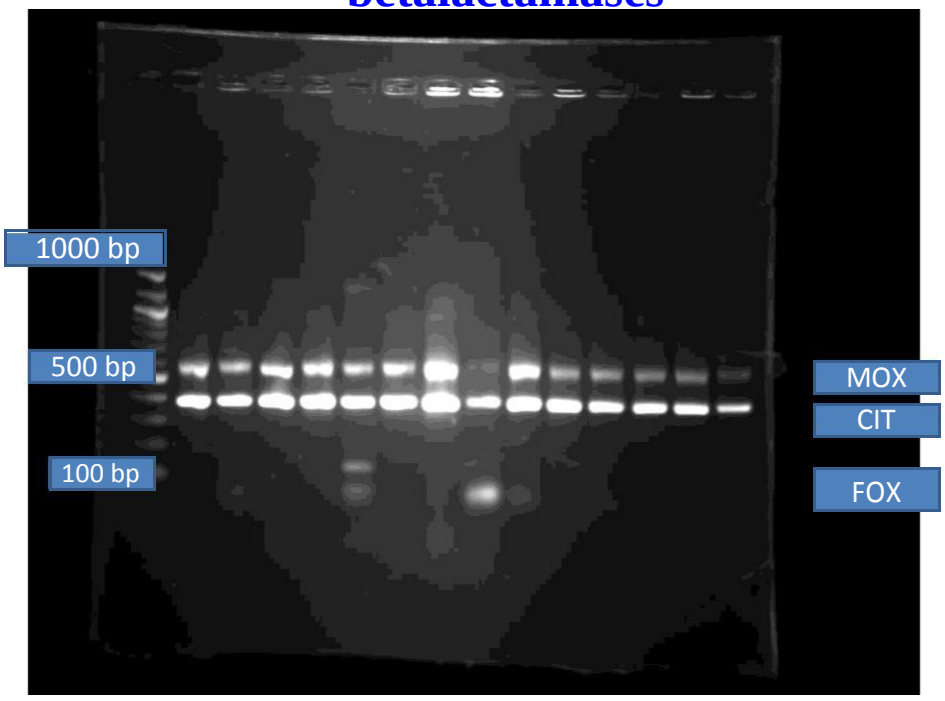


Fig – 13b: Gel picture of Genotypic detection of AmpC betalactamases



Results and Analysis

RESULTS AND ANALYSIS

Out of the 256 isolates of Enterobacteriaceae included in the study , most isolates were from urine specimen 30% (78) followed by blood 16% (40) and others as seen in Fig - 14

Fig – 14: Specimenwise distribution of Enterobacteriaceae included in the study

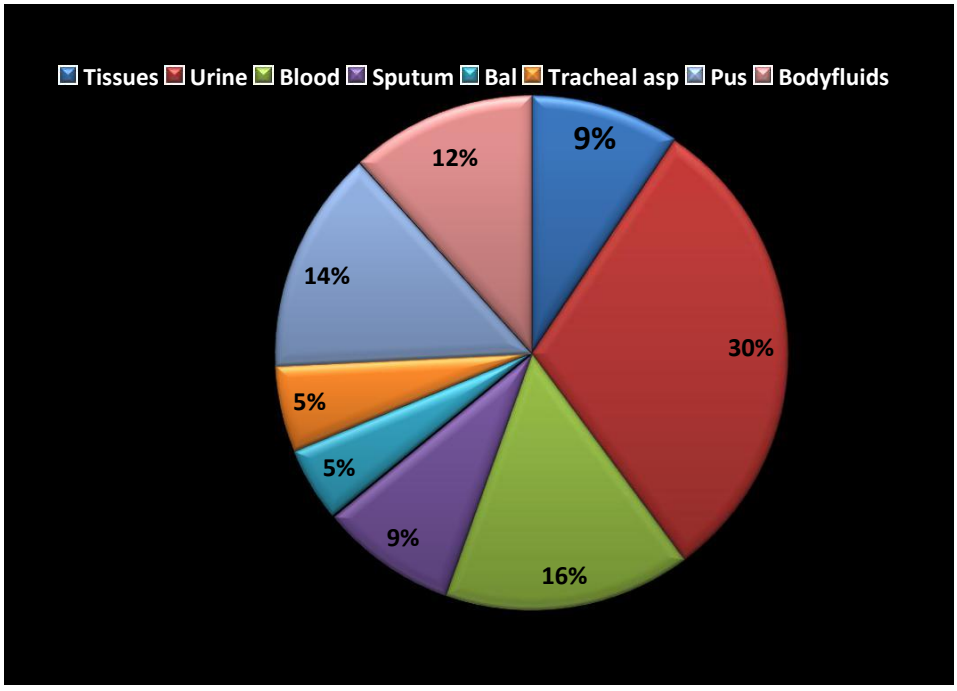
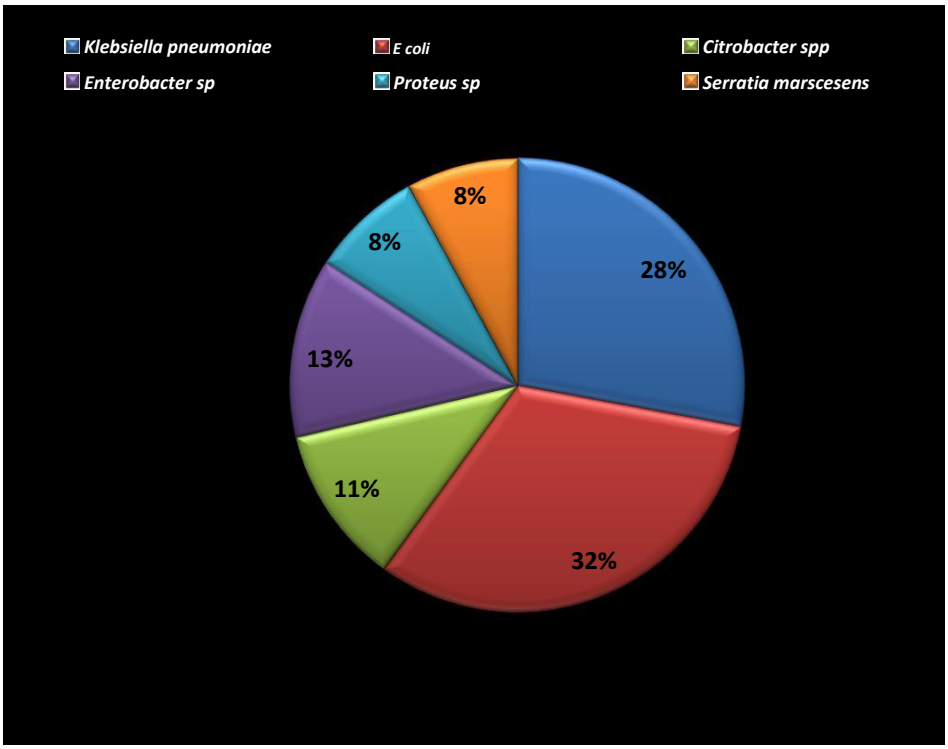


