EVALUATION OF CONVENTIONAL METHODS WITH MOLECULAR METHODS FOR THE DETECTION OF AmpC BETA LACTAMASE IN *ENTEROBACTERIACEAE* AMONG CLINICAL ISOLATES

Dissertation submitted to

THE TAMIL NADU DR.M. G. R MEDICAL UNIVERSITY

CHENNAI- 600032

In partial fulfillment of the requirement for the degree of

Doctor of Medicine in Microbiology (Branch IV)

M. D. (MICROBIOLOGY)

DEPARTMENT OF MICROBIOLOGY

TIRUNELVELI MEDICAL COLLEGE

TIRUNELVELI- 11

APRIL 2015

CERTIFICATE

This is to certify that the Dissertation **''EVALUATION OF CONVENTIONAL METHODS WITH MOLECULAR METHODS FOR THE DETECTION OF AmpC BETA LACTAMASE IN *ENTEROBACTERIACEAE* AMONG CLINICAL ISOLATES''** presented here in by **Dr.Meenakshi.C** is an original work done in the Department of Microbiology, Tirunelveli Medical College Hospital, Tirunelveli for the award of Degree of M.D.(Branch IV) Microbiology under my guidance and supervision during the academic period of 2012-2015.

L.D.THULASI RAM MS. (Ortho)
The DEAN
Tirunelveli Medical College,
Tirunelveli-627011

CERTIFICATE

This is to certify that the dissertation entitled, **''EVALUATION OF CONVENTIONAL METHODS WITH MOLECULAR METHODS FOR THE DETECTION OF AmpC BETA LACTAMASE IN *ENTEROBACTERIACEAE* AMONG CLINICAL ISOLATES''** by **Dr.C.Meenakshi**, Post graduate in Microbiology (2012-2015), is a bonafide research work carried out under our direct supervision and guidance and is submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai, for M.D. Degree Examination in Microbiology, Branch IV, to be held in April 2015.

Guide,
Department of Microbiology,
Tirunelveli Medical College,
Tirunelveli.

Dr. C . Revathy M.D.,
Professor and Head,
Department of Microbiology,
Tirunelveli Medical College,
Tirunelveli.

TIRUNELVELI MEDICAL COLLEGE

INSTITUTIONAL RESEARCH ETHICS COMMITTEE
TIRUNELVELI, STATE OF TAMILNADU, SOUTH INDIA PIN 627011
91-462-2572733-EXT; 91-462-2572944; 91-462-2579785; 91-462-2572611-16
online@tvmc.ac.in, tirec@tvmc.ac.in; www.tvmc.ac.in

CERTIFICATE OF REGISTRATION & APPROVAL OF THE TIREC REF NO: 434/MIC/2013/29.03.13

PROTOCOL TITLE: An Evaluation of Conventional Methods with Molecular Methods for Detection of AMPC Beta Lactamase in Enterobacteriaceae among Clinical Isolates

NAME OF PRINCIPAL INVESTIGATOR: C.Meenakshi

DESIGNATION OF PRINCIPAL INVESTIGATOR: Post Graduate Resident

DEPARTMENT & INSTITUTION: Department of Microbiology, Tirunelveli Medical College

Dear Dr. C.Meenakshi, *the Tirunelveli Medical College Institutional Ethics Committee (TIREC) reviewed and discussed your application during the IEC meeting held on* 29.03.2013.

THE FOLLOWING DOCUMENTS WERE REVIEWED AND APPROVED

1.	TIREC Application Form	☐
2.	Study Protocol	☐
3.	Department Research Committee Approval	☐
4.	Patient Information Document and Consent Form in English and Vernacular Language	☐
5.	Investigator's Brochure	☐
6.	Proposed Methods for Patient Accrual Proposed	☐
7.	Curriculum Vitae of the Principal Investigator	☐
8.	Insurance /Compensation Policy	☐
9.	Investigator's Agreement with Sponsor	☐
10.	Investigator's Undertaking	☐
11.	DCGI/DGFT approval	☐
12.	Clinical Trial Agreement (CTA)	☐
13.	Memorandum of Understanding (MOU)/Material Transfer Agreement (MTA)	☐
14.	Clinical Trials Registry-India (CTRI) Registration	☐

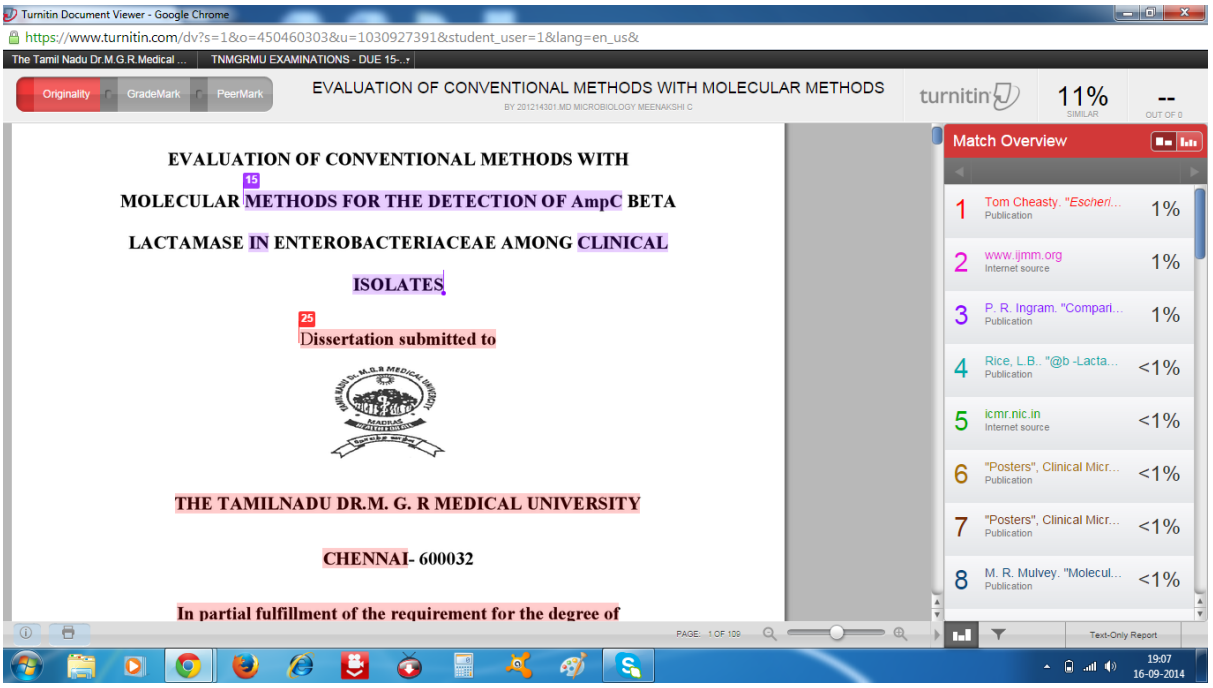
THE PROTOCOL IS APPROVED IN ITS PRESENTED FORM ON THE FOLLOWING CONDITIONS

1. The approval is valid for a period of 2 year/s or duration of project whichever is later
2. The date of commencement of study should be informed
3. A written request should be submitted 3weeks before for renewal / extension of the validity
4. An annual status report should be submitted.
5. The TIREC will monitor the study
6. At the time of PI's retirement/leaving the institute, the study responsibility should be transferred to a person cleared by HOD
7. The PI should report to TIREC within 7 days of the occurrence of the SAE. If the SAE is Death, the Bioethics Cell should receive the SAE reporting form within 24 hours of the occurrence.
8. In the events of any protocol amendments, TIREC 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. The PI must comment how proposed amendment will affect the ongoing trial. Alteration in the budgetary status, staff requirement 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. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented.
   d. 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 IEC, only then can they be implemented.
   e. Approval for amendment changes must be obtained prior to implementation of changes.
   f. The amendment is unlikely to be approved by the IEC unless all the above information is provided.
   g. Any deviation/violation/waiver in the protocol must be informed

STANDS APPROVED UNDER SEAL

Dr.K.Shanmugam MD
Registrar, TIREC
Tirunelveli Medical College, Tirunelveli – 627011
State of Tamilnadu, South India

Dr.V.Ramasubramanian MD DM
Member Secretary, TIREC
Tirunelveli Medical College, Tirunelveli – 627011
State of Tamilnadu, South India


Turnitin Document Viewer - Google Chrome
https://www.turnitin.com/dv?s=1&o=450460303&u=1030927391&student_user=1&lang=en_us&
The Tamil Nadu Dr.M.G.R.Medical ...
TNMGRMU EXAMINATIONS - DUE 15-...
Originality
GradeMark
PeerMark
EVALUATION OF CONVENTIONAL METHODS WITH MOLECULAR METHODS
BY 201214301.MD MICROBIOLOGY MEENAKSHI C
turnitin
11%
SIMILAR
--
OUT OF 0
EVALUATION OF CONVENTIONAL METHODS WITH
MOLECULAR METHODS FOR THE DETECTION OF AmpC BETA
LACTAMASE IN ENTEROBACTERIACEAE AMONG CLINICAL
ISOLATES
Dissertation submitted to
THE TAMILNADU DR.M. G. R MEDICAL UNIVERSITY
CHENNAI- 600032
In partial fulfillment of the requirement for the degree of
PAGE 1 OF 109
Match Overview
1 Tom Cheasty. "Escheri... Publication 1%
2 www.ijmm.org Internet source 1%
3 P. R. Ingram. "Compari... Publication 1%
4 Rice, L.B.. "@b -Lacta... Publication <1%
5 icmr.nic.in Internet source <1%
6 "Posters", Clinical Micr... Publication <1%
7 "Posters", Clinical Micr... Publication <1%
8 M. R. Mulvey. "Molecul... Publication <1%
Text-Only Report
19:07
16-09-2014

DECLARATION

I solemnly declare that the dissertation titled **"EVALUATION OF CONVENTIONAL METHODS WITH MOLECULAR METHODS FOR THE DETECTION OF AmpC BETA LACTAMASE IN *ENTEROBACTERIACEAE* AMONG CLINICAL ISOLATES''** is done by me at Tirunelveli Medical College hospital, Tirunelveli.

The dissertation is submitted to The Tamilnadu Dr.M.G.R.Medical University towards the partial fulfilment of requirements for the award of M.D. Degree (Branch IV) in Microbiology.

Place: Tirunelveli
Date:

Dr.C.Meenakshi,
Postgraduate Student,
M.D Microbiology,
Department of Microbiology,
Tirunelveli Medical College
Tirunelveli.

ACKNOWLEDGEMENT

I sincerely express my heartful gratitude to the Dean, Prof **Dr. L.D.Thulasiram M.S, (Ortho)** Tirunelveli Medical College, Tirunelveli for all the facilities provided for the study.

I am thankful to **Dr. N.Thirumani Pandian M.S., M.ch,** Vice Principal, Tirunelveli medical college for his encouragement during the study period.

I take this opportunity to express my profound gratitude to **Dr.C.Revathy,M.D,** Professor and Head, Department of Microbiology, Tirunelveli Medical College, whose kindness, guidance and constant encouragement enabled me to complete this study.

I am deeply indebted to **Dr.R.Nepolean M.D**, Professor, Department of Microbiology, Tirunelveli Medical College, who helped me to sharpen my critical perceptions by offering most helpful suggestions and corrective comments.

I am very grateful to **Dr. S. Poongodi @ Lakshmi, M.D.**, Professor, Department of Microbiology, Tirunelveli Medical College, for the constant support rendered throughout the period of study and encouragement in every stage of this work.

I wish to thank **Dr. V.Ramesh babu, M.D**., Professor, Department of Microbiology, Tirunelveli Medical College, for his valuable guidance for the study

I am highly obliged to Dr.B.Cinthuja M.D., Dr.G.Velvizhi, M.D., Dr.G.Sucila Thangam, M.D, Dr V.P Amudha M.D., Dr I.M Regitha M.D., Dr.S.Gowri M.D, Dr.M. Kanagapriya M.D, Assistant Professors, Department of Microbiology, Tirunelveli Medical College, for their evincing keen interest, encouragement, and corrective comments during the research period.

Special thanks are due to my postgraduate colleagues, Dr.V.G.Sridevi, Dr.R.Nagalakshmi, Dr.A.Uma maheshwari for never hesitating to lend a helping hand throughout the Study.

I would also wish to thank my junior post-graduate colleagues, Dr.T.Sankaranarayanan, Dr.P.Rajpriya, Dr.S.Suganya, Dr.M.Jeeva Dr.S.Ummer Sheriff, Dr.R.Shiny, Dr.S.Prarthana Dr.V.Indumathy and Dr.R.PoornaKala, Dr.P.Anbumathy for their help and support.

Special Thanks are due to the, Mr.V.Parthasarathy, Mr.V.Chandran, Mr.S.Pannerselvam, S.Santhi, S.Venkateshwari. M.Mali, S.Arifal Beevi, A.S.AbulKalam, A.Kavitha, K.Vadakasi, T.Jeya, K.Sindhu, K.Umayavel, Sreelakshmi, Mr.Manivannanand are other supporting staffs for their services rendered.

I extend my sincere thanks to Mr.Heber for his valuable guidance in statistics.

Finally, I extend my wholehearted thanks to my beloved parents, friends, family members, my husband Dr.K.Balasubramaniam and my daughter B.Subakrishna and my son B.Navaneeth Kumar for their silent sacrifices, support and words of encouragement which were the sources of inspiration for the successful completion of this study.

Finally I thank the Almighty for without Him nothing would have been possible.

CONTENTS

ABBREVIATIONS

ATCC	-	American type culture collection
CLSI	-	Clinical laboratory standard institute
CTX-M	-	Cefotaxime resistant gene
DNA	-	Deoxy ribonucleic acid
DD method	-	Disc Diffusion method
E. coli	-	*Escherichia coli*
EAggEC	-	Enteroaggregative*E.coli*
ESBL	-	Extended Spectrum Beta lactamase
EHEC	-	EnteroHaemorrhagic*E.Coli*
ETEC	-	Enterotoxigenic*E. coli*
EIEC	-	Enteroinvasive*E.coli:*
EPEC	-	Enteropathogenic E. Coli
HUS	-	hemo- lytic-uremic syndrome
IMP	-	Imipenamase
K. pneumonia	-	*Klebsiella pneumoniae*
KPC	-	*Klebsiella pneumoniae* carbapenemases
LPS	-	Lipopolysaccharide
MDR	-	Multi drug resistant
MH agar	-	Mueller hinton agar
MHT	-	Modified Hodge test

NDM	-	New Delhi metallo-β-lactamase
OXA	-	Oxacillin-hydrolyzing
PBP	-	Penicillin binding protein
PCR	-	Polymerase chain reaction
RNA	-	Ribonucleic acid
RT-PCR	-	Real Time Polymerase Chain Reaction
SHV	-	Sulfhydryl variable
SSi	-	Surgical site infections
TEM	-	Temorina
UTIs	-	Urinary tract infections
UPEC	-	Uropathic*E.coli*
VTEC	-	Verotoxigenic*E. coli*
VIM	-	Verona integron encoded metallo-β-lactamase

LIST OF TABLES

EVALUATION OF CONVENTIONAL METHODS WITH MOLECULAR METHODS FOR THE DETECTION OF AmpC BETA LACTAMASE IN *ENTEROBACTERIACEAE* AMONG CLINICAL ISOLATES

Aim of the study:

The aim of the study is the evaluation of conventional methods with molecular methods for the detection of AmpC beta lactamase in *Enterobacteriaceae* among clinical isolates in Tirunelveli medical college hospital.

Materials and Methods:

The study included 50 strains of various enterobacteriaceae species isolated from clinical samples(Urine,pus).These isolates were screened for AmpC β lactamase by Disc diffusion method with Cefoxitin, Modified hodge test,AmpC Disc test and the genotype (BlaAmpC) was confirmed by Polymerase chain Reaction.

Results:

A total of 50 (urine[n=45], pus[n=5]) non-duplicate Cefoxitin resistant isolates from clinical samples which includes *E.coli*(n=23), *Klebsiella pneumoniae*(n=21), *Klebsiella oxytoca(*n= 6) were taken for this study. Modified Hodge test detected 16 (32%)AmpC positive isolates indicated by clover leaf pattern.Among these ,eight isolates(34.8%) were *E.coli* and another eight isolates(38.1) were *Klebsiella pneumoniae*.

AmpC disc test detected 18 AmpC positive isolates.Among these, ten isolates(43.5%) were *E.coli* and seven isolates(33.3%) were *Klebsiella*

pneumoniae , one isolate(16.6%) was *Klebsiella oxytoca*.Real time PCR detected bla AmpC gene in twenty one (42%) of the 50 screen positive isolates .Among these,eleven isolates (47.8%)were *E.coli* ,10 isolates(47.6%) were *Klebsiella pneumoniae* .The sensitivity, specificity of Modified hodge test and AmpC disc test were 71.42%, 96.55%, and 80.95%, 96.55% respectively.

In AmpC-positive isolates, the resistance to third generation Cephalosporins was high, reaching 100% for Ceftriaxone and Ceftazidime and Ceftazidime+clavulanic acid and were resistant in 43%, 81%,75% and 80% respectively to Amikacin,Gentamicin,Nitrofurantoin and Norfloxacin .

Conclusion:

This study highlights the prevalence of AmpC enzyme production among clinical samples and also bla AmpC among AmpC producers.Modified Hodge test is simple to do and less costlier.AmpC disc test is to be considered as a diagnostic tool for AmpC detection in routine laboratory because of its high sensitivity, rapid and easy interpretation.In the present study, MDR among AmpC positive study isolates was high suggesting plasmid mediated spread.Dissemination of AmpC producers within the hospital or between the different regions of our country may become significant public health issue. Hence, recognition of AmpC may enhance hospital infection control rate by making the physician to think about the selection of suitable antibiotics.

Key Words : ***Enterobacteriaceae***, AmpC, MHT, AmpC disc, test, PCR.

1. INTRODUCTION

Gram negative rods belonging to *Enterobacteriaceae* are the pathogens frequently associated with sepsis, hospital acquired infection and infections involving urinary tract and gut[1]. In this family, *Escherichia coli* is the most common pathogen which causes Urinary tract infections, Appendicitis, Peritonitis, Post operative wound infections, Cholecystitis, Sepsis and Diarrhoea in infants & adults, Hemolytic uremic syndrome.[2] Another significant pathogen in this family, *Klebsiella pneumoniae*, is the cause of classic lobar pneumonia. *Enterobacteriaceae* are sensitive to broader-spectrum β-lactams, Quinolones, Aminoglycosides, Sulfonamides, Nitrofurantoin.[3] Now a days, antibiotic resistance is more common among isolates from human infections[1].In Gram-negative bacilli, the different mechanisms of drug resistance include Extended Spectrum Beta lactamase (ESBL) production, AmpC β lactamase production, porin deficiency and efflux mechanisms. Production of ESBLs and AmpC β-lactamases are the most common among the mechanisms of resistance to third generation Cephalosporins. Since late 1970s, AmpC β-lactamases have gained importance as one of the mediators of antimicrobial resistance in Gram negative bacilli.[4]

They are clinically important because they award resistance to broad spectrum Cephalosporins, β- lactam -β lactamase inhibitor combinations and Aztreonam.

AmpC β-lactamases are not inhibited by Clavulanic acid; but, they are inhibited by Cloxacillin.[5] These enzymes fit in to Ambler class C &Bush-Jacoby-Medeiros group 1.Plasmid mediated and chromosomal or inducible AmpC are two different types of AmpC β-lactamases. Organisms such as *Citrobacter freundii, Morganella morganii, Enterobacter cloaca, , Hafnia alvei* and *Serratia marcescens* contain Chromosomal AmpC enzymes and are inducible by Cefoxitin and Imipenem but weakly induced by the third or fourth generation Cephalosporins.[4]

The widespread dissemination of AmpC β lactamase genes on transferable plasmids is a continuing challenge.[6]Plasmid mediated ampC enzymes was first reported in 1988.[7] These enzymes are present in *K. pneumoniae, K. Oxytoca,E. coli, Enterobacter aerogenes, Salmonella enterica serotype Senftenberg,, Proteus mirabilis, M. morganii*. They vary from chromosomal AmpCs in uninducible nature and in association with broad multidrug resistance.[8]Plasmid-mediated AmpC genes are of special interest because they have ability to spread from one genus or species to different organisms. Plasmid-mediated AmpC enzymes containing strains have been isolated from hospitalized patients, and from outpatient clinics and rehabilitation centers.[9] National level prevalence of plasmid mediated

AmpC type resistance is unknown because studies did not examined the strains at the molecular level which is required to reveal the various mechanisms involved.