Fig – 15: Distribution of various Microorganisms (n-256) included in the study



Of the 256 cefoxitin resistant isolates included in the study *E coli* 32% (85) and *Klebsiella pneumoniae* 28.9% (74) were the predominant isolates followed by the others . (Fig – 15) & (Table –3)

TABLE- 3: Showing distribution of clinical isolates screened positive for Amp C beta lactamases by Cefoxitin screening test

ORGANISMS	CEFOXITIN RESISTANCE (%)
Klebsiella pneumoniae	74 (28.9)
E.coli	85 (32.2)
Citrobacter spp	30 (11.7)
Enterobacter spp	34 (13.2)
Proteus spp	21(8.2)
Serratia spp	12 (4.6)
TOTAL	**256 (100)**

Table – 4: Detection of AmpC betalactamases by various phenotypic methods

Organisms	DAT	CIAT	BAIT	CCDD	DST	M3D
Klebsiella (n=74)	56 (77%)	62 (86%)	64 (86.3%)	15 (20%)	59 (81%)	63 (87.5%)
Ecoli (n= 85)	40 (47%)	43 (50%)	56 (65.8%)	30 (35%)	30 (35%)	64 (75.2%)
Enterobacter aerogenes (n= 34)	15 (41%)	18 (50%)	24 (66%)	16 (44.4%)	15 (41.6%)	20 (55.5%)
Citrobacter Spp (n=30)	18 (60%)	19 (63%)	17 (56.6%)	5 (16.6%)	3 (10%)	19 (63.3%)
Proteus spp (n=21)	9 (42.8%)	12 (57%)	10 (47.6%)	5 (23%}	4 (19%)	11 (52%)
Serratia marcescense (n=12)	2 (15.3%)	3 (25%)	5 (41%)	1 (8.3%)	3 (25%)	5 (41%)
TOTAL (256)	140 (54.6%)	157 (61.3%)	176 (68.75)	72 (28.1%)	114 (44.53%)	182 (71.09)

Out of the 256 cefoxitin resistant enterobacteriaceae isolates 182 (71.09%) of the isolates were found to be AmpC producers by the gold standard Modified 3 D (M3D) assay . Boronic acid inhibition test (BAIT) detected 176 (68.75%) isolates as AmpC producers followed by Ceftazidime – Imipenem Antagonism test (CIAT) ,Disc Antagonism Test (DAT) , Double disc synergy test (DST) & Cloxacillin Combined Disc Diffusion test (CCDT) as seen in the table – 4.

TABLE – 5: Sensitivity and Specificity of various AmpC phenotypic detection methods

	Disc antagonism test	**Ceftazidime-imepenem antagonism test**	**Boronic acid inhibition test**	**Cloxacillin combined disc diffusion test**	**Double disc synergy test**
Sensitivity	86.72%	87%	93.96%	87.5%	86.72%
Specificity	66.4%	78%	93.24%	79%	66.6%
Accuracy	75%	83.5%	93.75%	84.37%	72%

Among the various phenotypic methods tested for AmpC producers, BAIT had the highest sensitivity (93.96%) and specificity (93.24%) followed by CIAT and CCDT with similar sensitivity and specificity .DAT and DST had the least sensitivity (86%) and specificity (66%) (Table – 5)

Table – 6:Distribution of Enterobacteriacae isolates subjected to molecular detection of AmpC genes

Organisms	M3 D POSITIVE	M3D NEGATIVE	TOTAL
Klebsiella pneumoniae	34	5	39
E coli	27	5	32
Citrobacter sp	7	4	11
Enterobacter sp	10	6	16
Proteus sp	4	2	6
Serratia sp	0	2	2
Total	**82**	**24**	**106**

About 106 randomly selected cefoxitin resistant enterobacteriaceae isolates with 82 positive for AmpC by M3D assay and 24 negative for M3D assay were further subjected to Molecular detection of te well identified 6 AmpC betalactamases gene groups (Table-6).

Table 7 : Positive for Amp C Betalactamase genes among strains positive and negative by M3D assay

Organisms	M3D (+ VE)		M3D (- VE)	
	No of isolates	AmpC genes (+ VE)	No of isolates	AmpC genes (+ VE)
Klebsiella pneumonia	34	30	5	2 (FOX)
E coli	27	21	5	0
Citrobacter sp	10	5	6	0
Enterobacter sp	7	3	4	0
Proteus sp	4	2	2	0
Serratia sp	0	0	2	0
TOTAL	**82**	**61(74.3%)**	**24**	**2 (8.3%)**

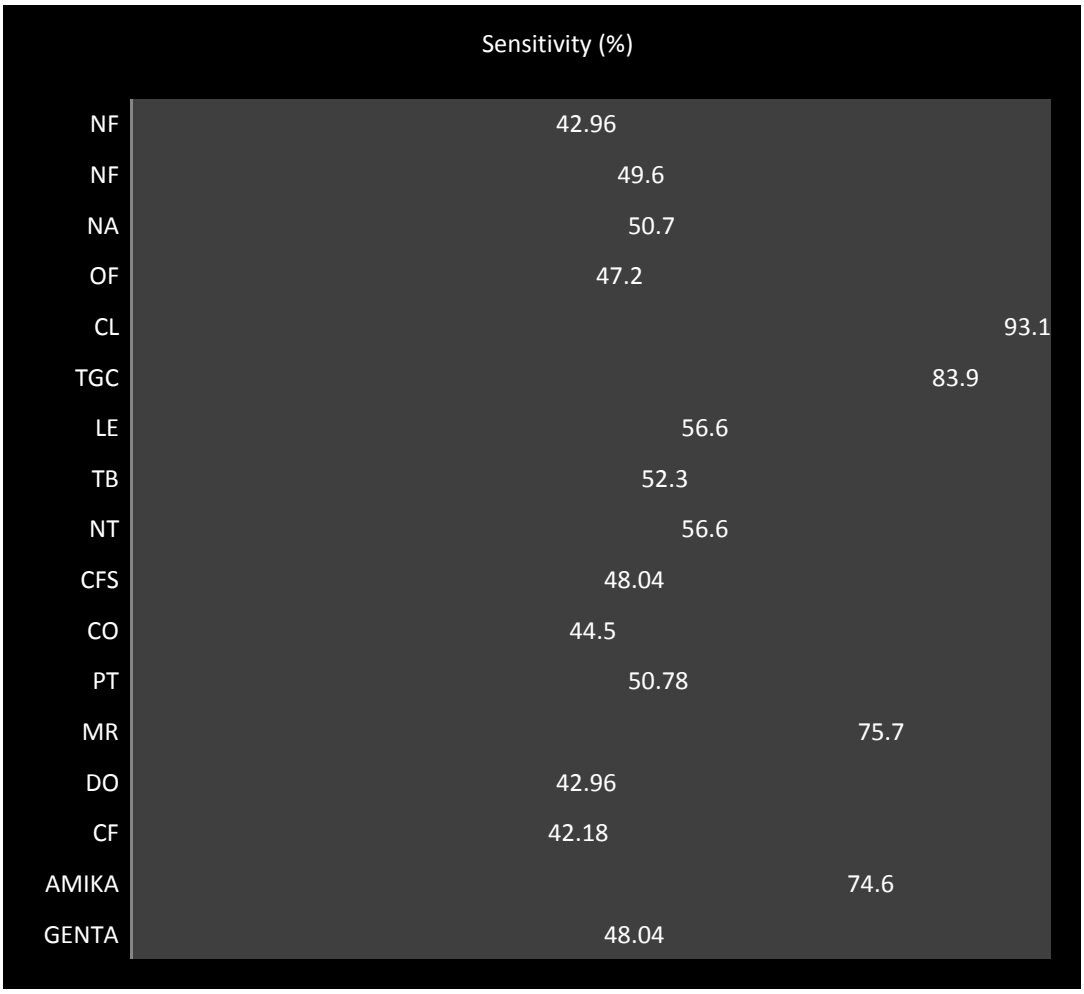
About 61 (74.3%) of the M3D assay positive isolates and 2 (8.3%) of the M3D assay negative isolates were found to harbor Amp C genes (Table –7)

TABLE -8 : Different AmpC Betalactamases gene groups among isolates that were M3D assay positive