One study from the United States observed 4 per cent of the *Escherichia coli* and 7 to 8.5 per cent of the *Klebsiella* species contained plasmid mediated AmpC type enzymes(2004).[10]In a study from Singapore,plasmid mediated AmpC was present in 26 per cent of study isolates, with CMY enzymes in *E.coli* and DHA enzymes in *K. Pneumoniae.*[11]

AmpC prevalence in *Klebsiella* and *E coli* species was 24.1%, 37.5% respectively in India.[12] In another Indian study from Karnataka, prevalence of AmpC was 3.3 per cent in *Klebsiella*isolates.[13]Detection of AmpC mediated resistance in Gram negative organisms poses a problem because the phenotypic tests may be confusing for wrong report which results in treatment failures. There are presently no CLSI criteria for AmpC detection.[7] For initial detection, screening with cefoxitin disc is recommended . But, it does not consistently reveal AmpC production. Some of the available phenotypic tests include Modified Hodge test (Yong et al., 2002), AmpC disc test (Sanghal et al) and inhibitor-based assays with boronic acid compounds (Tan et al., 2009) or cloxacillin (Brenwald et al., 2005).[14] Six plasmid-mediated AmpC families (MOX, CIT, DHA, EBC, FOX and ACC-1) were present among Gram negative organisms.[15]

CMY-2, which is the most common subtype of AmpC β-lactamases , is present all over the world .ACT-1 is an inducible subtype common in *E. coli* and *Klebsiella* species from Delhi region. These Genotypes were detected by PCR.[16]

Among the *Enterobacteriaceae* family, *E. Coli* and *Klebsiella pneumoniae* producing plasmid-mediated AmpC β-lactamases are accountable for nosocomial outbreaks of infection.[17]They have also been associated with treatment failure when compared with organisms without plasmid-mediated AmpC β-lactamases.[18] Pai *et al* reported a treatment failure rate of almost 52% for AmpC-containing *K. pneumoniae* associated with bloodstream infections. Rapid detection of AmpC beta lactamases is important to direct proper antibiotic therapy and for suitable infection control measures.[19]Therefore the present study was attempted to evaluate and compare phenotypic methods and molecular methods for the detection of AmpC beta lactamase among clinical isolates of *Enterobacteriaceae* in Tirunelveli medical college hospital.

2. AIMS AND OBJECTIVES

1. To screen the clinical isolates of *Enterobacteriaceae* for AmpC β lactamase production in Tirunelveli medical college hospital.
2. To evaluate phenotypic methods for the identification of AmpC enzyme producers.
3. To detect the prevalence of blaAmpC by PCR.
4. To assess the antimicrobial sensitivity pattern of the clinical isolates of *Enterobacteriaceae* .

3. REVIEW OF LITERATURE

3.1.Description of the *Enterobacteriaceae* family:

Enterobacteriaceae are a huge family of Gram-negative rods and the members of this family are either free-living or part of the normal flora of humans and animals[3].The family contains 40 genus and 150 named species and subspecies, along with named biogroups and unnamed organisms[1].

3.1.1.Important Genera& species

Escherichia coli,Klebsiella pneumoniae, Salmonella enterica S.Typhi, Salmonella Typhimurium, Salmonella Enteritids, Shigella, Citrobacter, Enterobacter, Serratia, Proteus, Providencia, Morganella, Yersinia Pestis, Yersinia enterocolitica, Yersinia Pseudotuberculosis are the significant species of *Enterobacteriaceae* family[2].

3.1.2.Clinical significance of *Enterobacteriaceae:*

They produce Septicemia(50%),Urinary tract infections(70%), and Intestinal infections, Nosocomial infections.[1]The most common cause of urinary tract infections (UTIs) in both community and health care settings are *Enterobacteriaceae* .[20]

JB Sharma *et al* reported that 2/3rd of the cultures from the patients with SSI(Surgical site infections) from North east India teaching hospital showed the growth of *Enterobacteriaceae.*[21]

Nosocomial sepsis in Neonates was caused by *Enterobacteriaceae* commensals of the newborn intestine.[22]

Beena antony *et al* isolated 18 *Enterobacter Cloacae* from neonates with septicaemic syndrome in Mueller medical college, Karnataka.[23]

3.2.*Escherichia*:

The genus consists of six species; *E. Coli,Escherichia albertii, Escherichia blattae, Escherichia fergusonii*, *Escherichia hermannii*, and *Escherichia vulneris.* All species have been isolated from human specimens except *E. blattae*, which is a commensal organism of cockroaches.[24]

3.2.1.*E. Coli*:

Theodore Escherich (German paediatrician) identified *E. coli* in his studies of the intestinal flora of infants in 1885.Castellani and Chalmers denoted the genus *Escherichia* and identified the type species *E. coli* (1919).This is the most common causative organism in human bacterial infections.[2]

3.2.2. Morpholgy:

They are rod in shape and 2.0 and 6.0 μm in length and 1.1 and 1.5 μm in width. All strains are motile by peritrichous flagella with the exception of the *E. coli 'inactive*' species. Polysaccharide capsules are common in *E. coli.* Different kinds of fimbriae are present in *E.coli* and are called as organs of adhesion.[25]

3.2.3.Cell wall:

Cell wall has an outer membrane composed of phospholipids, lipid A and proteins which is in turn surrounded by capsular polysaccharides.[25]

3.2.4.Antigens in *E. Coli:*

E. coli serotyping was done by determination of the O, K, and H antigens. Sometimes a fimbrial virulence factor is also present(F).

3.2.5. Virulence factors:

EPEC -Enteropathogenic E. coli :

1. bfpA - Bundle-forming pilus
2. dsbA- Disulfide isomerise.
3. eae- Intimin
4. per -Plasmid encoded regulator.
5. SepA/sepB

Verotoxigenic *E. coli* (VTEC) :

1)eae -Causes intimate adherence to epithelial cells; similar to eae of EPEC

2)vtx gene family A1B5 toxin (similar to Shiga toxin). A subunit removes one base specifically from eukaryotic 28S rRNA and results in inhibition of protein synthesis. B subunit is for binding to receptor on host cell .

EHEC-EnteroHaemorrhagic*E.Coli*:

hlyA - Enterohemolysin

ETEC -Enterotoxigenic *E. coli*:

1)LT -(Heat-labile A1B5 toxin).

2)ST- heat-stable toxin .

3)CFAs, CSs -colonization factor antigens or coli surface associated antigens [25]

S.Sharma *et al observed that* serum resistant factor was thegeneral virulence factor noted in 132 (86.8%) isolates and 36 (23.7%) isolates with Haemolysin,42 strains (27.6%) were hydrophobic and protease was produced only in 4 (6.9%) isolates of *E. Coli.*[26]

3.2.6. Extraintestinal infections:

Urinary tract infections are caused by the Uropathic *E.coli* (UPEC) Appendicitis, Cholecystitis, Sepsis and Peritonitis, Post operative wound infections are other infections by Enterobacteriaceae.[2]

BATantry *et al* described that 1980(67%)were urine culture positive out of 2842 samples for *Escherichia coli* which is followed by *Klebsiella pneumoniae* as the commonest cause of UTI. [27]

Enteropathogenic strains of *E. coli* have been found in the Neonatal Intensive Care Unit as the causative agents for neonatal sepsis.[22]

Marlieke E.A *et al* estimated that more than 8,000 deaths were associated with Third generation Cephalosporin resistant *E.Coli* blood stream infections in the European region in 2007.[28]

Asima Banu *et al* reported that 253(26.8%)urine culture among the total 943 culture positive urine samples and 23(4.3%) out of 538 culture positive sputum samples, 101(6.5%) out of 1534 culture positive exudate

samples, 2(1.2%) out of 166 blood samples were positive for E.coli in her study among the clinical isolates of Bangalore Medical college[29]

3.2.7. Intestinal infections:

These are caused by the pathovars EPEC, ETEC, EIEC, EHEC, and EaggEC.

EPEC:Enteropathogenic *E.coli*:[30]

This is a causative agent of diarrheal disease in young children, including neonates.

ETEC:Enterotoxigenic *E.coli:*

It is a causative agent of Traveller's diarrhea in industrialized countries, causing infection in 25 to 75% of cases.

EIEC:Enteroinvasive*E.coli:*

*G*enetic and clinical features of EIEC are similar with Shigella.

EHEC:Enterohaemorrhagic *E.coli.*(STEC)

These pathogens cause hemorrhagic colitis and haemolytic uremic syndrome (HUS). The most prominent serotype is O157:H7 .

EAggEC:Enteroaggregative*E.coli:* EAEC adheres to intestinal mucosa and induces toxic effects that result in diarrhoea.[30]

Andrej *et al* reported that 128 (90%) from total 143 EAEC strains were positive for gene encode anti-aggregation protein detected by PCR assay .[31]

Sanjucta Dutta detected Diarrhoeal *E.Coli* in 11.8% (452/3826) among Hospitalized Diarrheal Patients in Kolkata, India and observed that EAEC was more prevalent (5.7%) than ETEC (4.2%) and EPEC (1.8%) by Multiplex PCR assay.[32]

3.3.*Klebsiella*species :

Von Frisch identified a capsulated organism from rhinoscleroma patient in 1882.Friedlander cultivated another organism (Friedlander's bacillus)from the patients with pneumonia in 1883. Ozaena bacillus was discovered by Abel in 1896.[33]

Non existence of motility and the occurrence of polysaccharide capsule are the characteristic features of the genus *Klebsiella* .The colonies have mucoid character. Total seventy capsular types have been identified.Pili are also present on the surface and they are helpful for adherence to respiratory and urinary epithelium.[3]

Klebsiella pneumoniae (K,pneumonia subspecies pneumoniae, K. pneumoniae subsp. Rhinoscleromatis, K. pneumoniae subsp. Ozaenae), Klebsiella oxytoca, Klebsiella ornitholytica, Klebsiella terrigena are the significant species of this family.[33]

3.3.1. Virulence factors

The principal virulence factor for *K. pneumoniae* is its polysaccharide capsule. Some capsule types (K1 and K2) may be more important than

others. Inhibition of phagocytosis is the mechanism of virulence by capsule. Type 1 pili is implicated in adherence to host cells.[34]

3.3.2. Clinical significance of *Klebsiella* species:

K. pneumoniaeis the cause of classic lobar pneumonia. These strains cause wide spread colonization of hospital patients.[33], *Klebsiella* species exihibit high MDR pattern than other species of *Enterobacteriaceae*.[3]

K.oxytoca: It is an Indole positive organism discovered by Flugge in 1886.[33]*K. oxytoca* is the main pathogen in long-term-care facilities. [30]

K. rhinoscleromatis and ***K. Ozaenae***: Rhinoscleroma caused by *K. Rhinoscleromatis* is a slowly progressive mucosal upper respiratory infection. *K. ozaenae* causes chronic atrophic rhinitis.

Pneumonia,abdominal infection, UTI, surgical site infection and bacteremia are caused by Klebsiella species.[30]

In one study at Maharajah's institute of medical sciences, Andhrapradesh, India- 2008 to 2010, Dr.R.Sarathbabu *et al* observed that 24.36% culture were positive for *klebsiella pneumoniae* in sputum samples, 20.09% in urine samples and 24.82% in pus samples.[35]

Malik *et al* reported that Multi drug resistant *K. pneumonia*e were the commonest organism isolated in 30 neonates, UP, India, leading to 14.7% incidence of Klebsiella nosocomial infection.[36]

Francesco Casella *et al*(Italy) reported that two cases of Asiatic patients with *Klebsiella* were associated liver abscess evaluated at their institution. [37]

B. N. Harish *et al* observed that blood culture from 130 patients submitted from Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Pondicherry, were positive for *K. Pneumonia*e.[38]

Varsha Chaudhary *et al*reported *klebsiella* species as the causative agent in 1.5% of vaginitis in her study.[39]

3.4. Antimicrobial therapy:

Enterobacteriaceae are insusceptible to high concentrations of Penicillin G, Erythromycin, and Clindamycin, but they are susceptible to the broader-spectrum beta-lactams, Tetracycline, Chloramphenicol, Aminoglycosides, Sulfonamides, Quinolones, Nitrofurantoin.[3]

3.4.1.Beta – lactam antibiotics

Beta lactam antibiotics have beta lactam ring in their primary structure.

Beta – lactam drugs:

1)Penicillins,2)Cephalosporins ,3)Monobactams ,4)Carbapenems

Penicillins:[40]

Penicillins are subgrouped into

1) Penicillin G, PenicillinV.

Figure:1

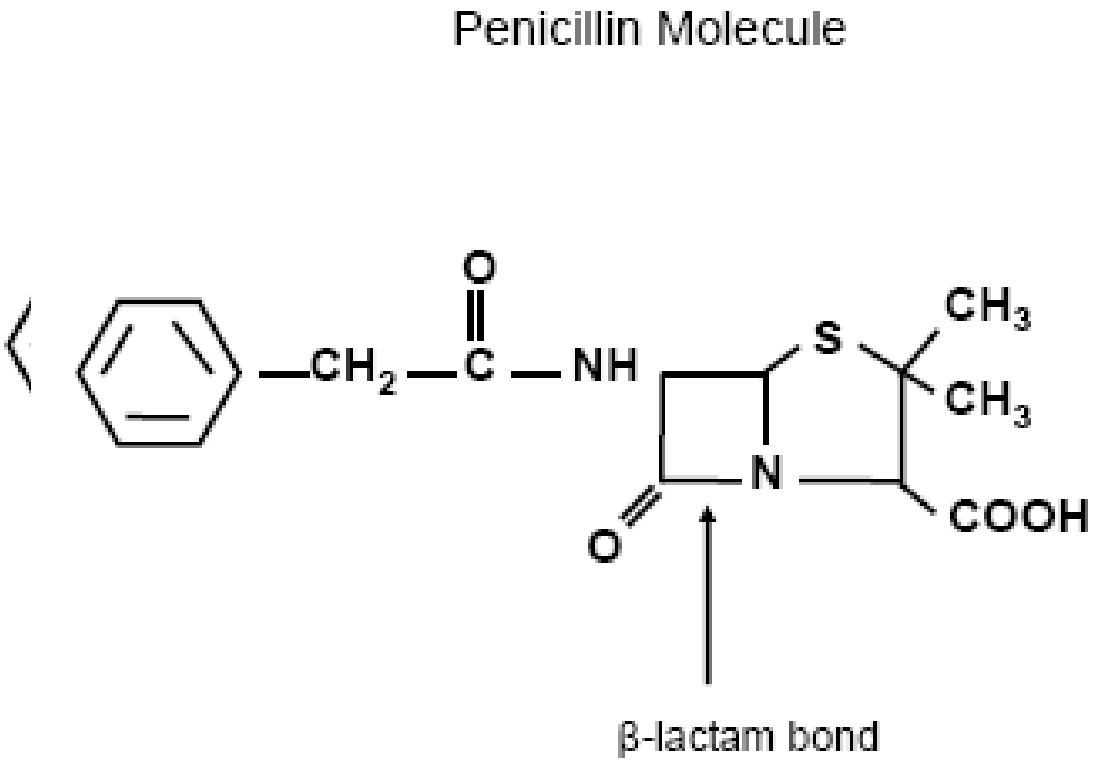
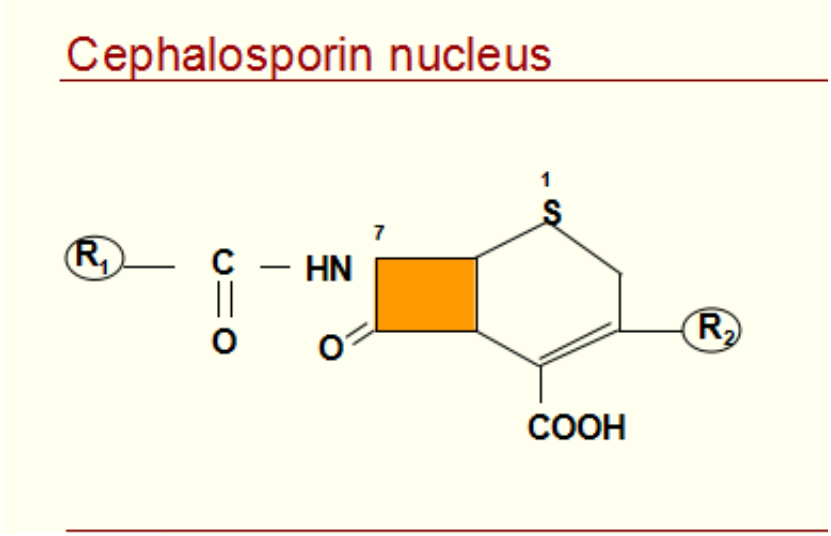


Figure:2



2) Penicillinase resistant penicillins: Methicillin, Nafcillin, Oxacillin, Cloxacillin.

3) Carboxypenicillins: Carbenicillin, Ticarcillin

4)Aminopenicillins: Ampicillin, Amoxicillin.

5)Ureidopenicillins: Mezlocillin, Piperacillin.

Cephalosporins :

There are five generations of Cephalosporins. They are:

- First generation: Cefazolin, Cephalothin, Cephalexin.
- Second generation: Cefuroxime, Cefaclor, Cefamycins (Cefotetan, Cefoxitin ,Cefamandole).
- Third generation: Cefotaxime, Ceftriaxone, Cefpodoxime, Ceftizoxime, Cefperazone, Ceftazidime.
- Fourth generation: Cefepime, Cefpirome.
- Fifth generation:Ceftabiprole

Carbapenems:

Imipenem, Meropenem, Ertapenem are comimg under this group.

Monobactams: Aztreonam

Mechanism of action:

The binding of the beta lactam to PBPs inhibit the synthesis of cell wall and leads to autolysis and death of the cell.[41]

β-LACTAMASE INHIBITORS :

1)Clavulanic Acid

2)Sulbactam

3) Tazobactam

are examples of the **β**-lactamase inhibitors.

3.4.2.Aminoglycosides:

Gentamicin, Kanamycin, Tobramycin, Streptomycin, Netilmicin, Neomycin, and Amikacin are classified as Aminoglycosides.

Mehanism of action:

They prevent the synthesis of protein by combining with 30S ribosome.

Macrolides:

Examples: Erythromycin, Azithromycin.

Mehanism of action:

It prevents the synthesis of protein by combining with 50S ribosome.

Tetracycline:

Mehanism of action:

It prevents the synthesis of protein by combining with 30S ribosome instead of t-RNA binding .

Chloramphenicol, **Lincosamides, Streptogramin**:

Mehanism of action:

They prevent the synthesis of protein by combining with 50S ribosome.

Quinolones:

e.g :Ciprofloxacin, Levofloxacin, Gatifloxacin, and Moxifloxacin

Mehanism of action:

They inhibit DNA gyrase (Inhibition of Replication)

Sulfonamides :

Mehanism of action:

They act by inhibiting Folic acid synthesis(Inhibit Dihydro terroate synthetase)

Trimethoprim act by inhibition of Folic acid synthesis(Inhibit Dihydro folate synthetase)

3.5. Antibiotic resistance patterns:

Intially, there was a negligible resistance among the species of *Enterobacteriaceae.* Now a days, antibiotic resistance in isolates of *Enterobacteriaceae* is a major hazard to victorious therapy of infection which is emerging in many parts of the world .[1]

3.5.1. Classification of resistance:

It can be broadly divided into

- Intrinsic resistance

- Acquired resistance

Intrinsic resistance;

This type of resistance is an inherited property of a species.

Example:

Citrobacter freundii- Cephalothin

Citrobacter koseri -Cephalothin, Carbenicillin

Edwardsiella tarda- Colistin

Enterobacter cloacae- Cephalothin

Escherichia hermannii -Ampicillin, Carbenicillin

Klebsiella pneumoniae- Ampicillin, Carbenicillin

Proteus mirabilis -Polymyxins, Tetracycline, Nitrofurantoin

Acquired resistance:

The following are the mechanisms of acquired resistance

- ✓ genetic mutation
- ✓ gene transfermechanims (Transformation,conjucation,Transduction),
- ✓ combined mutation & gene transfer methods.[41]

Mutations :

Incorporation of incorrect nucleotides occur during DNA replication randomly. Normaly a mutation will cause resistance to one class of antimicrobial agents, however changes affecting impermeability and efflux may result in a multiple resistance towards many classes of antimicrobial agents [42]

Horizontal gene transfer:

By processes like conjugation, transformation, and transduction resistance genes are able to spread from one bacterium to another.

Conjugation:

It is a process in which genes from one bacterium are transmitted to another bacterium. The process involves a donor that contain a transferable element and a recipient that does not. The donor produces a pilus that attaches the two cells. The outer membrane of the two cells fuse and DNA can be transferred from the donor to the recipient. Both plasmids and chromosomal parts can be moved.

Transformation:

It is the uptake of naked DNA from the environment. Cell lysis will release fragmented DNA that naturally competent bacteria can take up. Typically only short DNA fragments are exchanged.

Transduction:

It is a mechanism in which shift of host genes between two bacterium by bacteriophages.[43]

3.5.2. Resistance elements:

The resistance elements are of two types namely

- Plasmids
- Transposons

Plasmids:

Plasmids are genetic elements that replicate independently of the host chromosome. They do not have an extracellular form and exist inside cells as free, circular, double stranded DNA. Examples of plasmid-encoded genes are virulence factors and resistance genes.

Transposons:

These are elements of DNA with ability to move from one place to another within the genome. These elements are found in humans and all organisms. Transposases are the enzymes which promote the movement of DNA. Length of the transposons vary in range from about 1000 bp, only carrying the genes for the transposases, to larger elements harbouring other genes, including resistance encoding genes. [44]

Insertion sequence (IS) elements:

Insertion sequence (IS) elements are the smallest type of transposons found in bacterial cells. Their insertion into the genome of bacteria cause alterations that may result in resistance to antimicrobial agents.

3.6.Resistance mechanisms in *Enterobacteriaceae:*[41]

β-lactam antibiotics:

It includes β lactamase inactivation, alteration in PBP targets, impermeability. (Production of β-lactamases which destoy β-lactam ring, so antibiotic can't bind to PBP and interfere with cell wall synthesis).

Tetracycline:

These are efflux, altered target, impermeability and enzymatic inactivation

Chloramphenicol :

It is by enzymatic inactivation and impermeability

Quinolones:

They bring about resistance by altering target and development of impermeability

Sulfonamides :

By altering the target ,they develop resistance.

Trimethoprim :

Altered target, impermeability are the processes for the development of resistance.

Aminoglycosides:

- Enzymatic inactivation-Aminoglycoside modifying enzyme alters various sites on the aminoglycoside molecule so that the ability of drug to bind the ribosome is decreased.
- Decreased uptake of aminoglycosides due to change in number &character of porin channels.

3.7.Beta-lactamases:Production of β-lactamases isthe most universal Gram-negative resistance mechanism .[44]

These are enzymes that cause hydrolysis of beta-lactam drugs. As a result the cell is resistant to the action of the beta lactam drugs.

In gram-negative bacteria, the beta lactam drugs enter through the porin channels in to the cell and gets exposed to beta-lactamases in the periplasmic space. Before they reach their PBP targets, the beta-lactam molecules are destroyed by beta-lactamases.

The beta-lactamases are secreted extra cellularly into the surrounding medium by gram positive bacteria and destroy the beta-lactam molecules before they have a chance to enter the cell.

3.7.1..Classification of β-Lactamases:[40]

Two major classifications of β- lactamases:

1)Molecular classification (Ambler) system

2) Functional (Bush-Jacoby) classification system.

Molecular classification

Based on similarities in amino acid sequence, the Ambler classification separates β-lactamases into four classes (A to D) .

A, C, and D enzymes belong to Serine β-lactamases

B enzymes belongs to Metallo β lactamases

Functional Classification

(Bush-Jacoby classification system)

Four groups-1,2,3,4.(Group-2: 2a, 2b,2br , 2d, 2be, 2c, and 2f)

Group 1

CEPHALOSPORINASE:

These are not inhibited by clavulanic acid- the molecular class C.(AmpC Enzymes)

Group 2

It includes Penicillinases, cephalosporinase which are inhibited by clavulanic acid. There are two subclasses namely 2a and 2b. 2b is the broad spectrum β- lactamases

GROUP 2a :

This group has penicillinases. It comes under the Molecular Classification A.

BROAD-SPECTRUM (2b):

They have the ability to inactivate penicillins and Cephalosporins .It belongs to the category of Molecular Class A. It is sub classified into 2be and 2br.2be is named as extended spectrum and 2br is called as inhibitor resistant.

EXTENDED-SPECTRUM(ESBL)or2be:

They cause hydrolysis of third-generation Cephalosporins and monobactams and they are subjected to inhibition by clavulanic acid. It is grouped under A of Molecular Classification

INHIBITOR-RESISTANT (2br):

It develops reduced inhibition by clavulanic acid-susceptible to tazobactam. They are also placed in category of A of molecular classification.

GROUP 2c:

They have carbenicillinase. They cause hydrolysis of carbenicillin.They are in category C of the molecular classification.

GROUP 2d:- Molecular Class D or A

There are two enzymes in this group called cloxacillinase and oxacillinase.

CLOXACILLINASE :

The enzyme in this group cloxacillinase inactivates cloxacillin. These are weakly inhibited by clavulanic acid. They belong to either A or D of molecular classification.

OXACILLINASE:

Oxacillinase inactivates the oxazolylpenicillins like oxacillin, cloxacillin. Group D is their placement in molecular classification.

GROUP 2e:-

Cephalosporinase of this group causes hydrolysis of monobactams, they are inhibited by clavulanic acid .They are in category A .

GROUP 2f:

Carbapenamase belonging to this subtype is a serine-based enzymes.It stays in the group A of molecular classification.

Group 3: Molecular Class B

They have Metalloenzymes**.**Clavulanic acidhas no inhibitory action on these this enzyme.They are Zinc based beta-lactamases, these have the ability to hydrolyse Penicillins, Cephalosporins, and Carbapenems.

Group 4:

Penicillinase is the enzyme belonging to this category. They do fit into any category of molecular classification. Clavulanic acid cannot act on these enzymes also.

3.7.2.Genetic make up of β-Lactamases:

β-Lactamases may be chromosome, plasmid encoded enzymes and their production is in a constitutive or inducible manner. Integrons encoded enzymes are also present. Genetic elements containintegrase gene and antibiotic resistance genes and integration site are called Integrons[40].

3.7.3.Significant types of β-lactamase enzymes:

1) ESBLs--Extended-spectrum β-lactamases

2)Carbapenemases

3)AmpC-

- ✓ Plasmid-mediated
- ✓ Chromasomally-mediated

3.7.4. ESBL:

ESBLs are generally Bush group 2be&Ambler class A .

Epidemiology

ESBL isolates were discovered first in Western Europe in 1980.ESBLs are mainly found in *Kiebsiella pneumoniae, Kiebsiella oxytoca,* and *E.coli,* butalso isolated from *Enterobacter species, Salmonella enterica, Morganella ,Proteus rnirabilis, Serratia marcescens,* and *Pseudomonas aeruginosa.*

Mark E. Rupp *et al* observed that about 40 % of *Escherichia coli and Klebsiella pneumonia* were ESBL producers in many parts of the world.[45]

Arne Søraas *et al* reported that 342 *E. coli* and 17*K. Pneumoniae* among 359 urine samples were ESBL producers yielded from Vestre Viken Hospital Trust , South-Eastern part of Norway.[46]

Arif Hussain *et al* observed that 23% *Enterobacteriaceae* isolates were ESBL-producers .[47]

Substrate profile:

ESBLs are β-lactamases that hydrolyze Penicillins, third generation Cephalosporins, and Monobactam and are inhibited by Clavulanate, Sulbactam, and Tazobactam (β lactamase inhibitors) and are encoded by mobile genes.[48]

Families of ESBL:

There are three families(CTX-M, SHV, and TEM).

CTX-M β-lactamases:

K. pneumoniae, E. coli,Citrobacter freundii, Salmonella, Shigella species, , Enterobacter species, and *Serratia marcescens* are the pathogens that contain CTX-M family.CTX-M-15 is the most widely distributed ESBL type in India.[49]

TEM:

There are 130 TEM-type beta-lactamases and 50 SHV-type enzymes. In 1965, the TEM -1 β lactamase enzyme was first identified from an *E.coli* isolateof Temoniera patient in Greece. TEM-1 is the cause of 90% of Ampicillin resistance in *E. coli*. *E. coli* and *K. Pneumoniae* are the pathogens that contain TEM-type beta-lactamases .

SHV:

SHVhas a similar overall Structure with TEM-1.SHV stands for sulf hydryl variable. *K. Pneumoniae* contains SHV-1 beta-lactamase which is the cause of 20% of Ampicillin resistance.[40]

Mohammed Sahid *et al* observed that the prevalence of blaCTX-M, blaTEM, and blaSHV in 28.8%, 10.9% and 13.7% isolates of *Enterobacteriaceae* by Multiplex PCR, Uttar Pradesh, India.[50]

3.7.5.Carbapenemases

The types of Carbapenemases are

1)*Klebsiella pneumoniae* carbapenemases

2)Serine based carbapenamases

3)Metallo enzymes-(IMP Type carbapenemases, VIM Type carbapenemases)

4)OXA Type carbapenamases

***Klebsiella pneumoniae* carbapenemases**:

Klebsiella pneumoniae carbapenemases (KPC) confer resistance to third and fourth generation Cephalosporins and Carbapenems. KPC-producing *Enterobacteriaceae* were first reported in North Carolina in 1996.[51]

Serine based carbapenamases

Serine carbapenemases of Bush group 2f or class A type. (Sme-1, Sme-2, IMI-1, GES-2, and KPC-2) . Usually, class A carbapenemases hydrolyze Imipenem but are not resistant to Clavulanic acid inhibition .

Metalloenzymes:

These are Class B β-Lactamases (Bush Group 3 Enzymes).These β-lactamases require zinc for their action. Chelating agents (EDTA) prevent the action of Metallo enzymes. There are three sub classes.

1)B1 enzymes (IMP-1, VIM-2, and CcrA- one or two zinc ions)

2) B2 enzymes (e.g., CphA) - Accumulation of a second zinc ion inhibit their activiy.

3)B3 enzymes (e.g., L1) need two zinc ions. The majority of metallo-β-lactamases are chromosomally encoded.

Their appearance may be constitutive or inducible. The inducible metallo-βlactamases are seen in *B. cereus, Stenotrophomonas maltophilia, Aeromonas hydrophila, and Aeromonas jandaei.*[40]

VIM:

These metallo-β-lactamases are broad- spectrum enzymes and are active against most β-lactams, including Carbapenems. blaVIM is an integron-borne metallo- β-lactamase that is usually found in *Pseudomonas aeruginosa* isolates. This VIM metallo-β-lactamase has spread to other enteric bacilli (*E. coli, Enterobacter aerogenes, Enterobacter cloacae, and Klebsiella species.*).

IMP :

IMP metallo-β- lactamases have been found as part of integrons in the following bacteria: *P. aeruginosa, Pseudomonas putida, Serratia marcescens, Pseudomonas stutzeri, Acinetobacter baumannii, Pseudomonas fluorescens,*

K. pneumoniae, K. oxytoca, E. aerogenes, and Escherichia coli .Recently,NDM-1 (New Delhi metallo-β-lactamase) is a class B β-lactamase encoded by a mobile genetic element is also emerged.[40]

Prasanta Raghab Mohapatra *et al*(Chandigarh)observed that NDM-1 isolated from *Enterobacteriaceae* in Guwahati, Kolkata, Hyderabad, and New Delhi in India suggestsextensivedistribution.[52]

S.Nagaraj(2012)*et al* observed that blaNDM positive*K. pneumoniae* isolates were 75% and 5 isolates were blaVIM positive by PCR in south India.[53]

OXA Carbapenamases:

These Class Dβ-Lactamases are presentin *Enterobacteriaceae, Acinetobacter species, and P. aeruginosa.* These enzymes award resistance to variety of Penicillins.

They are faintly inhibited by Clavulanic acid. Several OXA β-lactamases have resemblance with an ESBL phenotype.

3.7.6. AmpC enzymes:

These enzymes belong to Bush-Jacoby-Medeiros group 1 and Ambler class C. Theseinactivate Penicillins, third generation Cephalosporins, and Aztreonam & Cephamycins and are resistant to inhibition by Clavulanate, Sulbactam, and Tazobactam (in the case of Tazobactam, the resistance to inhibition is usually less) but are inhibited by Cloxacillin and Phenylboronic acid .[40]

3.8.Emergence of Resistance in *Escherichia coli:*

Because of inadequate and empirical treatment with commonly used antibiotics, *E.Coli* has developed resistance against these drugs. This has resulted in difficulty to treat urinary tract infection.

MS.Kumar (Hyderabad)observed that ESBL producers were present in 19.8% of *Enterobacteriaceae* isolates in tertiary care hospital at India(2006).[54]

Asima banu *et al (*Bangalore*)* analysed that 94.2% were resistant to Ampicillin and least (0%) were resistant to Carbapenams followed by 15.6% to Netilmicin among 349 isolates of *E.coli* in 2011.[29]

E.coli had a higher percentage of resistance to cotrimoxazole(OP76%,IP79%)followed by cephalexin(OP72%,IP81%)and the lowest resistance to amikacin(OP11%,IP13%)and cefixime, *Klebsiella* species had somewhatdiverse susceptibility pattern of *E coli(*2012-Kashmir*)* .[27]

3.9. Antibiotic Resistance pattern in *K. Pneumoniae*:

Nosocomial outbreaks of infection in ICUs (intensive care units) was caused by Antimicrobial-resistant strains of *K. Pneumoniae* .[30]They are intrinsically resistant to Ampicillin. There is exchangeable resistance to third generation Cephalosporins because of the production of plasmid encoded Extended-Spectrum β- lactamases in nosocomial strains.[33]

R.Sarathbabu *et al*(Andrapradesh) denoted in his study(2012) that mainstream of the *Klebsiella* species were sensitive to Amikacin and the sensitivity to Amikacin was 75.56% in 2008, 70.37% in 2009,and 66.67% in 2010 for pus samples; 66.67% in 2008 and 78.31% in 2008,74.44% in 2009 and 71.60% in 2010 for urine samples; thus showing a gradual increase in

resistance and decrease in sensitivity .[35]*Klebsiella pneumoniae* carbapenemase (KPC) was first described in North Carolinarecently in 1996.[7]

3.10. AmpC enzymes

These enzymes belong to Ambler class C &Bush-Jacoby-Medeiros group1.

3.10.1. Substrate profile:

These β-lactamases inactivate Penicillins, third generation Cephalosporins & Cephamycins and Monobactams. These enzymes are resistant to inhibition by Clavulanate and Tazobactam, Sulbactam (the resistance to inhibition is usually less in the case of Tazobactam) but are inhibited by Cloxacillin and Phenyl boronic acid.[40]

3.10.2.Mechanism:

These β-lactamases have outsized active-site cavities which may permit them to bind the extended-spectrum Cephalosporins. This conformational expansion and flexibility enhance hydrolysis of oxyimino β lactams. These enzymesare distinguished from class A by this "substrate-assisted catalysis".

3.10.3.structural elements:

The structural elements are similar for class A enzymes. Near the N terminus of a long helix, the active-site serine (Ser64) is situated and lysine in the next helix (Ser64-Xaa-Xaa-Lys67). Tyr-Xaa-Asn (Tyr150) is

considered as second element pattern. The molecular size is from 38 to 42 kDa. [40]

3.10.4. Epidemiology:

The first class C β-lactamase structure determined was for the AmpC cephalosporinase of *Citrobacter freundii,* discovered by Oefner et al. The structures of P99 β-lactamase of *Enterobacter cloacae,* AmpC β-lactamase from *E. coli,* and *E.nterobacter cloacae* GC1 and *Enterobacter cloacae* 908R β-lactamases have been identified.

3.10.5. Repression & Expression

In Gram-negative bacilli producing class C enzyme, β-lactamase production is usually repressed. This repression has been identified first for *Enterobacter* species. The processes of cell wall production and breakdown are strongly correlated to Repression and activation. The repressor and the activator of ampC transcription is AmpR molecule.

Mulveymr *et al* reported that *E. coli* isolates resistant to cefoxitin were found with incorporation of IS10 and IS911 into the promoter region of the blaAmpC gene, leading to an over production of the enzyme in Canadian hospitals . [55]

3.10.6. Chromosomal AmpC enzymes :

Almost all gram-negative bacteria produce these enzymes in greater or lesser extent. Some pathogens do not contain this enzyme(*Salmonella,*

Klebsiella, Proteus mirabilis, Proteus vulgaris, and *Stenotrophomonas maltophilia*).

Chromosomally encoded (and inducible) enzymes are present in clinical isolates of *Citrobacter freundii, Enterobacter cloacae, Enterobacter aerogenes, Morganella morganii, Serratia marcescens,* and *Pseudomonas aeruginosa.*[40]

Expression of chromosomal AmpC in *E. coli* is not inducible, but some *E. coli* species constitutively express enzymes.*K. Pneumoniae* does not possess chromosomal AmpC.[56]

V.Supriya et al(Varanasi) in her study in 2008 observed that 7% of *Pseudomonas aeruginosa* were inducible AmpC producers among the total of 120 isolates.[57]

3.10.7.Plasmid-encoded AmpC:

The rapid global dissemination of *Enterobacteriaceae* harbouring plasmid- encoded AmpC enzymes implies a important clinical threat.[5]There are four groups.

- Group 1-plasmid-encoded AmpC cephalosporinases comprise those which derived from the chromosomal AmpC of *C. freundii*.
- Group 2 -correlated to the chromosomal AmpC of *Enterobacter cloacae* ,
- Group 3 - related to the AmpC of *P. aeruginosa*
- Group 4- enzymes fit in to the CMY-1 β-lactamase .

Epidemiology:

Plasmid encoded AmpC enzymes was first reported in 1988.[7]

In United states, the largest outbreak was observed with MIR-1-producing *K. pneumoniae* isolates from 11 patients at the Miriam Hospital over a period of 9 months .[59]These enzymes are present in *K. pneumoniae, E. Coli, E. aerogenes, K. Oxytoca, Salmonella enterica serotype Senftenberg, Proteus mirabilis, M. Morganii.*The loss of porin channels in clinical isolates with plasmid-encoded AmpC enzymes may result in resistance to carbapenems.[9]Difference between chromosomal AmpCs and these enzymes are its uninducible character and organization with broad multidrug resistance.[8]

Xuan Qin *et al* (Washington) observed that blaCMY-2 was the most common genotype in AmpC producing in E. coli isolates.[60]

3.10.8.Prevalence:

Global prevalence:

Plasmid-encoded AmpC β-lactamases have been observed in many gram-negative organisms from every parts of the world. Slike polsfuss *et al (*Switzerland) observed that33 *E.Coli* among the total 38 isolates were AmpC-producing pathogens. [61] Xuan Qin *et al(*Washington)observed that 36 (0.45%) isolates among the total 8.048*Enterobacteriaceae*isolates were AmpC producing pathogens.[60]

Azza A.Elsharkawy *et al(* Egypt)evaluated that2.6% were pAmpC producers among 38 *Klebsiella pneumoniae* strains by phenotypic and Genotypic tests. [62]

Nevine Fam *et al (*Egypt*)*detected AmpC genes in 28.3% (17/60) of the study isolates including *E. Coli* and *Klebsiella* by PCR.[63]

Chelsie *et al*(USA) reported that 4% were AmpC producing pathogens among the 120 clinical isolates comprised of *K. pneumoniae, Klebsiella oxytoca, and E. coli* isolates by PCR .[15]

Akujobi*et al* (Nigeria) reported that 56.25%of *E.coli* and 43.75% of *Klebsiella* isolates were AmpC producing pathogens in Nigeria in 2012.[4]

Hai-Fei Yang et al (2012)observed that 5 of the 146 *Serratia marcescens* isolates from 34 hospitals in Anhui, China harboured pAmpC genes.[64]

One study by Fatima *et al(*Iran*)* showed that out of (73) Gram-negative bacteria, only 5 (6.8%) isolates produced AmpC β- lactamase in 2012.[65]

Şerife Altun *et al(*Turkey*)*observed that Amp-C beta-lactamase was present in 4 (33%), 7 (46.7%) of *E. coli, Klebsiella spp* respectively in 2012 by Inhibitor based test.[58]

Yusuf *et al* (Kano-nigeria) reported that *Morganella* species (50.0%), *Enterobacter* spp (18.8%), *K. aerogenes*(16.7%), *K. pneumoniae* (16.4%) ,

P. mirabilis (15.7%) and *E. coli* (8.6%) were positive for AmpC enzyme production. [66]

3.10.9. Prevalence in India:

In 2003, based on phenotypic tests, the reports from Delhi showed33.3 per cent of Gram-negative bacteria (GNB) to be AmpC enzyme producing pathogens .[67]In 2005, the figures from Delhi showed 6.7 per cent of GNB were AmpC enzyme positive isolates.[68]V.Hemalatha *et al* described that the AmpC production was in 7 (9.2%) of *E.coli* and *K.pneumoniae* isolates from SRM University,Chennai in 2006 by Phenotypic method.[69]

Deepika *et al(*2007) detected that 40% of *E. Coli* were AmpC enzyme positive isolates from Subharti Medical College, Uttar Pradesh using Phenotypic methods.[70]

Parveen et al(Jawaharlal Institute of Postgraduate Medical Education & Research, Puducherry) concluded that plasmid-encoded AmpC genes was found in 92 (38.1%) clinical isolates, which included *K. pneumoniae* (n=32) and *E. coli* (n=60)among the 241 total isolates(*E.coli*-132, *K.pneumoniae*-109) from five Indian hospitals detected by PCR in 2010.[71]

Anand Manoharan *et al(*2010*)* in his study showed plasmid mediated AmpC β lactamases in 12.5 per cent isolates with 5.2 per cent of commonly reported genotypes collected from five Indian tertiary care centres.[13]

Mohamudha Parveen R *et al(*2010*)* concluded that 23.5%of E.coli and 74.4% of Klebsiella isolates were positive for AmpC enzymes from

Jawaharlal Institute of Post Graduate Medical Education and Research, Pondicherry.[72]

S.Peter *et al* concluded that 21/51 (41%) *E. coli* isolates from Institute of Medical Microbiology, Switzerland were true AmpC producers by phenotypic and genotypic tests in 2011 .[73]

Vijaya Shivanna *et al*(Andrapradesh-2011) described that *E.coli* (20.5%), *K.pneumoniae* (5.5%) and *Acinetobacter* species (3.5%) were AmpC positive isolates.[74]

Varsha Gupta *et al(*2012*)* reported that 20%of *K. pneumoniae* isolates were AmpC producing pathogens in University of Madras.[8]Sasirekha *et al* (2013)reported that 19.8% of *E.coli* and 18.2% of *Klebseilla s*trains were AmpC enzyme producers in Bangalore.[19]

B. L. Chaudhary *et al (*2013*)*concluded that 14(6.94%) were AmpC producer by confirmatory method(Inhibitor based method)among201 *klebsiella* isolates collected from MGM Hospital Kamothe, Navi Mumbai.[75]

Rajesh Bareja *et al(*2013 -Hariyana*)* said that 18.3% of *Escherichia coli*, 13.4% of *Klebsiella* species were AmpC-β-lactamase producers among 129 isolates.[76]

Sridhar Rao PN *et al (*Karnataka*)*reported that 35 (50%) *E.coli* isolates were AmpC enzyme producers among the 62 screen-positive isolates. [77]

3.10.10.DISTRIBUTION;

T.y.Tan *et al* (singapore) observed that the preponderance of AmpC-positive isolates were from urine cultures.[11]

Slike polsfuss *et al* (Switzerland) concluded that the mainstream of AmpC producing pathogens were isolated from urine (52.6%), wound (7.9%) respiratory tract (18.4%) specimens .[62]

Nevine Fam *et al* observed that the AmpC positive isolates were recovered from urine specimens (63%), pus (17%), sputum (12%) and body fluids (8%). Specimens were collected from patients (21.7%) admitted in ICU and (16.6)attending outpatient clinic and admitted in nephrology, urology, surgery and gastroenterology wards (61.7 %).