M3D + ve Organisms	**FOX**	**MOX**	**DHA**	**CIT**	**EBC**	**ACC**	**CITMOX**	**FOX+ MOX+ CIT**	**FOX+ MOX+ CIT+ACC**	**TOTAL**
Klebsiella	8	5	2	2	0	0	5	4	4	**30**
E coli	5	3	2	2	0	1	2	3	3	**21**
Citrobacter	2	1	0	0	0	0	2	0	0	**5**
Enterobacter sp	1	0	0	0	0	0	0	0	2	**3**
Proteus sp	1	0	0	0	0	1	0	0	0	**2**
Serratia sp	0	0	0	0	0	0	0	0	0	**0**
Total	**17**	**9**	**4**	**4**	**0**	**2**	**9**	**7**	**9**	**61**

Of the 63 isolates positive for AmpC genes, Fox (55.5%) was the commonest gene group identified which were also found in 2 (8.3%) M3D assay negative, followed by MOX (53.9%) , CIT(46%) and others . More than one genes were present in 26(41.2%) of the isolates (Table-7 & 8)

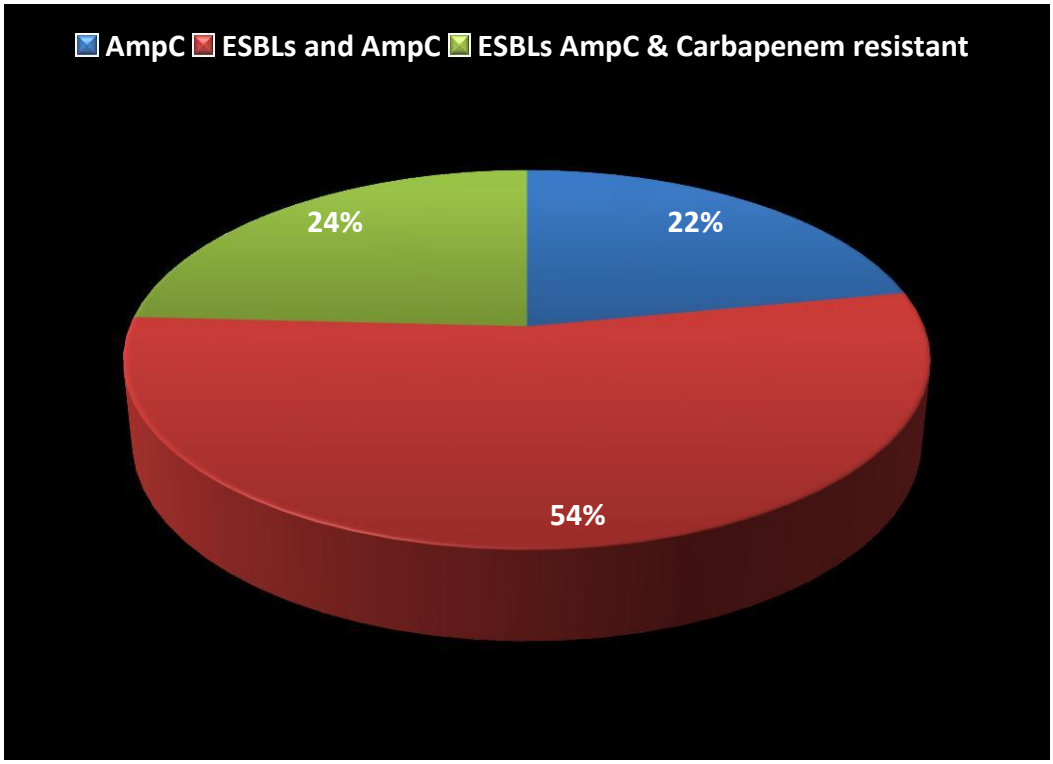
Fig – 16 : Antibiotic sensitivity pattern of the AmpC betalactamase producing isolates



The Antibiotic susceptibility pattern of the AmpC producing enterobacteriaceae showed colistin to be the most sensitive (93.1%) followed by Tigecycline (83.9%), Meropenem (75.7%) , Amikacin (74.6%) and others (Fig -16)

Ceftazidime and ceftazidime clavulunic acid helped in the phenotypic detection of ESBLs production among the AmpC producers .(54%) . along with those that were resistant to carbapenems (24%). Fig -17.

Fig -17: AmpC producing isolates occurring with ESBLs and Carbapenem ressistance



Discussion

DISCUSSION

Despite the discov**e**ry of Amp C β lactamases at least 3 decade ago Confusion still exists about the importance of their resistance mechanisms, optimal test methods and appropriate reporting protocols.[1] Increasing prevalence may be due to exposure to previous empirical cephalosporin therapy which is a known selective pressure for increasing AmpC production among them.[2]Failure to detect these enzymes has contributed to their uncontrolled spread and sometimes to therapeutic failures.[1]

Although there are no CLSI guidelines for phenotypic methods to screen and detect AmpC activity in Enterobacteriaceae, several methods have been developed for the detection of AmpC, namely, the modified three-dimensional test,[44] inhibitor based methods employing Boronic acid,[44] and others . Reduced susceptibility to Cefoxitin is one of the screening methods for putative AmpC enzyme detection.[42] ACC types seem to be the only known enzymes that can be missed by cefoxitin screening.[**42**]