Sasirekha et al reported that 69.3 % of the AmpC producing pathogens were from females than males (30.7 %)[19]

B. L. Chaudhary *et al (*2013) reported that the AmpC prevalence was highest in Pus (52.63%) followed by ET tube (12.5%), urine (7.40%), sputum(7.14%).[77]

Akujobi*et al* (Nigeria) observed that the percentage of AmpC positive isolates from urine samples was significantly higher than those from other samples. Wound samples had the least percentage distribution of the isolates which are pure AmpC producers (11.11%).[4]

Smitha O. Bagali *et al(*Karnataka*)* described that 12(50%) were from urine specimens, 7(29%) from pus among the total 24 AmpC producing strains of*E. Coli.*[78]

3.10.11.Risk factors:

- Previous exposure to antibiotics, predominantly third generation Cephalosporins and Fluoroquinolones.
- Presence of severe disease
- Usage of invasive medical equipment (urinary catheters, endotracheal tubes, and central venous lines,nasogastric feeding tubes)
- Previous surgery
- Recent hospitalisation
- Prolonged hospital stay
- Intensive care units are recognised as “risk units” due to the high selective pressure in combination with susceptible patients.[7]

Kenneth h rond et al observed that risk factors for AmpC-producing species of *Klebsiella pneumoniae*comprisecare in ICU, insertion of urinary catheter, central venous catheterization and prolonged hospital stay and prioruse of antimicrobial agents, mainly third generationCephalosporins and β- lactamase enzyme inhibitor combinations.[18]

3.10.12. Detection methods:

Screening with Cefoxitin disc is suggested for initial detection. However, it does not constantly designate AmpC enzyme production. Kirby-bauer disc diffusion method is used to detect Cefoxitin susceptibility.

Coudron et al worn the cut-off point for disc diffusion with Cefoxitin (zone diameter < 18 mm) for screening of isolates for the detection of AmpC enzyme production .[67]

Phenotypic tests:

The modified Hodge test (Yong et al., 2002) and AmpC disc test(Sinhal *et al*,2005*)*,Tris-EDTA disc test (Black et al., 2005a), modified three dimensional test(Vikas Manchanda et al,2002), Inhibitor-based assays - boronic acid compounds (Tan et al., 2009) or cloxacillin (Brenwald et al., 2005) are available. [14]

Modified (Cefoxitin) Hodge test:

E.coli ATCC 25922is streaked on MHA plates as lawn culture. Cefoxitin (30 μg) disc arekept in the centre of the plate.Test isolateis streaked from periphery to the rim of disc. 3 mm or more of "diagonal" growth in the cloverleaf pattern is positive for AmpC production. Isolates that had no or minimal distortion of the cefoxitin zone are considered to be negative for AmpC production . [79]

Paul *et al* reported that Sensitivity of MHT was 73%and Specificity of MHT was 95%.[14]

AmpC disc test(Saline disc test)

Moistening of the sterile plain disc with 20 µl of sterile salineis done. Then the saline disc is inoculated with a the test organism. MHA plate with lawn of *E.coli* ATCC 25922 is then prepared. Cefoxitin discis kept on the MHA plate. The disc with test organism is inverted and placed close to the Cefoxitin disc.[78]

Interpretation:

After incubation, the plate is examined for indentation or flattening of zone margin that indicates the positive result.

This test is easier to perform and can be used in routine microbiology laboratories. [74]According to Paul et al, AmpC disc test had a sensitivity of 86%)and a specificity of 94%.[14]

Tris-EDTA disc test

The procedure is the same as Saline disc test.But Tris EDTA disc is used to induce AmpC enzyme production in this test.[80]Paul *et al* observed that the sensitivity of Tris-EDTA test was 97% and specificity of this test was 98%.[14]

According to Justim elley study, the sensitivity of TE inhibition disc test was85% and specificity was 90%.[81]

Modified three dimensional test:

Crude enzyme extract is prepared by centrifugation and repeated freeze–thawing of bacterial suspension. *E.coli* (ATCC 25922) is streaked on

MHA plate as lawn culture. Cefoxitin (30 mg) disc is kept in the centre of the plate. Linear slits (3 cm) with circular wells are made at 5 mm distance. The wells are encumbered with the enzyme extract in 10 μL .Incubation is done at 37°C overnight. Clear distortion of zone of inhibition of Cefoxitin is diagnostic of positive isolates. The isolates with no distortion are considered as Negative isolates. But this method has some limitations like requisite of a applicator, trouble in filling the slits, proper incubation of the plates to avoid leak of the suspension.[67]

Inhibitor-based tests:

Antibiotic discs(6 mm) are supplemented with either boronic acid compounds or cloxacillin which are commercially available . Inoculation of MH agar is done with the test isolate and both unsupplemented and supplemented discs are kept. Incubation done for 16–18 h at 35 ^{o}C and the increase in zone size around the supplemented disc compared to the unsupplemented disc is recorded. Phenylboronic acid (400 μg) with cefoxitin (30 μg) and cloxacillin (200 μg) with cefoxitin (30 μg) are used.

Paul *et al* observed that sensitivity of Phenylboronic acid (400 μg)+ Cefoxitin was 66% and Specificity of 98%. [14]

Philip E. Coudron *et al(*virginia*)* observed that the inhibitor based test(boronic acid) was a useful method to identify plasmid-encoded AmpC β-lactamase .[82]

Noyal Mariya Joseph *et al* observed that the phenylboronic acid-cefoxitin disc test had a sensitivity of 72.9 per cent, specificity of 45.4 per cent.[83]

Molecular methods: Multiplex PCR is the "gold standard" test for plasmid-encoded AmpC enzyme detection by utilizing six primer pairs.[9]

Gene family of AmpC:

Six plasmid-encoded AmpC families (MOX, CIT, DHA, EBC, FOX and ACC-1) are present.[15] CMY-type β-lactamase belonging to CIT family is the most general type among them.[84]

Escherichia coli and *Klebsiella pneumoniae* contain major proportion of plasmid-encoded AmpCgenes. These are copied from the chromosomal *ampC* genes of *Enterobacter cloacae, Citrobacter freundii, Hafnia alvei and Morganella morganii* .[56]CMY-2, which is the most common subtype of AmpC enzyme present all over the world and ACT-1 which is an inducible subtype are found to be prevalent in *E. coli* and *Klebsiella* species .[16]

Justin ellem et al reported that the prevalence of plasmid mediated AmpC in Australia is47% for DHA and 53% for CMY type.[81]

Tenover *et al* observed about 58% of the positive isolates by inhibitor based test were positive for the presence of AmpC genes by multiplex PCR.[85]

3.10.13. Significance of AmpC detection:

The presence of plasmid-encoded AmpC hide the detection of ESBL and KPC-producing pathogens by routine phenotypic methods. This leads to

problems in surveillance and infection control policies. Another problem encountered is the false positive susceptibility pattern of the AmpC enzyme producer leading to increased incidence of treatment failure. There are no guidelines by the CLSI to identify these enzymes.[15]Patients may receive inappropriate antibiotics and become seriously ill or colonised, increasing the possibility of cross-infection due to undetection of AmpC producers.[86]

Hanna E. Sidjabat *et al* observed that symptomatic infection likely to occur in patients with CMY-type AmpC β-lactamase–producing *E. Coli*.[84]

Isolates producing AmpC β enzyme are insusceptible to currently available β-lactamase inhibitors and resistant to additional β-lactams and they are feasible for budding resistance to carbapenems. AmpC carries the danger of extension to other organisms through plasmid arbitration within a hospital or geographic region.[9]

3.10.14.Treatment:

Carbapenems:

It is the Choice of drug for treating the AmpC producing bacteria.(e.g., Imipenem, Meropenem, Ertapenem)

Fluoroquinolones:

If there is an in vitro susceptible to Fluoroquinolones, this drug is used.

Tigecycline

Tigecycline is an analogue of the semisynthetic antibiotic Minocycline and is a broad spectrum antibiotic that acts by binding to the 30S ribosomal subunit and by inhibition of protein translation in bacteria.

For interpretation of sensitivity testing of Tigecycline, there is no CLSI guidelines.It is a another option for treatment of AmpC infections, but clinical experience is incomplete .[9]

Timocillin(6-alfa-methoxy derivative of ticarcillin):

For this drug, clinical experience is incomplete.

4. MATERIALS AND METHODS

This study was undertaken in Clinical microbiology laboratory of Tirunelveli Medical College, Tirunelveli for a period of one year from April 2013 to May 2014.

- To detect the prevalence of AmpC β lactamase production among *Enterobacteriaceae* isolates from clinical samples (Urine,pus).
- To evaluate phenotypic methods by screening with Cefoxitin disc diffusion test and Modified Hodge test,AmpC disc test.
- To identify blaAmpC gene by Real-Time PCR.
- To find out various risk factors associated with the study group.
- To assess the Antimicrobial susceptibility pattern of the clinical isolates of *Enterobacteriaceae* **.**

4.1. Materials

4.1.1.Sample collection and processing

A total of 50 (urine[n=45], pus[n=5]) non-duplicate Cefoxitin resistant isolates from clinical samples which includes *E.coli*(n=23), *Klebsiella pneumoniae* (n=21), *Klebsiella oxytoca(*n= 6) were taken for this study.

The features helping in identification of *E.coli are*

Morphology on Gram stained smear

Colony appearance on nutrient agar

Colony appearance on sheep blood agar

Colony appearance on MacConkey agar

Motility: Motile on Hanging drop procedure

Positive catalase test

Negative oxidase test

Hugh – Leifson Oxidation – Fermentation test – Fermentative pattern

Nitrate reduction to nitrite

Indole production

Negative citrateutilization test

Negative for urea hydrolysis

Acid/acid with gas in Triple sugar iron agar

Methyl red- positive

Voges – Proskaeur- negative

Presence of lysine decarboxylase

ONPG positive

The *Klebsiella pneumonia* & *Klebsiella oxytoca* isolates were identified by:

Morphology on Gram stained smear

Colony appearance on nutrient agar

Colony appearance on sheep blood agar

Colony appearance on MacConkey agar

Capsule demonstration by negative staining

Non - motile

Positive catalase test

Negative oxidase test

Fermentative pattern in Hugh – Leifson Oxidation – Fermentation test

Nitrate reduction to nitrite

Indole not produced (Indole produced by *Klebsiella oxytoca)*

Citrate utilized

Urea hydrolyzed slowly

Acid/acid with gas in Triple sugar iron agar

Methyl red negative

Voges – Proskaeur positive

Presence of lysine decarboxylase

ONPG positive

4.1.2. Ethical clearance

Because, this study involved the clinical samples from the patients, ethical clearance was obtained from ethical committee of Tirunelveli medical college before the commencement.

4.1.3. Proforma

From all patients, a proforma was prepared with details like name, age, sex, ward, clinical diagnosis, risk factors, surgical intervention, hospital stay and other parameters relevant to the study.

4.1.4. Storage of Sample :

The Gram negative isolates were sub-cultured on to nutrient agar slope and stored at 2 to 8˚C. The isolates were sub-cultured every fortnight.

4.1.5. Safety precautions:

With aseptic precautions, all the procedures were carried out in a Biosafety cabinet .

4.2. METHODS

4.2.1.Antibioticsensitivity test

All the strains were subjected to Disc Diffusion method(Kirby bauer method) to detect Cefoxitin resistance and their antibiotic sensitivity pattern.

4.2.2.DD method

DD method was performed by Kirby-Bauer method using Muller-Hinton agar with the following antibiotic discs (HiMedia Laboratories, Mumbai, India).

Gentamicin(10µg)

Amikacin (30µg)

Norfloxacin(10 µg)

Nitrofurantoin(300 µg)

Ceftriaxone(30µg)

Ceftazidime(30µg)

Ceftazidime with clavulanic acid(30/10 µg)

Cefoxitin(30 µg)

Imepenem (10µg)

Discs were stored in a tightly sealed container with dessicant at 2°C to 8°C. They were allowed to equilibrate at room temperature for one to two hour before opening the container to minimize condensation and to reduce the possibility of moisture affecting the concentration of antimicrobial agents.

4.2.3.Muller Hinton agar

The ingredients of the media was purchased from HiMedia Laboratories, Mumbai, India .Media was prepared as per the instruction guidelines from manufacturers. The media was placed in a hot air oven with their lids a jar for 10–15 minutes, plates were dried before inoculation.

4.2.4.Preparation of Inoculum :

Inoculum was prepared by direct colony suspension method by taking four to five well isolated colonies from 18-24 hours culture, in Muller Hinton broth to achieve a turbid suspension.

4.2.5.Standardization of Inoculum :

Comparison of the inoculum suspension with 0.5 McFarland standard suspension by positioning the tube side by side against a white card

containing several horizontal black lines was done. Comparison of turbidities by looking at the black lines through the suspensions was done. After standardization, the inoculum suspension was used within 15 minutes of preparation.

4.2.6.Principle of DD test:

The principle of DD depends upon the formation of a gradient of antibiotic concentrations as the antibiotic agent diffuses radially into the agar. The drug concentration decreases at increasing distances from the disc. The drug concentration at a specific point in the medium is unable to inhibit the growth of the test organism, at a critical point, zone of inhibition is formed.

4.2.7.Procedure:

- After standardization of bacterial suspension, the suspension was vortexed to make sure that it was well-mixed.
- By using a sterile swab, inoculation was done on Muller hinton agar .
- By rotating the plate to 60^0, streaking was done in three directions to ensure uniform distribution.
- Drying of the plate was done for three to five minutes. Antibiotic discs were evenly placed on the inoculated plate by using sterile needle mounted in a holder.

- 15 mm from the edge of the plate, the disc was kept and the minimum distance of 2.5 cm was maintained between two discs. Only six discs were applied on a 90mm plate.
- To ensure the adequate contact between the disc and the agar,slight pressure was applied on the disc and incubation was done at a temperature of 35˚C aerobically for 24 hours.
- Under transmitted light, measurement of the inhibition zone was done by using an antibiotic scale which included the disc's diameter.

4.2.8 .Interpretation of results:

The millimeter reading for each antibiotic agent was compared with that in the interpretive tables of the CLSI guidelines and results were interpreted as either susceptible, intermediate or resistant.

For Cefoxitin discs, zone size of ≥ 18mm was taken as sensitive while zone size of ≤ 15mm was taken as resistant. (Table .1).

Table.1. Interpretation of Antibiotic susceptibility test

S. No.	Antibiotic disc	Disc strength	Susceptible (mm)	Intermediate (mm)	Resistant (mm)
1.	Amikacin	30µg	≥17	15 – 16	≤14
2.	Gentamicin	10µg	≥15	13 – 14	≤12
3.	Nitrofurontoin(urine isolates)	300µg	≥16	11 – 15	≤10
4.	Norfloxacin(urine isolates)	10 µg	≥17	13 – 16	≤12
5.	Ceftriaxone	30µg	≥26	23-25	≤22
6.	Ceftazidime	30µg	≥18	14 – 18	≤15
8.	Cefoxitin	30µg	≥18	15 – 17	≤15
8.	Imepenem	10 µg	≥23	20-22	≤19

4.3.Phenotypic tests for detection of AmpC β-lactamases

Modified Hodge test, AmpC disc test were done on the Cefoxitin resistant strains for the identification of AmpC enzymes.

4.3.1.Modified Hodge test:

Principle:

If AmpC enzyme producers are present, it permits the growth of Cefoxitin susceptible strain (E.coli ATCC 25922) .This can be viewed as an indentation resembling cloverleaf pattern.

Procedure:

- 0.5 ml of the 0.5 McFarl and solution was mixed with 4.5 ml of MHB or saline to prepare 0.5 McFarland dilution of the *E.coli* ATCC 25922.
- *E.coli* ATCC 25922 in 1:10 dilution was streaked on MHA plates as lawn culture.
- Cefoxitin (30 mg) disc was kept in the middle of the plate. Test isolate was streaked from periphery to the rim of disc and incubated.
- 3 mm or more of “diagonal” growth in the cloverleaf pattern was positive for AmpC production. Isolates that had no or minimal distortion of the Cefoxitin zone were considered to be negative for AmpC production. On a single plate with one drug, four organisms can be tested.

4.3.2.AmpC disc Test:

- Moistening of the sterile plain disc with 20 µl of sterile saline was done.
- Then the saline disc was inoculated with the test organism.
- MHA plate with lawn of E.coli ATCC 25922 was prepared.
- Cefoxitin disc was kept on the MHA plate.
- The disc with test organism was inverted and placed close to the Cefoxitin disc.

Interpretation:

After incubation, the plate was examined for indentation or flattening of zone margin that indicates the positive result. Negative result was indicated by absence of indendation.

4.4.Real-Time PCR

The Cefoxitin resistant isolates were further tested for blaAmpC gene by Real-Time PCR by the kit purchased from Helini Biomolecules, Chennai, India and procedure followed according to the manufacturer's instructions.

4.4.1. Safety precautions

All the procedures were done in a Biosafety cabinet Level-2 with aseptic precautions.

4.4.2.Equipments

Refrigerated centrifuge

Vortex mixer

Thermo cycler (Biorad CFX 96)

Computer for data storage

4.4.3.DNA extraction

Each spin column(silica based) was recovered up to 20µg of DNA and yielded purified DNA of more than 30 kb in size. Isolated DNA was used directly for PCR reaction.

Components of extraction

Phosphate buffered saline

Binding buffer

Digestion buffer

Proteinase K

Lysozyme

Internal control template

Isopropanol

70%Ethanol

Elution buffer

Spin columns with collection tube

Storage and stability

The kit was stored at 37˚C.Proteinase K and Lysozyme was stored at -20˚C.

4.4.4.Sample preparation

Four to five colonies of *Enterobactericeae* isolate was inoculated into 1.5 ml of normal saline in a 2ml of microcentrifuge tube. Centrifugation was done for five minutes at 8000 rotations per minute. After discarding the supernatant, the remaining bacterial pellet was used.

4.4.5.Principle of extraction

Lysis of cells was done using Proteinase K and chaotropic salt was used for inactivation of nucleases. Nucleic acids of the bacterial cells have the property to bind to glass fibres in the spin column. In a series of rapid "wash and spin" steps, bound nucleic acid are purified to take away other contaminants of the cells. Nucleic acids were removed from the glass fibre by the process of salt elution. The above procedure has an advantage of rapid purification without using organic solvent extractions and DNA precipitation.

4.4.6. Extraction procedure

- ✓ All the steps were done at room temperature.
- ✓ The bacterial pellet was suspended in 200µl of phosphate buffered saline and dislodged the pellet by brief vortex for 30 seconds.
- ✓ 180µl of Digestion buffer and 20µl Lysozyme were added to the pellet and brief vortex done for 10 seconds.
- ✓ Incubation done at 37^0c for 15 mts.

- ✓ Binding buffer of 200µl and 20µl of proteinase K& 5µl of internal control template was added to the suspension and incubated at 56°C for 15 minutes in a water bath.
- ✓ 300µl of Isopropanol was added and this was mixed by inverting several times.
- ✓ Entire sample was pipetted into a spin column.
- ✓ Centrifugation was done for three times at 12000 rpm and about 500µl of 70%Ethanol was added between centrifugation after discarding the flow through. Finally one more centrifugation at 13,000 rpm to discard the residual ethanol. The spin column was transferred to a fresh 1.5ml microcentrifuge tube.
- ✓ 75µl of the Elution buffer (pre-warmed to 56˚C) was added to the centre of the spin column membrane. Care was taken not to touch the membrane with pipette tip.
- ✓ It was incubated for two minutes at room temperature and centrifuged for one minute at 13,000 rpm.
- ✓ The spin column was discarded and purified DNA was stored at -20°C.

4.4.7. PCR amplification

Key ingredients for amplification:

Probe PCR Mastermix

The probe mix contains the essential components for PCR amplification like DNA polymerase and deoxynucleotides.

Bla AmpC (CMY-2) primer & probe mix

The blaAmpC (CMY-2)primer & probe mix consists of TaqMan probe which is fluorescent labelled with FAM, forward primer and reverse primer.

Forward primer- 5'-CGGTGAAACCCTCAGGAATGAGTT-3'

Reverse Primer- - 5'-GCGGAACCGTAATCCAGGTAT-3'

eddProbe - - 5'-ACGAAGAGGCAATGACCAGGACGC-3'

Internal Control template

The internal control template consists of TaqMan probe which is fluorescent labelled with HEX, forward primer and reverse primer. The reason for adding the internal control is to make sure that PCR inhibitors are not present in the extracted sample DNA and the performance of PCR mix ingredients are good. When there is no amplification in internal control, it indicates that PCR inhibitors are present in the sample and efficiency of the nucleic acid purification is not optimum. It helps to rule out false negative results.

AmpC positive template

To be used for positive control mix.

Nuclease free water

It was used in negative control mix.

PCR amplification kit storage

The kit was stored at -20˚C.

bla AmpC detection mix

The bla AmpC detection mix for the samples consisted of

i. probe PCR master mix 10µl

ii. AmpC/internal control primer probe mix 10µl,

iii. purified DNA sample 5µl ,a total volume of 25µl.(Table.4.2)

For positive control mix, 5µl of positive control template was added instead of sample DNA and for negative control mix, 5µl of nuclease free water was added instead of sample DNA.(Table 3& 4)To prevent cross contamination, initially negative control, followed by samples and finally positive control was added. After adding all the ingredients, they were centrifuged and placed in the thermo cycler for PCR reaction to occur.

Table.2. bla AmpC detection mix for samples

S.No	Components	Volume
1.	Probe PCR Master Mix	10µl
2.	AmpC /Internal Control Primer Probe Mix	10µl
3.	Purified DNA sample	5µl
4.	Total reaction volume	25µl

Table.3:bla AmpC Positive control mix

S.No	Components	Volume
1.	Probe PCR Master Mix	10µl
2.	AmpC/Internal Control Primer Probe Mix	10µl
3.	Positive control template	5µl
4.	Total reaction volume	25µl

Table:4. bla AmpC Negative control mix

S.No	Components	Volume
1.	Probe PCR Master Mix	10µl
2.	AmpC/Internal Control Primer Probe Mix	10µl
3.	Negative control template	5µl
4.	Total reaction volume	25µl

4.4.8.Basic steps in amplification

For initial denaturation for Taq enzyme activation, the temperature is increased to 95˚C for five minutes initially.

Denaturation- By increasing the temperature 95˚C for 20 seconds, template DNA strand issplit in to two complementary strands.

Annealing- By decreasing the temperature to 55˚C for 20 seconds, two specific oligonucleotide primers get attached to the DNA template complementarily.

Extension- Increasing the temperature to 72˚C for 20 seconds, each primer is extended by DNA polymerase at the 3’ terminus and the complementary strands are synthesized along 5’ to 3’ terminus of each template DNA using deoxynucleotides in the reaction mixture.

Then two double stranded DNA copies are produced by allowing single template DNA strands to bind with the complementary DNA strands .

To amplify further, each copy of DNA is used as template. The doubling of products in every cycle for a total of 40 cycles leads to final PCR products having 2n copies of template DNA. Data collection was made at the end of extension and the computer produces the cross threshold (Ct) value by calculating the fluorescence emitted at the end of each cycle. (Table 5)

Table.5.Amplification profile for bla AmpC gene

	Step	Time	Temp
	Taq enzyme activation	5min	95^0 C
40cycles	Denaturation	20sec	95^0 C
	Annealing/ Data collection	20sec	55^0 C
	Extension	20sec	72^0 C

Ct value

When Ct value was less than 38, it was considered as positive for bla AmpC gene.

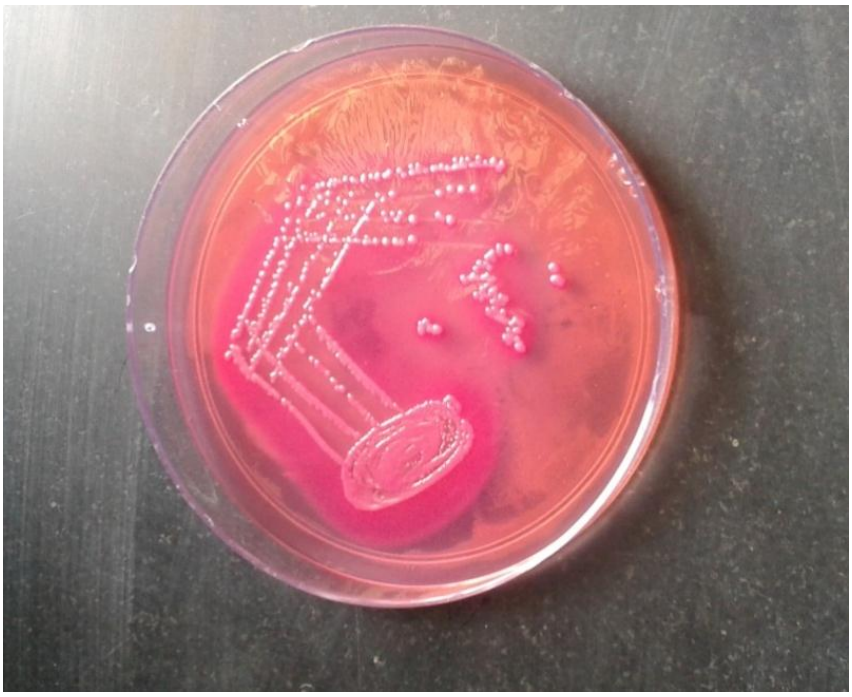
Samples that cross the threshold line at or after 38 cycles, should be retested.. (Table 6)

Negative result if no amplification occured.

Table.6 Interpretation of results

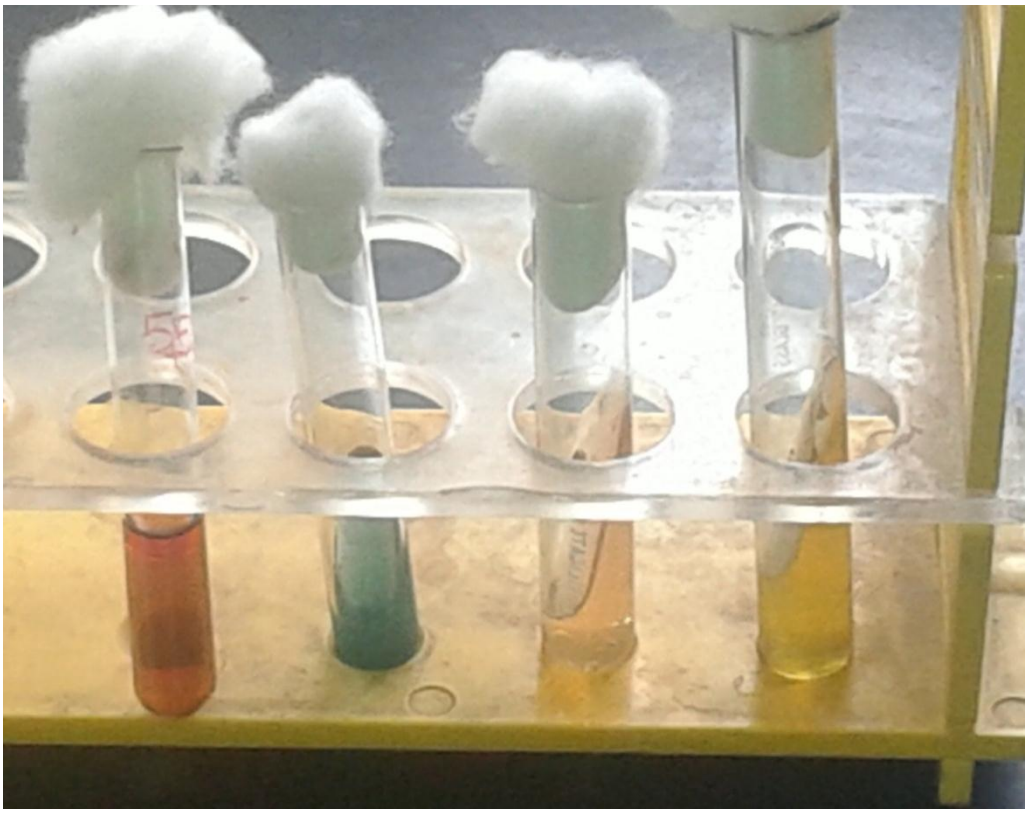
Test Sample	Negative control	Internal control	Positive control	Interpretation
+ ve	-ve	+ ve	+ ve	+ ve
- ve	-ve	+ ve	+ ve	-ve
- ve	-ve	- ve	- ve	Repeat
Positive	Positive	Positive	Positive	Repeat

4.Colony appearance of *E.coli* on MacConkey agar

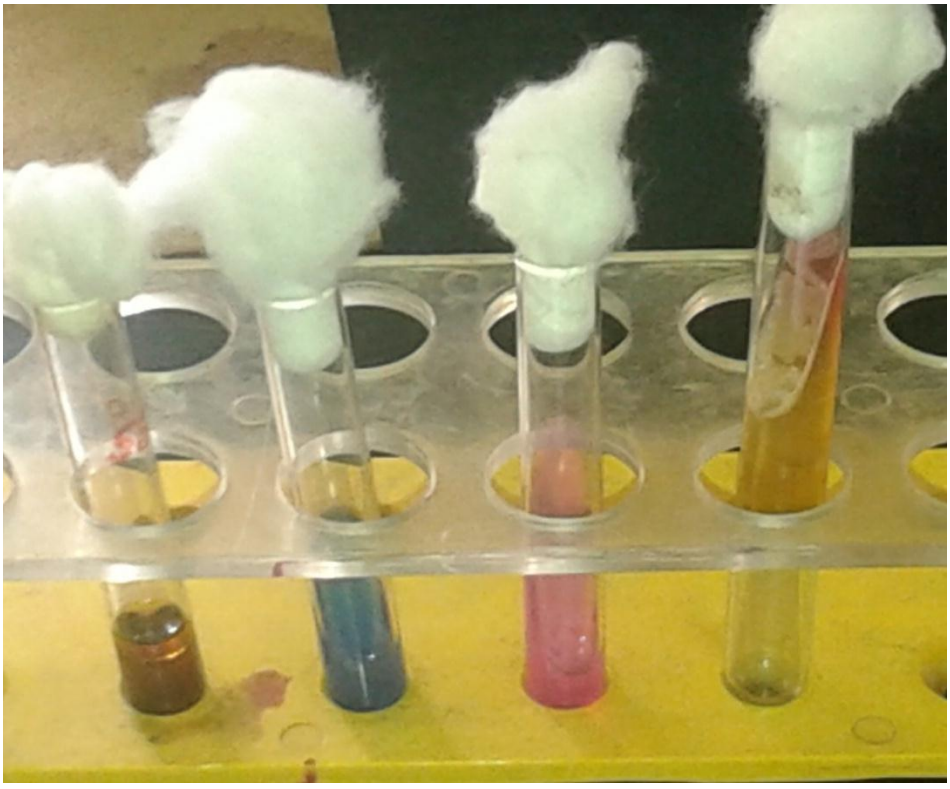


5. Colony appearance of *Klebsiella pneumoniae* on MacConkey agar

6.Biochemical Reactions of *E.coli*



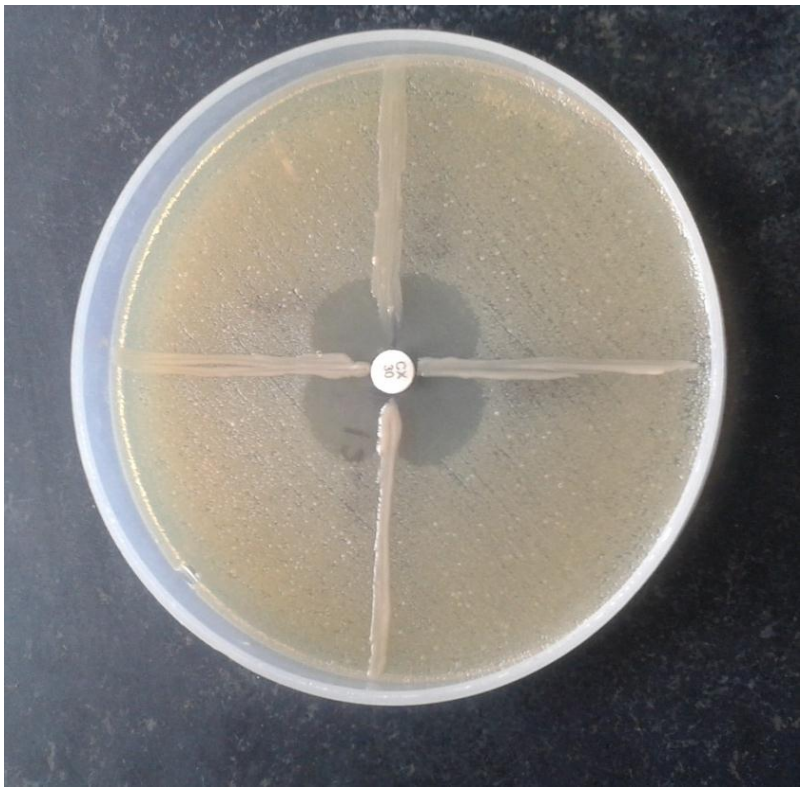
7.Biochemical Reactions of *klebsiellapneumoniae*



8.Antibiotic susceptibility test by Disc Diffusion method(Kirby bauer method)