In the present study, out of the 256 Cefoxitin-resistant enterobacteriaceae isolates, only **182 (71.9%)** were found to be positive for AmpC production by the Modified three dimensional assay. Amp C negative Cefoxitin resistance may be attributed to ESBLs and MBL production or non-

enzymatic mechanism such as porin channel mutation.[48] Overexpression of chromosomal AmpC gene due to mutation in the promoter or attenuator regions.[48] The other reason is that Cefoxitin has been demonstrated as a substrate to active efflux pump in clinical isolates.[48]

The microorganism that were predominantly AmpC producers were *Klebsiella pneumonia, E coli,* , followed by *Citrobacter sp , Enterobacter sp , Proteus sp Serratia marscence.* These findings were consistent with most researchers .[42, 44,45,46,47]

Among the five phenotypic methods tested against modified three dimensional assay , the boronic acid inhibition test had the highest sensitivity **(93.96%)** and specificity **(93.24%)** compared to the others **(Table-5).** The use of phenylboronic acid in combination with cefoxitin as a phenotypic screening method may be a better tool for laboratory diagnosis and confirmation of AmpC producing Enterobacteriaceae.[47]

However, Both the modified three dimensional test, and boronic acid test fail to distinguish between plasmid-mediated AmpC production and derepressed hyperproduction of chromosomal AmpC.[49] Detection of AmpC beta lactamases in *Klebsiella sp, Citrobacter koseri,* or *Proteus mirabilis* is

confirmatory for plasmid mediated AmpC production because these organisms lack chromosomal AmpC betalactamses .[42]

Inspite of many phenotypic tests, PCR is considered the gold standard which is not available for routine diagnostic laboratories. In this study, among the 106 isolates tested , 61 Amp-C genes groups were detected among 74.3% of the positive phenotypic isolates and 2.8% of the negative isolates.

Discrepancy between genotypic and phenotypic tests may be due to the following reasons: The presence of more AmpC beta-lactamases genes that continue to expand beyond those contained in the six families of genes covered by PCR.[46] The other explanation is that the isolates were most likely to be hyper-producers due to over-expression of chromosomal AmpC gene.[46] False negative results may be explained by the fact that the genes are detected by PCR but not effectively expressed phenotypically.[46]

FOX group genes (including FOX-1) were the predominant type in all isolates (55.5%) followed closely by MOX group gene (including CMY-1) (53.9%) then CIT group genes (including CMY-2) (46%) compared to the result of Fam et al.[50], who reported that, in clinical isolates of Enterobacteriaceae from Cairo, Egypt Amp-C genes were detected in 28.3% of the study population including *E. coli, Klebsiella* and *P. mirabilis.* CMY-2

enzyme was found disseminating in all 6 AmpC-positive *E. coli* and in 6/10 of *Klebsiella* species. Only one *K. pneumoniae* isolate harbored CMY-4 while DHA-1 was detected in 3 *Klebsiella* and in one *P. mirabilis* isolate. On the other hand the result of Montgomery et al.[51]who reported that 22 AmpC genes were detected in 25.8% of the positive cefoxitin screened isolates of which 40.9% belonged to each of the MOX and the FOX families, 13.6% belonged to the EBC family, and 4.5% belonged to the CIT family.

In a study by Soha A El Hady ,[51] both CMY-1 and CMY-2 were the most common genes detected in their region at Egypt ,DHA-type enzymes have been previously identified in Taiwan [53] and in China [54]. In Korea DHA-, CMY/MOX-, and ACT-1/MIR-1-type enzymes have also been identified [55].While in the United States, in addition to the types mentioned above, DHA-, ACT-1/MIR-1, and FOX-type enzymes have been identified [56].

In Japan, MOX-1, CMY-9,CMY-19, CFE-1, CMY-2 and DHA-1 have been found in clinical isolates [57.] Enzyme type CMY-2 is widely distributed geographically. It has been reported in Algeria, France Germany, Greece, India, Pakistan, Taiwan, Turkey, United Kingdom and the United States. Several studies for the detection of AmpC b-lactamase producers in many countries (Saudi Arabia, Taiwan, Korea, North and South America) revealed geographical discrepancy in AmpC b-lactamase types [58]

In our study, in 41.26% of the isolates, there were more than one gene. Nine isolates *K. pneumoniae*, nine isolates had both MOX (CMY-1) & and CIT(C isolates had three genes CMY-1, CMY-2 and FOX-1 and Nine more had CMY-1, CMY-2 , FOX-1 and ACC . For E. coli 6 isolates have both CMY-1 and CMY-2, 3 isolates have CMY-1, FOX-1 and 2 isolates have the three genes CMY-1, CMY-2 and FOX-1. Similar findings were reported by Soha A El Hady et al and Wassef et al [46]. These Plasmid-mediated AmpC β-lactamases pose a big challenge to infection control due to the fact that the AmpC gene can be expressed in larger amounts and has high transmissibility to other bacterial species. [46]

Enterobacteriaceae-producing both AmpC and ESBL have been increasing reported worldwide. As per CLSI guidelines [42],when using the new interpretive criteria, routine ESBL testing is no longer necessary before reporting results (i.e. it is no longer necessary to edit results for cephalosporins, aztreonam, or penicillins to resistant). [59] However, ESBL testing may still be useful for epidemiological or infection control purposes.