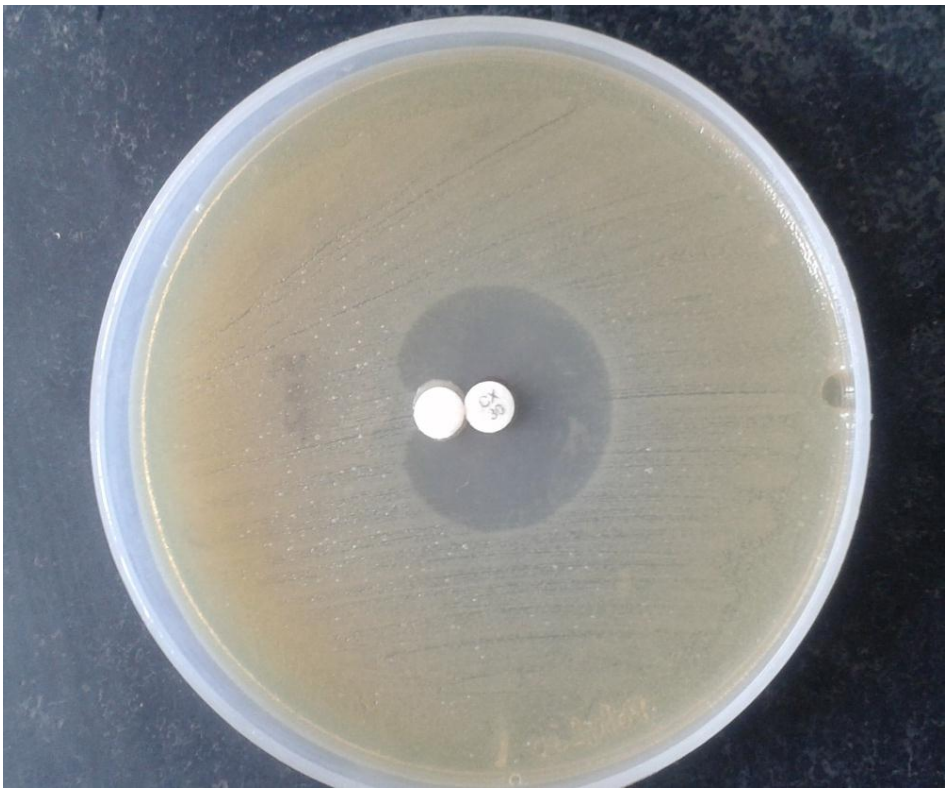
9.Modified Hodge test

Positive test

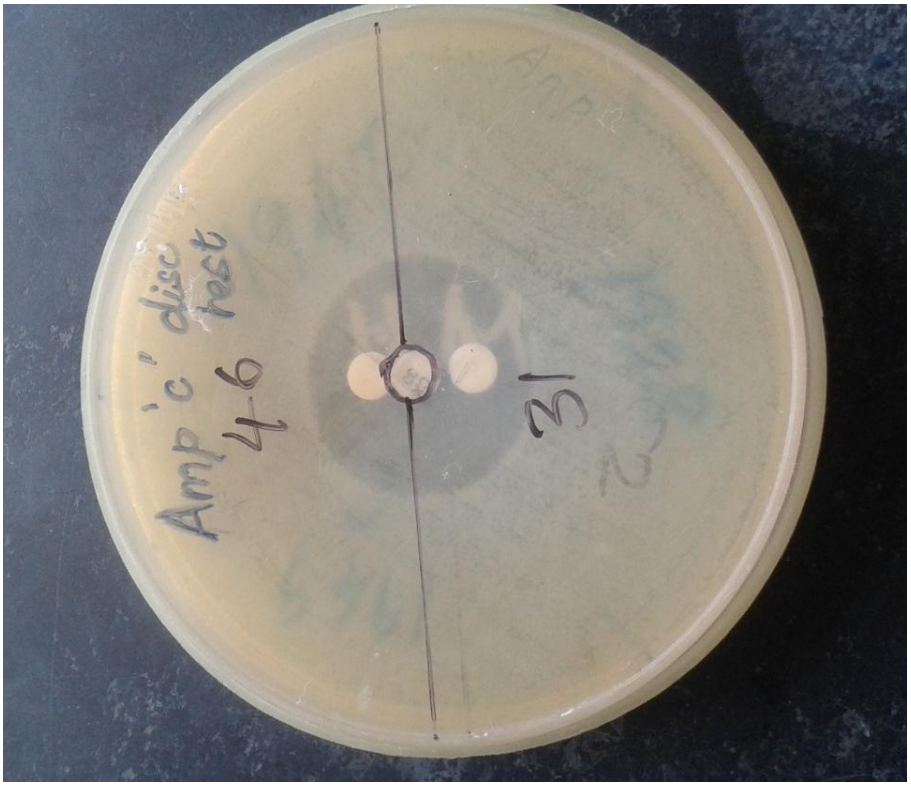


Negative test

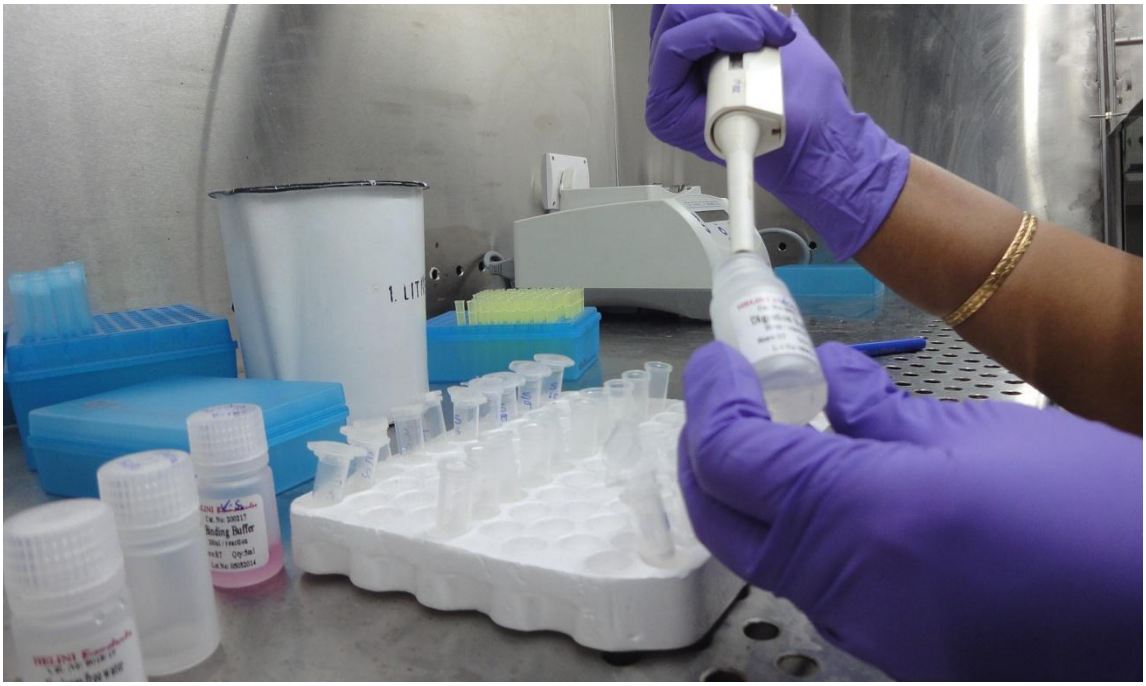
10.AmpC producer



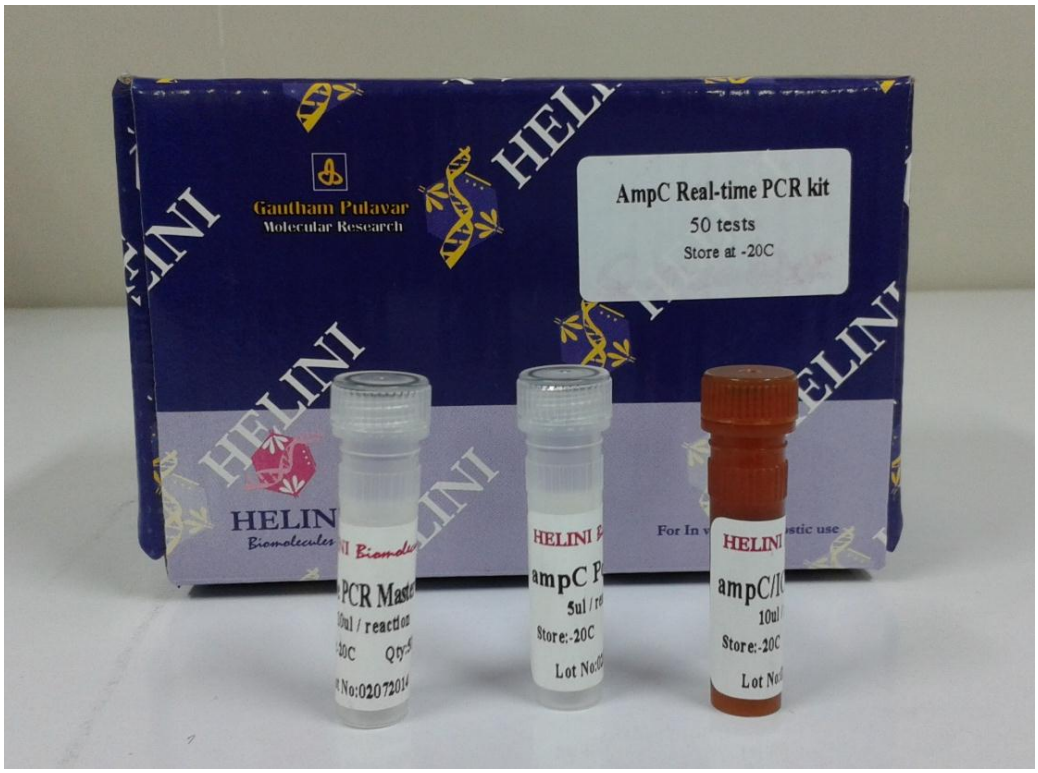
AmpC Non producer

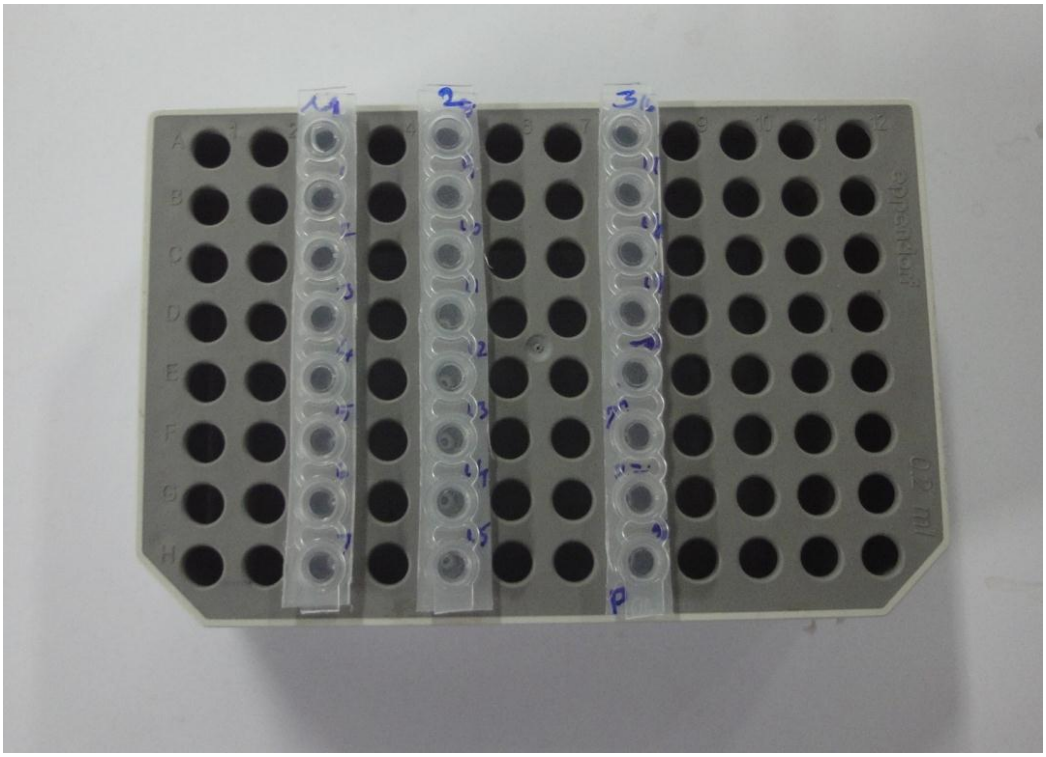


11.DNA Extraction test



12.PCR Amplification kit





13.Thermo cycler with loaded samples

5. RESULTS

5.1. Study samples

The study was undertaken in Clinical microbiology laboratory of Tirunelveli Medical College, Tirunelveli for a period of one year between April 2013 to May 2014. A total of 50 (urine[n=45], pus[n=5]) non-duplicate Cefoxitin resistant Gram negative isolates from clinical samples which included *E.coli*(n=23), *Klebsiella pneumoniae*(n=21), *Klebsiella oxytoca(*n= 6) were taken for this study. Modified Hodge test and AmpC disc test were done on the strains and detection of *bla* AmpC was done by Real-Time PCR. The risk factors and antibiotic sensitivity patterns of the isolates were further analysed.

5.2. Statistical Analysis

Data regarding the subjects were described in terms of percentages. The susceptibility, resistant and intermediately susceptible were described in terms of percentages. The statistical analysis was done using with the help of the IBM SPSS statistics 20. Chi square test, Fischer exact test and Mcnemer test were used to find out ‘p’ value. If the p value is less than 0.05 ,it is significant

5.3. Analysis by age and sex

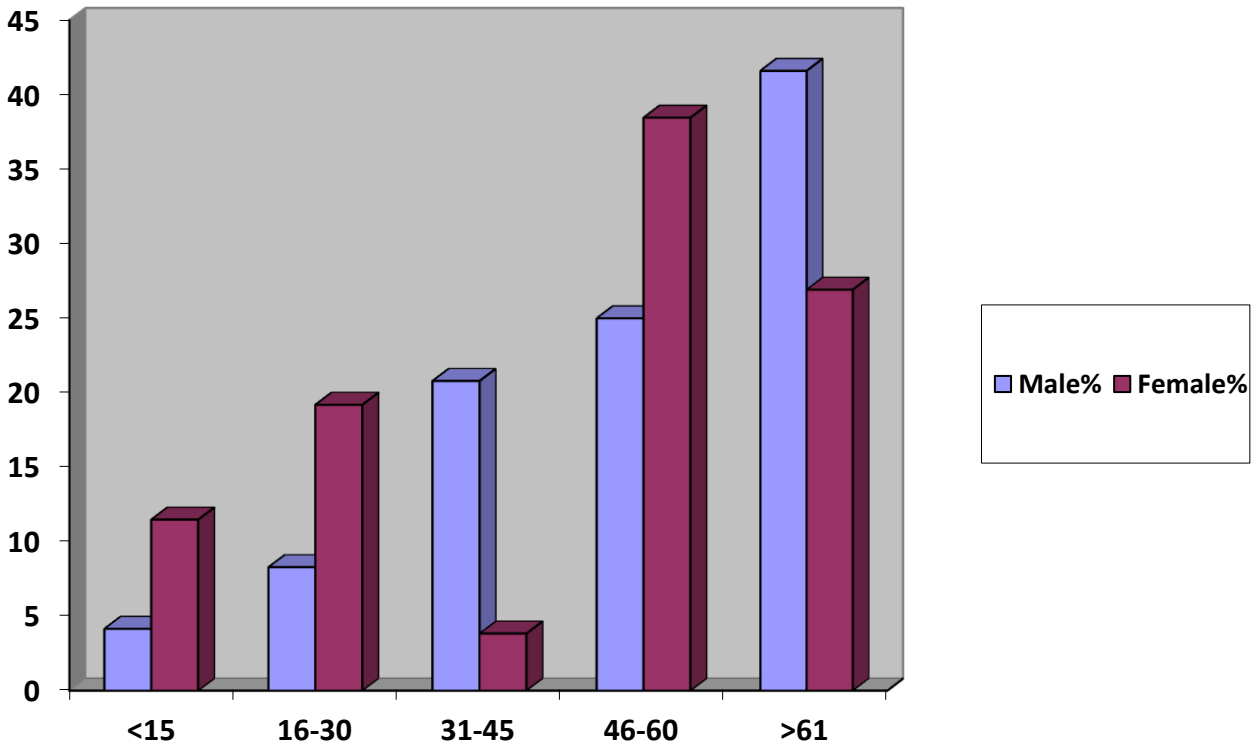
Table- 7: Sample distribution by age and sex

Age (years)	Male		Female		Total	
	No	%	No	%	No	%
≤ 15	1	4.2	3	11.5	4	8
16 – 30	2	8.3	5	19.2	7	14
31 – 45	5	20.8	1	3.8	6	12
46 – 60	6	25	10	38.5	16	32
≥61	10	41.6	7	26.9	17	34
Total	24	100	26	100	50	100

Out of Cefoxitin Resistant 50 isolates, 24 isolates (48%) were from males and the remaining 26 isolates (52%) were from females. A total of 4 (8%) isolates, fell in the study group of ≤ 15 years of which, 1 isolate (4.2%) was from male and 3 isolates (11.5%) were from females. Out of the 7 (14%) isolates in the 16-30 years age group, two isolates (8.3%) were from males and five isolates (19.2%) were from females. A total of 6 (12%) isolates were in the 31-45 age group, of which, five isolates (20.8%)were from males and one

isolate (3.84%) were from females. A total of 16(32%) isolates were in the 46-60 years group, out of which six isolates (25%) were from males and ten isolates (38.5%) were from females. Out of seventeen isolates in persons above 61 years, ten isolates (41.6%) were from males and seven isolate (26.9%) were from females(Table:7).

Fig – 15 : Analysis of samples by age and sex



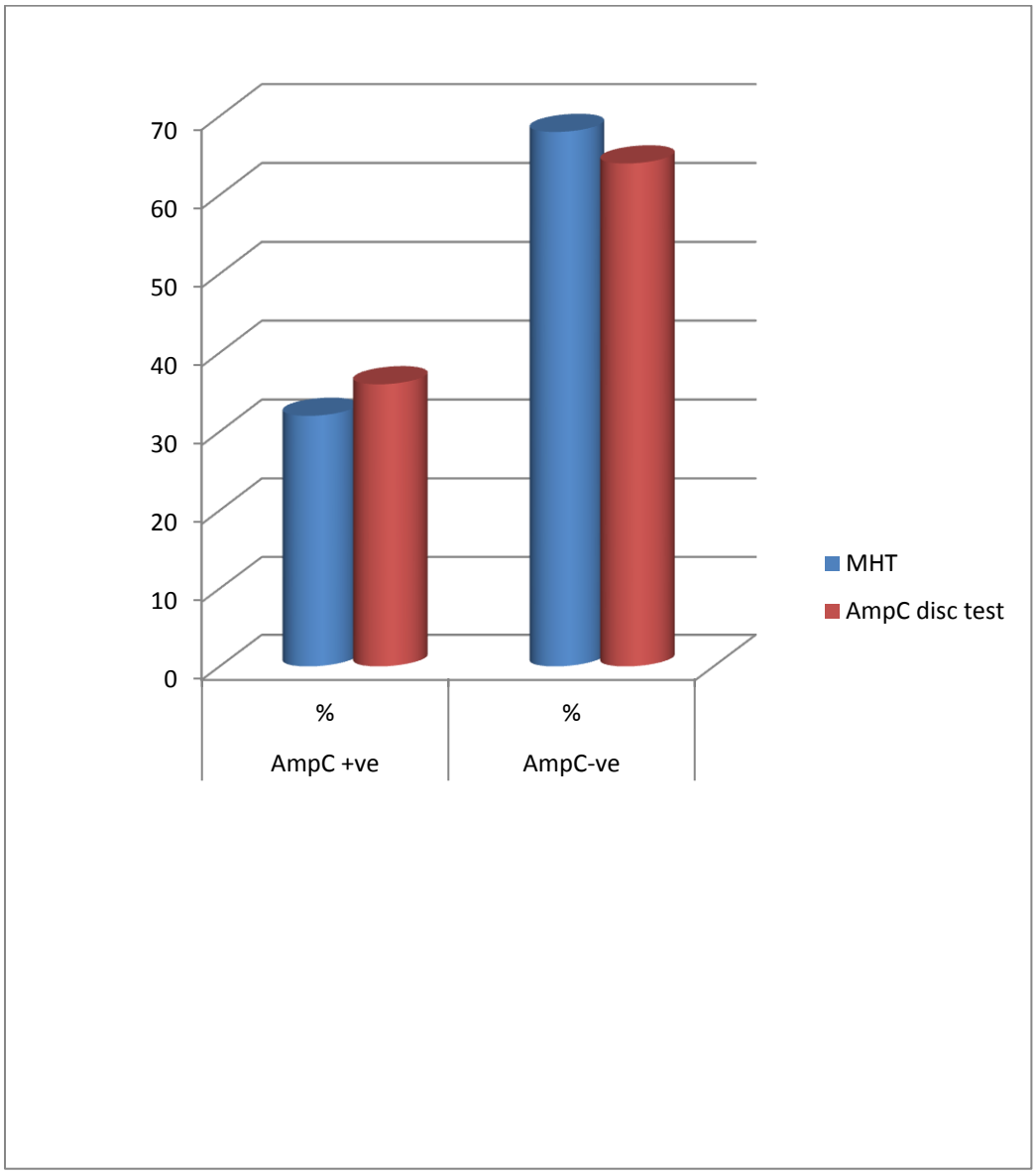
5.4:Analysis of various methods for AmpC detection

Table- 8: Comparison of MHT and AmpC disc test

Method	AmpC Positive		AmpC Negative	
	No	%	No	%
MHT	16	32	34	68
AmpC disc test	18	36	32	64

All the 50 Cefoxitin resistant isolates were evaluated for AmpC enzyme production by Modified Hodge test and AmpC disc test.Of these,16 gave positive results by MHT and 18 gave positive results by AmpC disc test.

Figure-16:Comparison of MHT and AmpC disc test



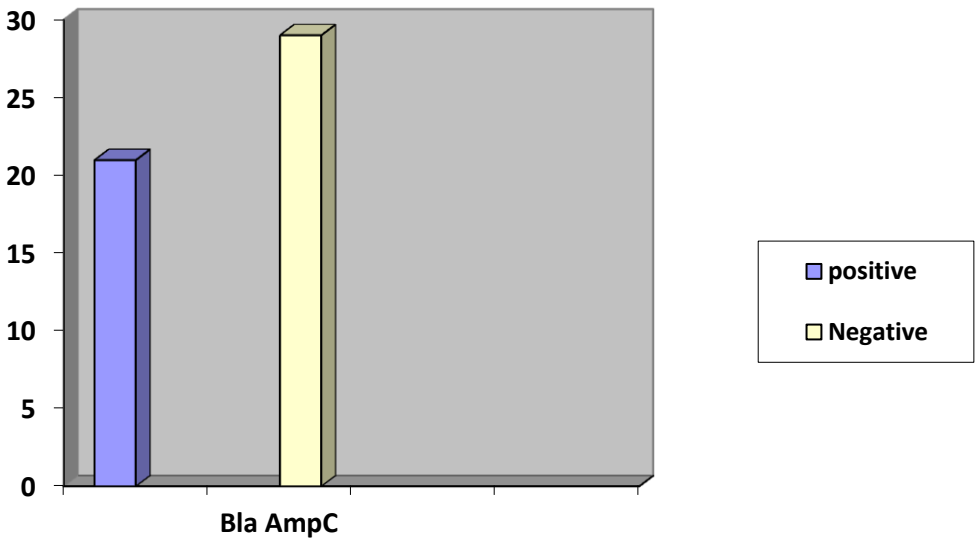
5.5: Prevalence of *bla* AmpC among Cefoxitin resistant isolates

Table -9: Prevalence of *bla* AmpC among Cefoxitin resistant isolates

PCR	CRI	%
Positive	21	42
Negative	29	58
Total	50	100

Among the 50 cefoxitin resistant isolates, *bla* AmpC was present in twenty one (52 %) by Real Time-PCR. (Table. 9).

Figure: 17. Prevalence of bla AmpC among Cefoxitin resistant isolates



5.4. Distribution of positive isolates by MHT,AmpC disc test and PCR

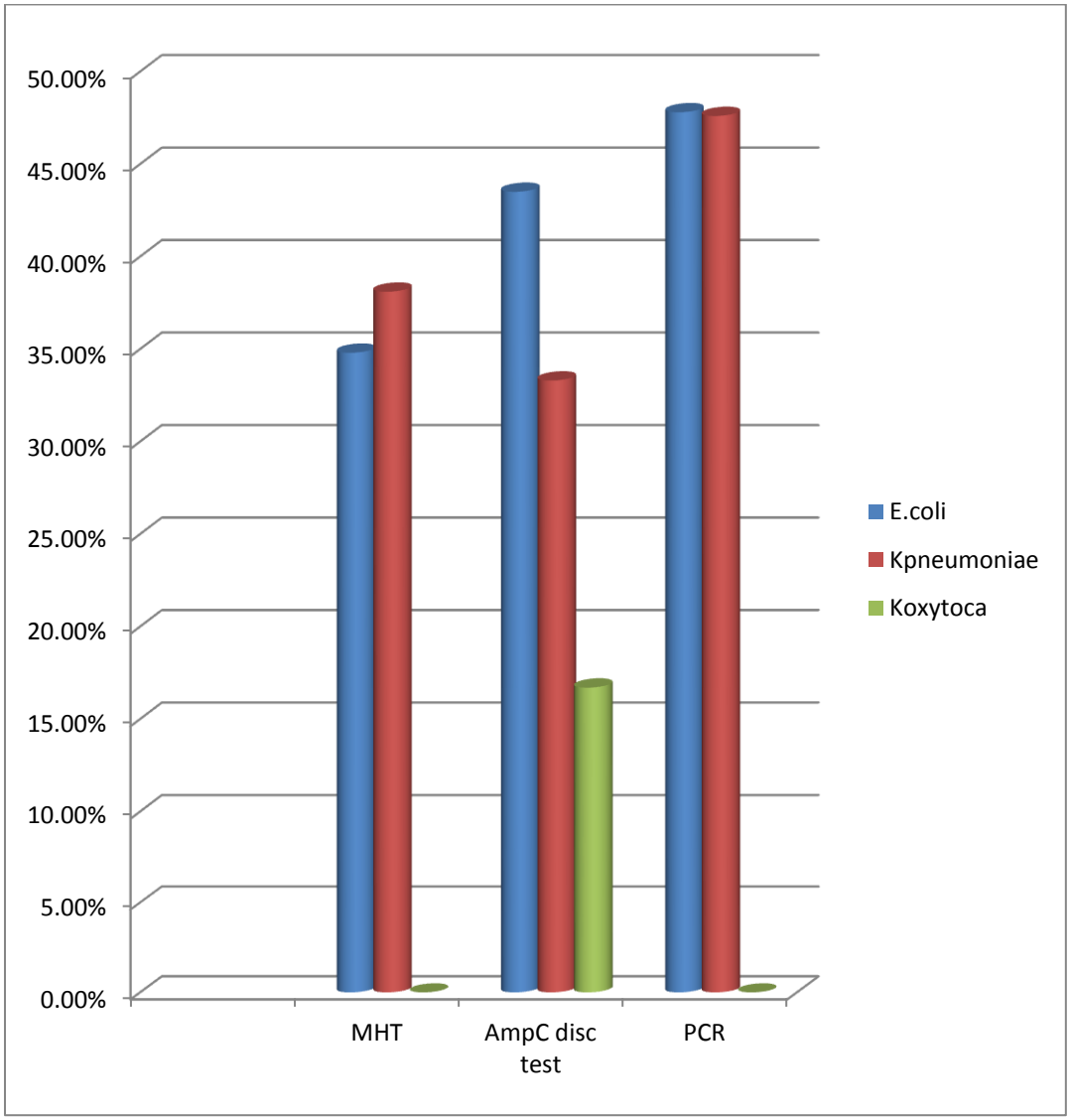
Table .10 Distribution of positive isolates by MHT,AmpC disc test and PCR

Positive Isolate	*E.coli*	*K.pneumoniae*	*K.oxytoca*
MHT	8(34.8%)	8(38.1%)	0
AmpC disc test	10(43.5%)	7(33.3%)	1(16.7%)
PCR	11(47.8%)	10(47.6%)	0

Among 16 MHT positive isolates,eight isolates(34.8%) were *E.coli* and another 8 isolates(38.1%) were *Klebsiella pneumoniae*. Among eighteen AmpC disc test positive isolates,ten isolates(43.5%) belong to *E.coli* and seven isolates(33.3%) were *Klebsiella pneumoniae*,One isolate(16.7%) was *Klebsiella oxytoca*.Eleven isolates(47.8%) were *E.coli* and ten isolates(47.6%) were *Klebsiella pneumoniae* among the 21 PCR positive isolates.

Figure.18. .Distribution of positive isolates by MHT,AmpC disc test,PCR

.



5.7. Correlation of MHT and PCR

Table- 11: Comparison of MHT and PCR

MHT	PCR	
	Positive	Negative
Positive	15	1
Negative	6	28
Total	21	29

The sensitivity, specificity of MHT were 71.42%, 96.55%, and positive predictive value and negative predictive value of MHT were 93.75% and 82.35% respectively.(Table :13)According to Mcnemar test(Value-0.375), Modified hodge test is not more sensitive than PCR(Gold standard).