In this study, co-production was observed in 54% of isolates. This is comparatively higher than that reported by Devaraju[60] (24%) and Nasir *et al.*[61] (11.5%). ESBLs detection tests may be insufficient in situations where high-level expression of AmpC may mask recognition of ESBL. Cefepime is a more reliable detector in the presence of AmpC as it is stable to the enzyme and will

thus demonstrate synergy arising from inhibition of ESBL by Clavulanate in the presence of AmpC[62] Some studies suggest the use of Cefepime-Clavulanate Etest as a suitable substitute.[62]

Carbapenems the drugs of choice for treating ESBLs and Amp C producing bacteria was found to have 75% sensitivity only . Colistin had the highest sensitivity followed by Tigecycline and Amikacin. Colistin is never administered as a monotherapy and caution exercised with increasing tigecycline and Colistin intrinsically resistant isolates such as *Proteus sp* which are Amp C producers

Summary

SUMMARY

With the increasing use of β-lactam drugs and introduction of various inhibitor combinations AmpC β-lactamases have emerged and are being reported globally among enterobacteriaceae with varying prevalence rates.

- Though AmpCs are associated with serious life threatening illness, detection of Amp C β-lactamases are very difficult and not routinely tested .

- This study aimed at comparing various phenotypic methods and also look for various AmpC gene clusters using multiplex PCR.

- About 256 non repeat Enterobacteriaceae clinical isolates that were cefoxitin resistant were included in the study conducted during the period Jan 2016 – July 2017.

- Urinary isolates were the predominant , followed by blood isolates and others

- *E coli* 32%(85)was the predominant isolate followed by *Klebsiella pneumonia 28.9%(74), Enterobacter sp , Citrobacter sp , Proteus sp* and *Serratia marscesens*.

- All the isolates were subjected to phenotypic detection of AmpC by 6 methods such as Modified three dimensional (M3D) assay , Boronic acid Inhibition test (BAIT), Ceftadidime – imipenem antagonism test (CIAT), Disc Antagonism test (DAT), Double disc synergy test (DST) and Cloxacillin Combined disc diffusion test (CCDT)

- About 182 (71.09%) of the isolates were found to produce AmpC AmpC.Cefoxitin resistant betalactamase negative maybe attributed to porin mutations, over expression of chromosomal AmpC.and active efflux mechanism .

- Amp C was found to be produced highest among *Klebsiella pneumoniae* (87.5%) followed by *E coli* (75.2%) and others

- BAIT test had the highest sensitivity (93.96%) and specificity (93.24%) comparable to M3D assay.

- About 106 randomly selected Cefoxitin resistant Enterobacteriaceae with 82 positive for AmpC by M3D assay and 24 negative for M3D assay were subjected to multiplex PCR for 6 AmpC gene clusters such as MOX, CMY-2, DHA, ACC, MIR/ACT, FOX.

- Sixty three of the 106 isolates were found to harbor one / more Fox (55.5%) was the commonest gene group identified , followed by MOX (53.9%) , CIT(46%) and others .

- Two M3D negative isolates were tested positive for FOX.

- More than one genes were present in 26(41.2%) of the isolates . Nine isolates were found to have the genes FOX,MOX, CIT, ACC.

- Antibiotic sensitivity testing by routine Kirby bauer's disc diffusion showed colistin to have the highest sensitivity (93%) followed by Tigecycline 83.9%, Meropenem 75.7% Amikacin 74.6%.

- AmpC coexisting with ESBLs was found among 54% of the isolates tested and 24% isolates also was found to be carbapenem resistant.

- Identifying the types of AmpC aid in hospital infection control and help physicians to prescribe the most appropriate antibiotic.

- Continual surveillance of AmpC resistance mechanisms that assist appropriate antibiotic therapy and better patient outcomes and also reduces antibiotic resistance through better infection control practices.

- Sequencing and typing the strains may be required to better understand the genetic relatedness and the molecular epidemiology of their resistance mechanism

Conclusion

CONCLUSION

Clinical laboratories needs to be upgraded with appropriate tools and qualified staff to recognize newer drug resistances emerging among Enterobacteriaceae. Amp C betalactamases producing bacteria are being frequently isolated from clinical samples and these enterobacteriaceae isolates also harbouring ESBLs and Carbapenemases is a concern, as they increase the complexity of their detection . Boronic acid inhibitor method using Cefoxitin reliably detected AmpC phenotypically. The most prevalent AmpC genes belonged to FOX, MOX and CIT in our study . The dissemination of these plasmid-mediated resistance genes within the hospital is an important public health issue. Identifying the types of AmpC may aid in hospital infection control and help the physician to prescribe the most appropriate antibiotic, thus decreasing the selective pressure, which generates antibiotic resistance. Sequencing and typing the strains may be required to better understand the genetic relatedness and the molecular epidemiology of their resistance mechanism. The study emphasizes the necessity for continual surveillance of resistance mechanisms that assist appropriate antibiotic therapy and better patient outcomes and also reduces antibiotic resistance through better infection control practices.