5.8. Correlation of AmpC disc test and PCR

Table -12: Comparison of AmpC disc test and PCR

AmpC disc test	PCR	
	Positive	Negative
Positive	17	1
Negative	4	28
Total	21	29

The sensitivity, specificity of AmpC disc test were 80.95%, 96.55% and positive predictive value and negative predictive value were 94.44% and 87.55% respectively in AmpC disc test. (Table 14)According to Mcnemar test(value-0.375), AmpC disc test is not more sensitive than PCR(Gold standard)

5.9. Distribution of *bla* AmpC gene positive isolates by age and gender

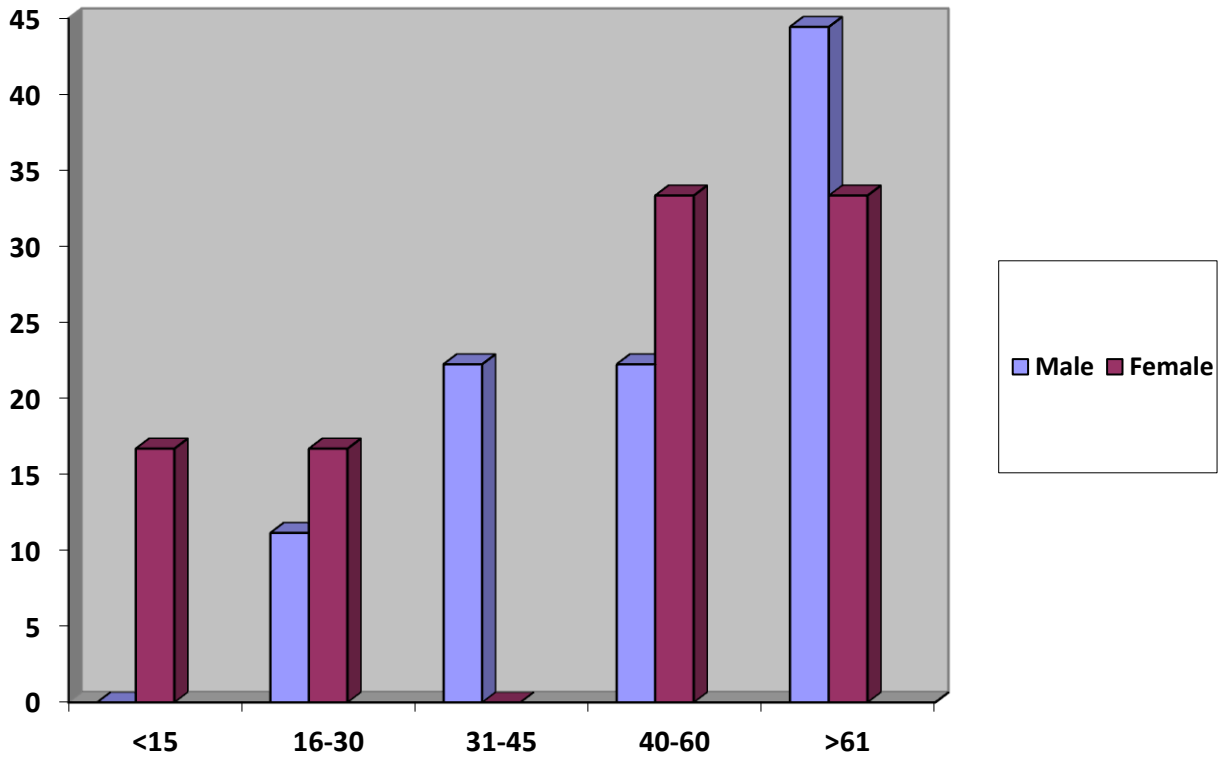
Table 13: *bla* AmpC gene positive isolates by age and gender

Age in years	PCR positive isolates			
	Male		Female	
	No	%	No	(%)
≤ 15	0	0	2	16.66
16 – 30	1	11.11	2	16.66
31 – 45	2	22.22	0	O
46 – 60	2	22.22	4	33.33
≥61	4	44.44	4	33.33
Total	9	42.85	12	57.14

Table 13 shows the distribution of PCR positive isolates by age and gender distribution. Most of the PCR positive isolates (57.14%) were from females. In the age group of below 15 years, two isolates (16.66%) were from females, no males were in this group. One isolate (11.11%) was from male and two females(16.66) were in the 16-30 years age group. In the 31-45 years age group, two isolates (22.22%) were from males and no females were in this group. Above 61 years four isolates (44.44%) were from males.(Figure.19

&Table .13) The mean age of female was 46.3 years and that of male was 53.7 years among AmpC positive isolates.

Figure:19: Distribution of *bla* AmpC gene positive isolates by age and sex



5.10. Distribution of *bla* AmpCgene positive isolates among various samples

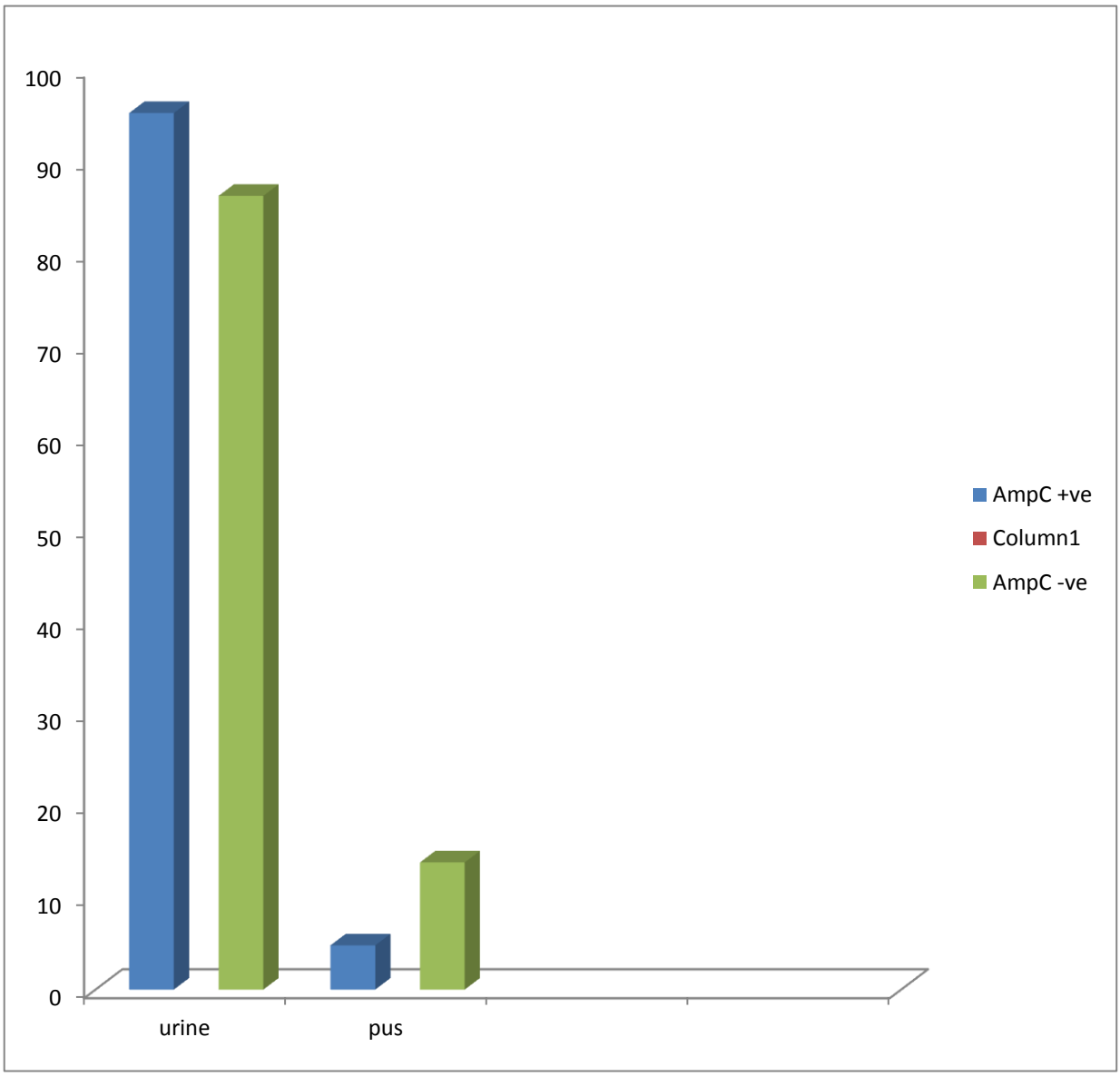
Table- 14: Distribution of *bla* AmpCgene positive isolates among various samples

Samples	AmpC positive		AmpC negative	
	No	%	No	%
Urine	20	95.2	25	86.2
Pus	1	4.8	4	13.8
Total	21	100	29	100

Among 21 AmpC positive isolates,20(95.2%) were isolated from Urine samples and one isolate (4.8) from pus.

Among 29 AmpC negative isolates,25(86.2%) were isolated from Urine samples and four isolates(13.8) from pus (Table-14)

Figure- 20: Distribution of *bla* AmpC gene positive isolates among various samples



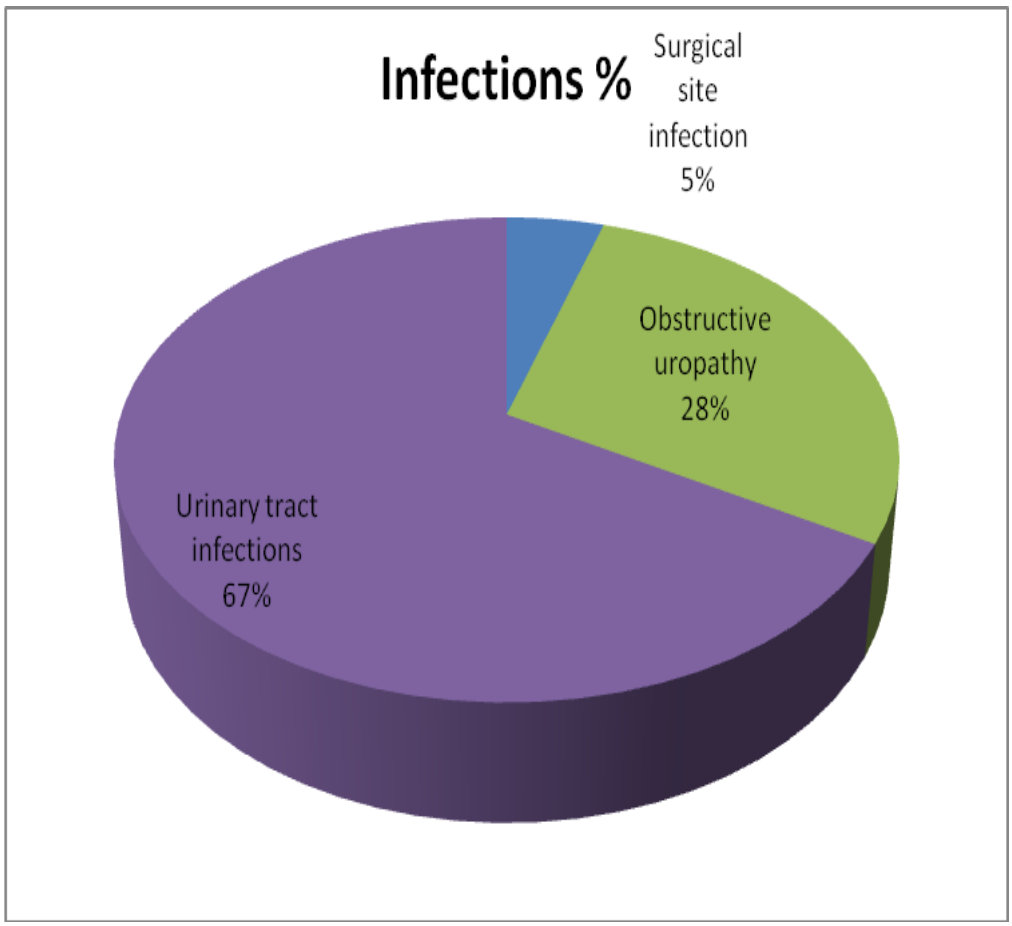
5.11: Association of *bla* AmpC gene producers with infections

Table- 15:Categorization of *bla* AmpC gene producers on infection basis

Infections	AmpC producers		AmpC non producers	
	No	%	No	%
Surgical site infection	1	4.8	2	6.9
Wound infection	0	0	2	6.9
Obstructive uropathy	6	28.6	1	3.4
Urinary tract infections	14	66.7	24	82.6
Total	21	100	29	100

In the present study, majority of the AmpC producers are associated with urinary tract infections i.e. 14 (66.7%). One isolate was recovered from surgical site infection (4.8%), Six isolates(28.6) from obstructive uropathy cases. (Fig.21 & Tab.15)

Fig -21: Association of infections with of *bla* AmpC gene producers



5.12. Risk factors:

5.12.1. Device related infections

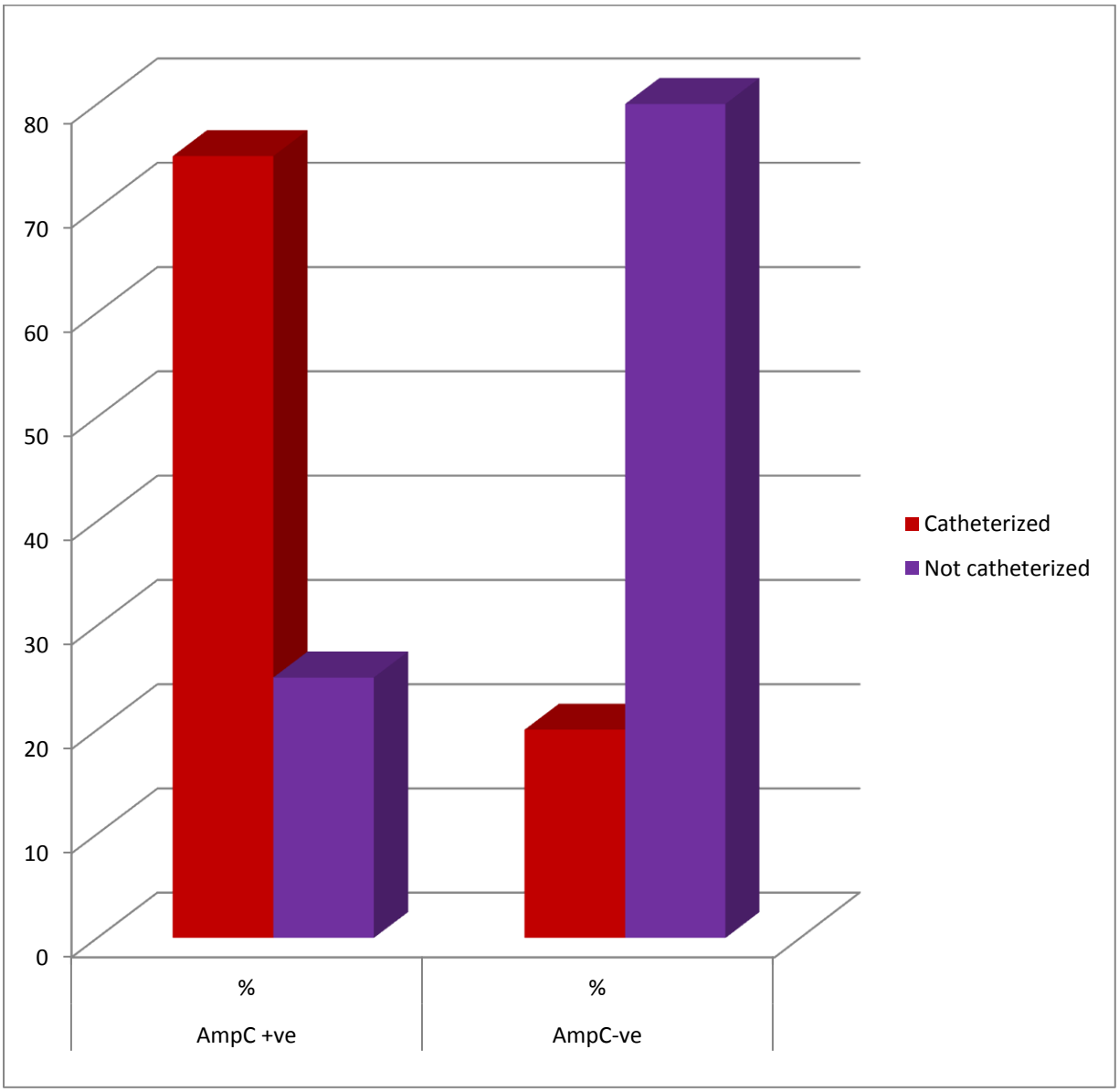
Table -16: Association of catheterization with *bla* AmpC gene producers among urine samples

Risk factors	AmpC producers		AmpC non producers	
	No	%	No	%
Catheterized	15	75	5	20
Not catheterized	5	25	20	80
Total	20	100	25	100

Urine samples for 75%of AmpC producers and 25 % of AmpC non producers were obtained from catheterized patients. There was a statistically significant association between catheterization and AmpC producers in urine samples.

(P = 0.0001) (Tab.16 & Fig. 22)

Figure- 22:Association of catheterization with with *bla* AmpC gene producers among urine samples



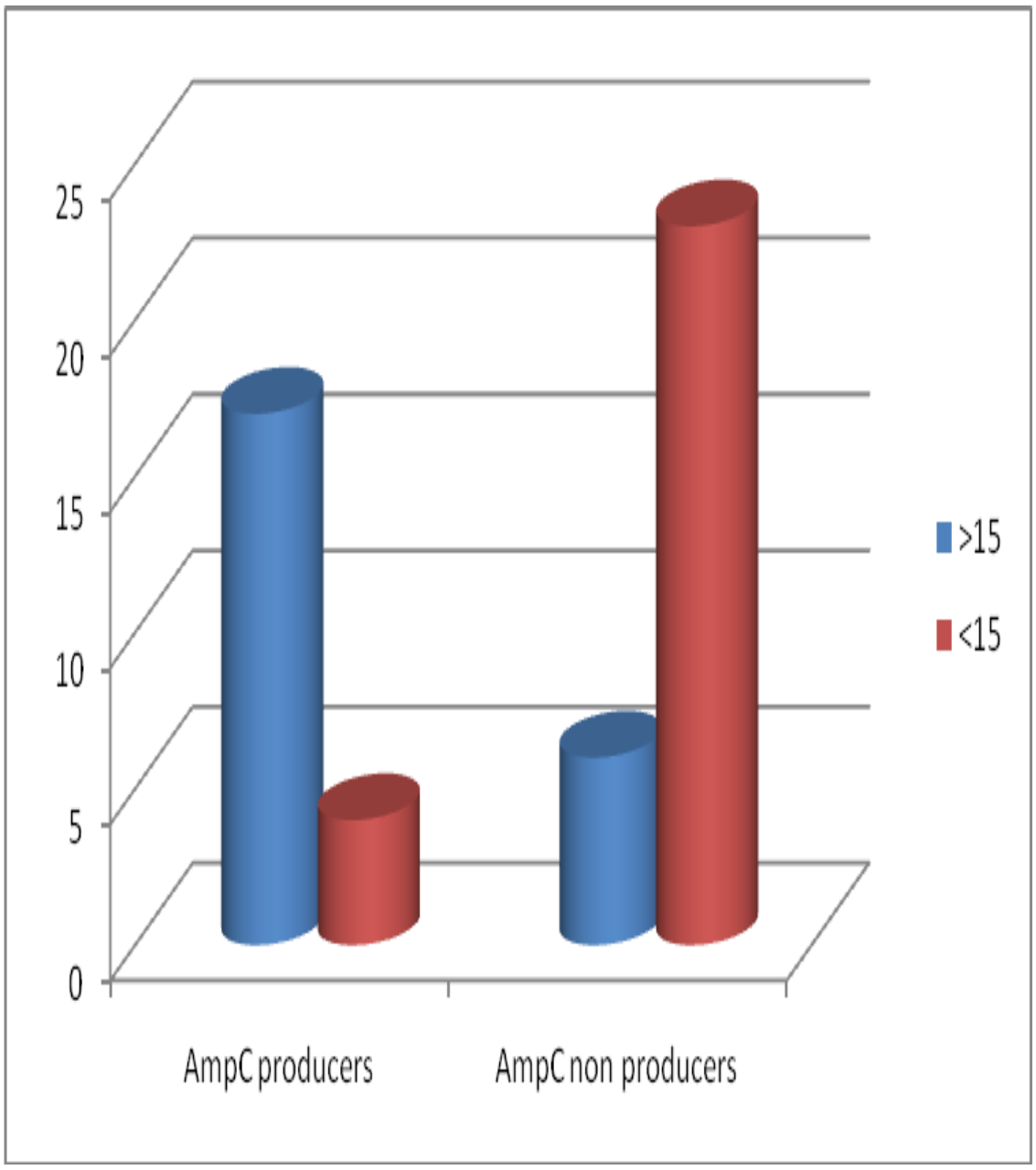
5.12.2. Duration of hospital stay

Table- 17:Duration of hospital stay among AmpC positive isolates

Duration in days	AmpC producers		AmpC non producers	
	No	%	No	%
>15	17	80.95	6	20.7
<15	4	19.04	23	79.31
Total	21	100	29	100

A total of four(19.04%) and seventeen (80.95%) of the AmpC positive isolates were from patients with less than 15 days stay in hospital and more than 15 days respectively.The association of AmpC positive isolates with the duration of stay in hospital was statistically significant [$P < 0.05$] (Table.17 & fig.23)