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Appendix

ABBREVIATIONS

AmpC	-	Betalactamase enzyme.
BAIT	-	Boronic acid inhibition test.
CIAT	-	Ceftazidime-Imepenem inhibition test.
CCDT	-	Cloxacillin Combined disc diffusion test.
DAT	-	Disc antagonism test.
DST	-	Double Disc synergy test.
M3DT	-	Modified 3 dimension test.

Annexure

URKUND

Urkund Analysis Result

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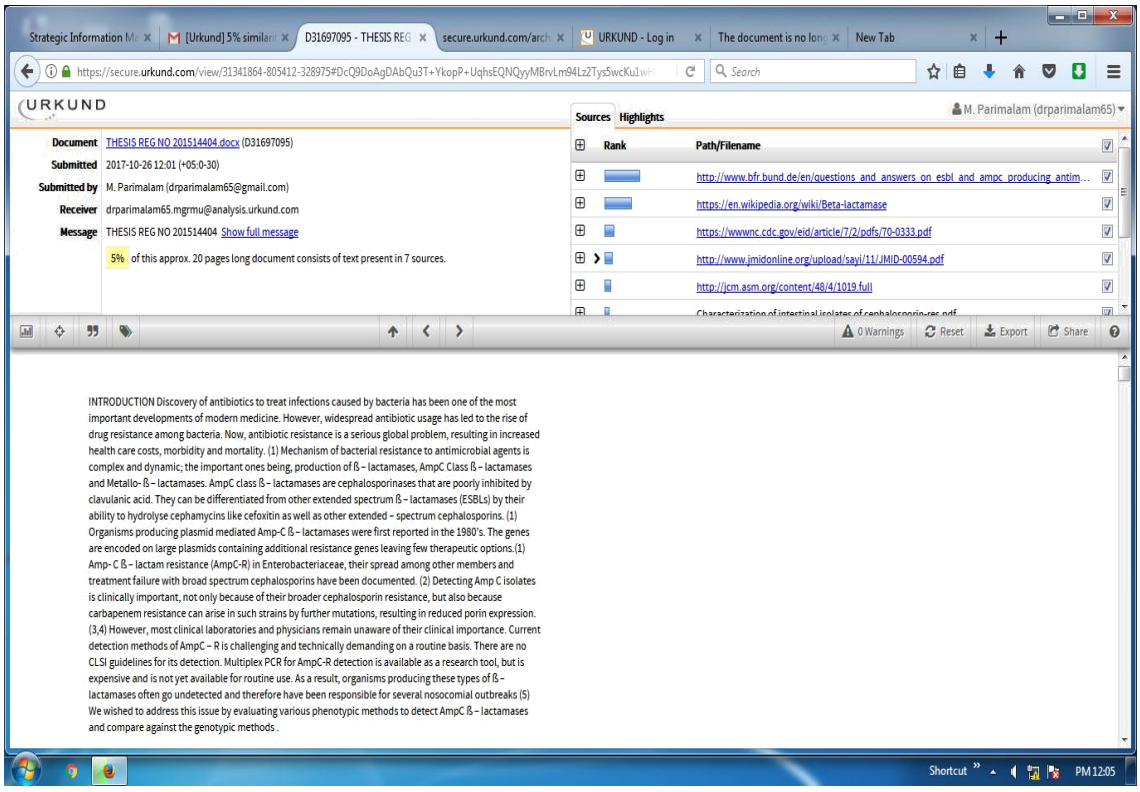
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INTRODUCTION Discovery of antibiotics to treat infections caused by bacteria has been one of the most important developments of modern medicine. However, widespread antibiotic usage has led to the rise of drug resistance among bacteria. Now, antibiotic resistance is a serious global problem, resulting in increased health care costs, morbidity and mortality. (1) Mechanism of bacterial resistance to antimicrobial agents is complex and dynamic; the important ones being, production of ß – lactamases, AmpC Class ß – lactamases and Metallo- ß – lactamases. AmpC class ß – lactamases are cephalosporinases that are poorly inhibited by clavulanic acid. They can be differentiated from other extended spectrum ß – lactamases (ESBLs) by their ability to hydrolyse cephamycins like cefoxitin as well as other extended – spectrum cephalosporins. (1) Organisms producing plasmid mediated Amp-C ß – lactamases were first reported in the 1980's. The genes are encoded on large plasmids containing additional resistance genes leaving few therapeutic options.(1) Amp- C ß – lactam resistance (AmpC-R) in Enterobacteriaceae, their spread among other members and treatment failure with broad spectrum cephalosporins have been documented. (2) Detecting Amp C isolates is clinically important, not only because of their broader cephalosporin resistance, but also because carbapenem resistance can arise in such strains by further mutations, resulting in reduced porin expression. (3,4) However, most clinical laboratories and physicians remain unaware of their clinical importance. Current detection methods of AmpC – R is challenging and technically demanding on a routine basis. There are no CLSI guidelines for its detection. Multiplex PCR for AmpC-R detection is available as a research tool, but is expensive and is not yet available for routine use. As a result, organisms producing these types of ß – lactamases often go undetected and therefore have been responsible for several nosocomial outbreaks (5) We wished to address this issue by evaluating various phenotypic methods to detect AmpC ß – lactamases and compare against the genotypic methods .
Shortcut
PM 12:05

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

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

Following points must be noted:

1. IHEC should be informed of the date of initiation of the study
2. Status report of the study should be submitted to the IHEC every 12 months
3. PI and other investigators should co-operate fully with IHEC, who will monitor the trial from time to time
4. At the time of PI's retirement/intention to leave the institute, study responsibility should be transferred to a colleague after obtaining clearance from HOD, Status report, including accounts details should be submitted to IHEC and extramural sponsors
5. In case of any new information or any SAE, which could affect any study, must be informed to IHEC and sponsors. The PI should report SAEs occurred for IHEC approved studies within 7 days of the occurrence of the SAE. If the SAE is 'Death', the IHEC Secretariat will receive the SAE reporting form within 24 hours of the occurrence
6. In the event of any protocol amendments, IHEC must be informed and the amendments should be highlighted in clear terms as follows:
   a. The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
   b. Alteration in the budgetary status should be clearly indicated and the revised budget form should be submitted
   c. If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval
   d. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented
   e. If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IHEC and only then can they be implemented
   f. Any deviation-Violation/waiver in the protocol must be informed to the IHEC within the stipulated period for review
7. Final report along with summary of findings and presentations/publications if any on closure of the study should be submitted to IHEC

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

Thanking You,

Yours Sincerely,

SECRETARY
PSG IMS&R
COIMBATORE-641004.
INSTITUTIONAL HUMAN ETHICS COMMITTEE

Dr Sudha Ramalingam
Alternate Member - Secretary
Institutional Human Ethics Committee

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

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To
Dr M Parimalam
Postgraduate
Department of Microbiology
Guide: Dr J Jayalakshmi
PSG IMS & R
Coimbatore

Ref: Project No.15/414

Date: December 29, 2015

Dear Dr Parimalam,

Institutional Human Ethics Committee, PSG IMS&R reviewed and discussed your application dated 21.12.2015 to conduct the research study entitled *"Occurrence and detection of AmpC β-lactamases among clinical isolates of Enterobacteriaceae at a tertiary care hospital"* during the IHEC meeting held on 24.12.2015.

The following documents were reviewed and approved:

1. Project Submission form
2. Study protocol (Version 1 dated 21.12.2015)
3. Confidentiality statement
4. Application for waiver of consent
5. Data collection tool (Version 1 dated 21.12.2015)
6. Current CVs of Principal investigator, Co-investigators
7. Budget

The following members of the Institutional Human Ethics Committee (IHEC) were present at the meeting held on 24.12.2015 at IHEC Secretariat, PSG IMS & R between 10.00 am and 11.00 am:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Mr. R. Nandakumar	BA., BL	Legal Expert, Chairperson	Male	No	Yes
2	Dr. S. Bhuvaneshwari (Member-Secretary, IHEC)	MD	Clinical Pharmacology	Female	Yes	Yes
3	Dr. S. Shanthakumari	MD	Pathology, Ethicist	Female	Yes	Yes
4	Dr D Vijaya	M Sc., Ph D	Basic Medical Sciences (Biochemistry)	Female	Yes	Yes

The study is approved in its presented form. The decision was arrived at through consensus. Neither PI nor any of proposed study team members were present during the decision making of the IHEC. The IHEC functions in accordance with the ICH-GCP/ICMR/Schedule Y guidelines. The approval is valid until one year from the date of sanction. You may make a written request for renewal / extension of the validity, along with the submission of status report as decided by the IHEC.

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

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November 11, 2016

To
Dr M Parimalam
Postgraduate
Department of Microbiology
Guide/s: Dr J Jayalakshmi
PSG IMS & R
Coimbatore

The Institutional Human Ethics Committee, PSG IMS & R, Coimbatore - 4, has reviewed your proposal on 11th November 2016 in its expedited review meeting held at IHEC Secretariat, PSG IMS&R, between 10.00 am and 11.00 am, and discussed your request to renew the approval and to include two co-investigators for the study entitled:

"Occurrence and detection of AmpC β-lactamases among clinical isolates of Enterobacteriaceae at a tertiary care hospital"

The following documents were received for review:

1. Request for renewal dated 07.11.2016
2. Status report

After due consideration, the Committee has decided to renew the approval for the above study.

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

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Mr R Nandakumar (Chairperson, IHEC)	BA., BL	Legal Expert	Male	No	Yes
2	Dr. S. Bhuvaneshwari (Member-Secretary, IHEC)	MD	Clinical Pharmacology	Female	Yes	Yes
3	Dr S Shanthakumari	MD	Pathology, Ethicist	Female	Yes	Yes
4	Dr Sudha Ramalingam	MD	Epidemiologist, Ethicist Alt. member-Secretary	Female	Yes	Yes
5	Dr D Vijaya	M Sc., Ph D	Basic Medical Sciences (Biochemistry)	Female	Yes	Yes

The approval is valid for one year (29.12.2016 to 28.12.2017).

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

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

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

Yours truly,

MEMBER SECRETARY
PSG IMS&R
COIMBATORE-641004.
INSTITUTIONAL HUMAN ETHICS COMMITTEE

Dr S Bhuvaneshwari
Member – Secretary
Institutional Human Ethics Committee

Proposal No. 15/414