Fig – 23:AmpC positive isolates by duration of hospital stay



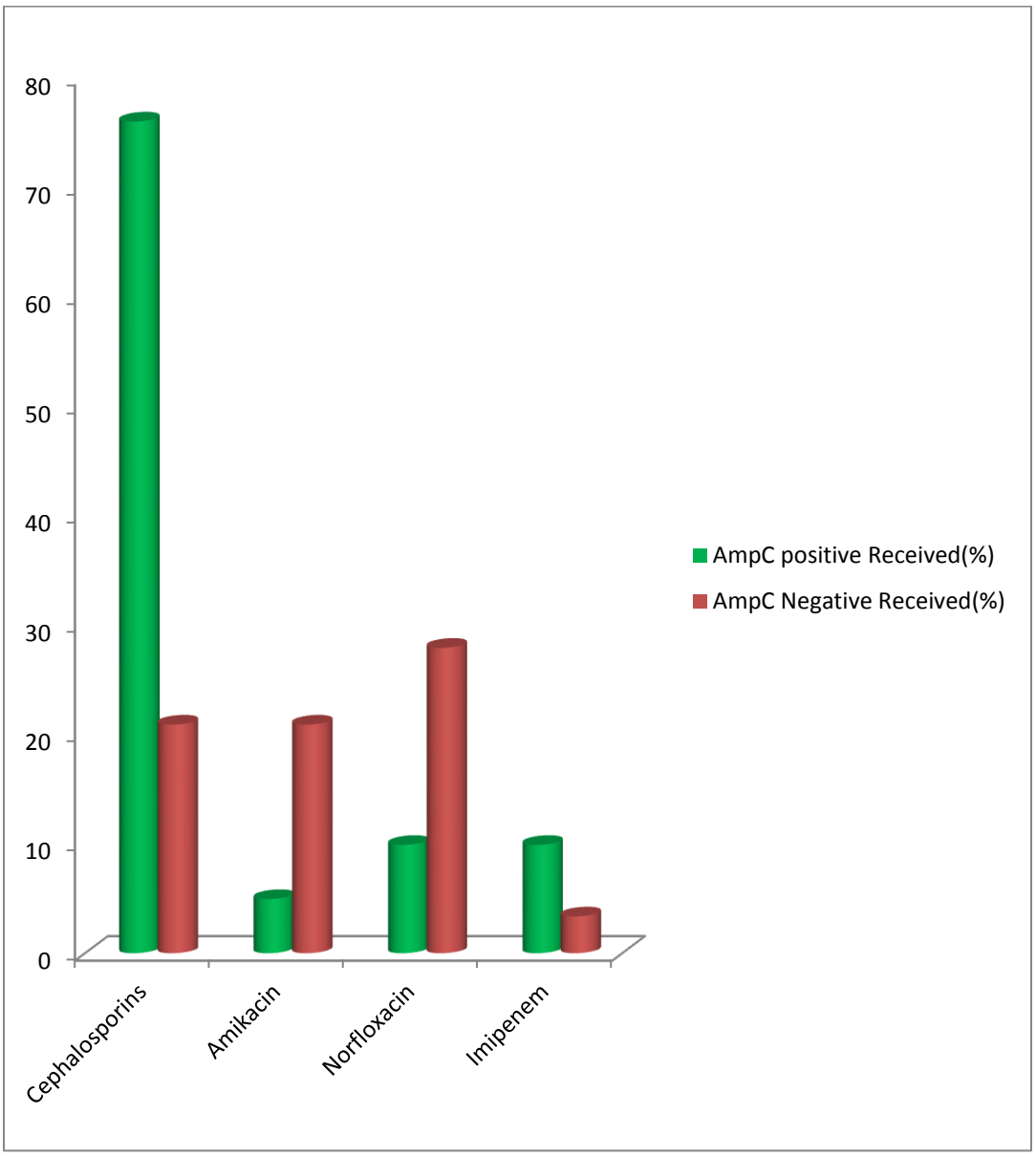
5.12.3. Exposure to antibiotics:

Table -18: Adminstration of Antibiotics among AmpC positive and AmpC negative isolates

Antibiotics	AmpC producers		AmpC non producers		Significance P<0.05
	Recieved	%	Recieved	%	
Cephalosporins	16	76	6	21	Significant
Amikacin	1	5	6	21	Not Significant
Norfloxacin	2	10	8	28	Not Significant
Imipenem	2	10	1	3.4	Not Significant

A total of sixteen (76%) patients had received third generation Cephalosporins among AmpC positive isolates.One (5%) patient had received aminoglycosides among AmpC positive isolates.A total of 2 (10%) patients had received fluoroquinolones among corresponding AmpC positive isolates. Totally two patients (10%) had received carbapenems among AmpC positive isolates.There was statistically significant difference in exposure to third generation Cephalosporins among AmpC positive and AmpC negative isolates. (P <0.05) (Tab.18 & Fig.24)

Figure- 24: Administration of Antibiotics among AmpC positive and AmpC negative isolates



5.13. Antibiotic Susceptibility pattern of *bla* AmpC positive and *bla*AmpC negative isolates :

Table-19: Antibiotic Susceptibility pattern of *bla* AmpC positive and *bla* AmpC negative isolates

Susceptibility	AmpC producers		AmpC non producers		Significance P<0.05
	R	%	R	%	
Amikacin	9	43	4	14	Significant
Gentamicin	17	81	18	62	Not Significant
Norfloxacin	16	80	8	32	Significant
Nitrofurantoin	15	75	10	40	Significant
Cephalosporins	21	100	29	100	Not Significant
Carbapenems	0	0	0	0	-

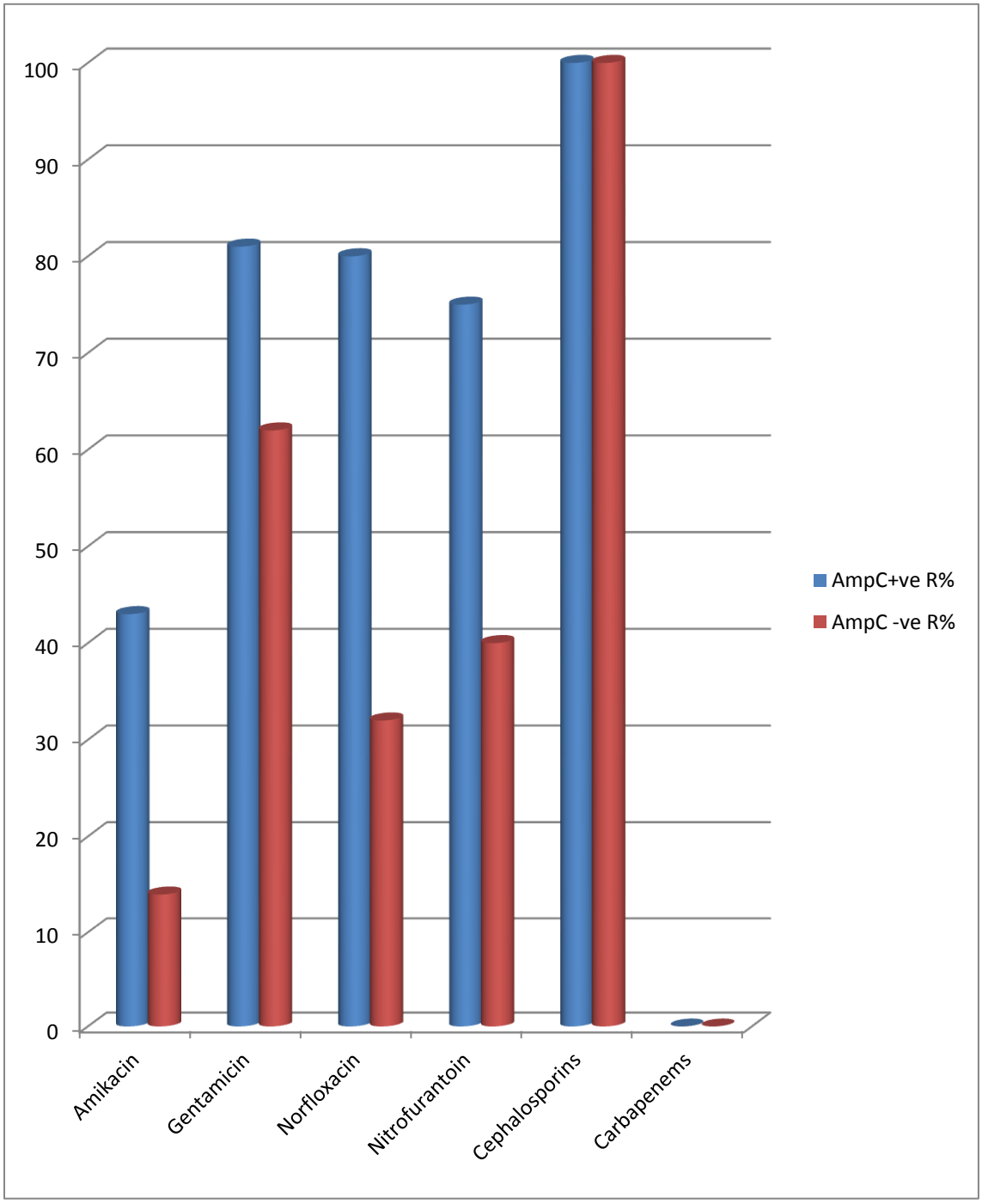
Resistance to Amikacin was noted among 9 (43%) AmpC positive and 4(14%) AmpC negative isolates. Among 20 AmpC positive and 25AmpC negative urine isolates, 15 (75%) isolates &10(40%) were resistant to Nitrofurantoin.A total of 16 (80%) AmpC positive urine isolates and eight

(32 %) AmpC negative isolates were resistant to Norfloxacin (5µg). Among the AmpC positive and AmpC negative isolates, seventeen (81%) &18(62%)were resistant to Gentamicin ,

In AmpC-positive and AmpC negative isolates, the resistance to third generation Cephalosporins was high, reaching 100% for ceftriaxone and ceftazidime and ceftazidime+clavulanic acid. All the isolates were sensitive (100%) to Imepenem (10µg) and none of them were resistant to the drug among AmpC positive and AmpC negative isolates.(Table:20,Figure: 26)

There was statistically significant difference in the susceptibility pattern of Amikacin,Nitrofurantoin, Norfloxacin among AmpC positive and AmpC negative isolates($P<0.05$).

Figure-25: Antibiotic Susceptibility pattern of *bla* AmpC positive and *bla* AmpC negative isolates



6. DISCUSSION

In the past few decades there has been a rise in the incidence of resistance to β-lactam antibiotics in bacterial pathogens.Among the various resistance mechanisms, production of β-lactamases is the most widespread and effective mechanism . Plasmid mediated AmpC-β-lactamase is a new threat worldwide as they mediate resistance to a broad spectrum of antibiotics. Among the *Enterobacteriaceae* family, *Klebsiella pneumoniae* and *E.Coli* which produce plasmid-mediated AmpC β-lactamases are responsible for nosocomial outbreaks of infection and colonization. As there are no CLSI guidelines, detection of *AmpC* β-lactamases is a challenge to microbiological laboratories and molecular detection is not also possible in all laboratories.However, proper recognition of AmpC producing *E. coli* and *Klebsiella* species is important for clinical management and epidemiological surveillance. [71.]

6.1. Phenotypic methods:

Various range of tests from enzyme extraction methods have been described in the literature,but these are consuming too much of time and difficult for routine use. Inhibitor based tests are also have been reported , but these inhibitors may not be readily available. So, simple methods by using available materials are used to detect AmpC enzyme in this study.

6.1.1. Modified hodge test and AmpC disk test:

All the 50 cefoxitin insusceptible isolates were evaluated by Modified hodge test and AmpC disk test for the production of AmpC enzyme. Of these, 16 and 18 isolates were positive by MHT and AmpC disk test respectively. Among 16 MHT positive isolates ,eight isolates(34.8%) were *E.coli* and another 8 isolates(38.1%) were *Klebsiella pneumoniae*. Similarly, Neelam Taneja *et al*[5]observed that 40% of *E.coli* isolates were positive for AmpC enzyme production by MHT.

Among eighteen AmpC disc test positive isolates,ten isolates(43.5%) belong to *E.coli* and seven isolates(33.3%) were *Klebsiella pneumoniae*,One isolate(16.7%) was *Klebsiella oxytoca*.Smitha O. Bagali *et al*[78] and Vijaya Shivanna *et al*[74]denoted that 24% and 20%of *E.coli* isolates were positive for AmpC enzyme production by AmpC disc test respectively.

6.2. Molecular methods:

Detection of *bla* ampC Gene(CMY-2) by RT-PCR

The advantages of molecular methods over phenotypic methods are identification of multiple AmpC gene types and shorter detection time. However, molecular methods may miss unusual gene types and require trained personnel and costlier equipments. For the identification of blaAmpC gene, Polymerase chain reaction have been used principally in research laboratories and reference centers. Goerge A jacoby *et al* concluded that multiplex PCR was the current "gold standard" for plasmid-mediated AmpC β lactamase detection.[9]

In this study, *bla* AmpC(CMY-2) was present in twenty one of fifty (42%) Cefoxitin resistant isolates. Similarly, various observations by Tanushree banu *et al* [16], Ty.Tan *et al*[11],Mai m helmy *et al*[89] Tenover *et al*[85]have observed that CMY-2 subtype of AmpC β-lactamases are the predominant type in *E. coli* and *Klebsiella* isolates.

6.3. Comparison of Phenotypic methods with molecular methods

Detection of AmpC enzyme by MHT and AmpC disc test were evaluated for sensitivity and specificity against RT-PCR as reference test .In the present study, the sensitivity and specificity of MHT were 71.42%and 96.55% respectively and the sensitivity of AmpC disc test was 81% and specificity was 96.55%. Similarly, Paul *et al* reported that Sensitivity of MHT was 73%and Specificity of MHT was 95%,and the sensitivity & specificity of AmpC disc test were 86% and 94%respectively. [14]

R.K.Manojkumar *et al* (Imphal) have observed that the sensitivity of AmpC disc test was 73.9%.[88]In contrast, another study by Yong D *et al*[79] reported that the sensitivity and specificity of the MHT were 100% and 94.9%, respectively.

In current study, AmpC disc test identified more positives than MHT. Similarly Tanushree banu *et al* found that the correlation between the AmpC disk test and the modified Hodge test is not perfect and the AmpC disk test identified more positives in their subset of isolates .[16]

Sometimes MHT shows false negative with AmpC producers as MHT uses integral cells, which may not liberate β-lactamases as efficiently.[18]It was found that the sensitivity of the Amp C disk test was more (81%) when compared to MHT(71.4%)and this test was a simple, convenient test and required no special inhibitors. The present study suggests that the AmpC disc test may be used for routine detection of the AmpC β lactamase in a clinical laboratory where the molecular methods are not available. But Phenotypic tests do not differentiate between chromosomal AmpC genes and plasmid mediated AmpC genes. Hence, genotypic characterization is considered as the gold standard .[71]

6.4. Prevalence of *bla* AmpC gene

In the present study, the prevalence of AmpC producers was 47.8% in *E.coli* and 47.6% in *Klebsiella pneumoniae* among the 50 Cefoxitin resistant clinical isolates by PCR . This is comparable with the study done by Akujobi *et al*[4] from Nigeria who reported that the prevalence of AmpC in *E.coli* and *Klebsiella pneumoniae* isolates was up to 56.25% and43.75% respectively in 2012.Similarly, Şerife Altun*et al*[58] from Turkey in 2013 found that 33% of *E.coli* and 46.7% of *Klebsiella pneumoniae* isolates were positive for *bla*AmpC gene. Parveen R. Mohamudha[71](Puducherry) reported that the prevalence of AmpC production in *E. coli* and *K.pneumoniae* isolates was 68.5% and 31.4% respectively in 2010.[71] Similarly, Sridhar Rao PN *et al*

[77](Karnataka) reported 48.38% of *E.coli* and 59.1% of *Klebsiella pneumoniae* as AmpC β lactamase enzyme producers in 2006.[77]

R.K. Manojkumar Singh *et al* (2011)from Imphal observed that the prevalence of AmpC β lactamase production in *E. coli* and *K.pnumoniae* isolates was 29.2%and22.9% respectively which was lower than the prevalence obtained by the present study.[88] The high prevalence of AmpC producers in our study is due to exposure of previous cephalosporin therapy whether empirically or according to the hospital antibiotic policy.

6.5. Distribution of *bla* AmpC positive isolates according to age and gender

In this study, most of the isolates (57.14%) were from females. The mean age of female was 46.3 years and that of male was 53.7 years among AmpC positive isolates. Similarly, Sasirekha et al (2013) reported that 69.3 % of the AmpC producing pathogens were from females .[32]

6.6. Distribution of *bla* AmpC positive isolates according to site of infection

Majority of the infections were associated with urinary tract infections i.e. 14 (66.7%). one isolate from surgical site infection (4.8%), six isolates (28.6 %) from Obstructive uropathy cases. The same distribution was noted in various observations done by T.y.Tan *et al*[11], Slike polsfuss*et al*[61] and Nevine Fam *et al*,[63] Smitha O. Bagali *et al*[78] who have concluded that the majority of the AmpC producing pathogens were isolated from urine. B.L.Chaudhary *et al* (2013) reported that the prevalence of AmpC positive isolate was highest in Pus (52.63%) followed by urine (7.40%). This is contrast to the present study. [75]

6.7. Risk factors

6.7.1. Device related infections

In the present study, there was a statistically significant association between catheterization and AmpC producers. Neelam taneja *et al*[5]in 2008(North india) found that inserting Foley's catheter is a risk factor for AmpC associated infections. Goerge A jocoby *et al* from Israel in 2009 also found that Foley's catheter insertion is a definite risk factor for AmpC associated infection.[9]

6.7.2. Duration of hospital stay

In this study, all AmpC positive (100%) strains were from inpatients and 80.95% of AmpC positive isolates were from patients with more than fifteen days stay in hospital .There was a statistically significant association between hospital stay and AmpC production. Similarly, Kenneth h rond *et al* observed that the hospital stay of AmpC positive patients was significantly longer than patients in the control group.(P = .047) [18]

Similarly, Various studies by Vikas Manchanda *et al*[67], Chakraburty*etal*[87], Rajesh pareja *et al* [76]who reported that AmpC positive isolates were mainly limited to hospitalized patients only.

But in contrast , one observation by Goerge A jacoby *et al* have reported that AmpC producers were also isolated from outpatient clinics, which indicate the presence of AmpC in the community .[9]

6.7.3. Antibiotic usage

An extensive use of β-lactam antibiotics in hospital and community has produced a major problem leading to increased morbidity, mortality and health care costs. Exposure to different classes of antibiotics like third generation Cephalosporins, Aminoglycosides, Fluoroquinolones, Nitrofurantoin and Carbapenems were analysed among AmpC positive and AmpC negative patients.

Among these antibiotics, exposure to Cephalosporins was statistically significant among corresponding AmpC positive patients. The high prevalence of AmpC producers in our study is due to exposure of previous Cephalosporin therapy whether empirically or according to the hospital antibiotic policy. Similar reports are available from various studies by Goerge A chocoby *et al*[9],Nevine fam *et al*[63]and they have confirmed that prior management with antibiotics, particularly combinations of Cephalosporins and β-lactamase inhibitors are significantly associated with infection by AmpC positive isolates.

Arindam Chakraborty *et al*[87] reported that combining Cephalosporins with Penicillins and addition of β lactam with a β-lactamase inhibitor are potential risk factors for *AmpC* induction. Limited use of antimicrobial agents, predominantly, broad-spectrum Cephalosporins,β-lactamase inhibitor combinations, Fluoroquinolones are suggested to prevent AmpC associated infection.Prolonged antibiotic treatment should be avoided.

6.8. Multidrug resistance:

In AmpC-positive isolates, the resistance to third generation Cephalosporins was high, reaching 100% for Cefotaxime and Ceftazidime and Ceftazidime+Clavulanic acid and they were resistant to Amikacin, Gentamicin, Nitrofurantoin and Norfloxacin in 42.9%, 81%,75% and 80% respectively.

AmpC-negative isolates were resistant to Cephalosporins i.e.100% to Cefotaxime, Ceftazidime and Ceftazidime+Clavulanic acid and13.8%, 62.1%,40% and32% were resistant to Amikacin, Gentamicin, Nitrofurantoin and Norfloxacin respectively. This is in accordance with the studies by Nevine fam *et al*,[90] Deepika handa *et al* [70]Who observed that the AmpC positive isolates showed high resistance to third generation Cephalosporins(100%). In contrast, Kenneth H rond *et al* reported that 36%and 56% of their AmpC-producing isolates were reported as susceptible to Cefotaxime and Ceftazidime, respectively.[18]In the current study, AmpC-positive isolates showed resistance to Norfloxacin (80%) and to Amikacin (42.9%)which were lower than those reported (94.1% and41.2% respectively)by Nevine fam *et al.*2008. In contrast, Deepika handa *et al* (Utthrapradesh-2007) observed that resistance among the AmpC positive isolates to Norfloxacin and Amikacin were 66.7% and 20.8% respectively.[70]

Similarly, Arindam chakraborty *et al*[87] in 2010 demonstrated that AmpC producers were multidrug resistant, with Amikacin- 40%, Gentamcin- 73%, Norfloxacin- 51%.

Fortunately all isolates retained susceptibility to Imipenem. It is in accordance with other studies by Varsha gupta *et al,* V.Hemalatha *et al*[69], Mohammad Soltan Dallalol *et al*[91] who reported that the susceptibility pattern of their isolates showed100 per cent susceptibility to Imipenem.

Amikacin is the second most common sensitive drug after Imipenem. So, these drug resistant organisms have limited therapeutic options and necessitated the increased use of Carbapenems.

In the present study, MDR among AmpC positive isolates was 33.3% which is due to plasmid mediated spread. Similarly, Arindam chakraborty *et al*[87] in 2010 demonstrated that 35% of AmpC producers were multidrug resistant.

In contrast, Mohammad Soltan Dallalol *et al* found that 70% of the AmpC positive isolates exhibited a multidrug resistance phenotype.[91]

6.9. Treatment

In the present study, it was found that treatment with Carbapenems was successful in nineteen(90.5%) of the corresponding AmpC positive patients. This is in accordance with the studies by Mai me helme *et al*[89],

Neelam taneja *et al* suggested that Imipenem and Meropenem were the best treatment option in treating serious infections caused by AmpC producing isolates.In the current study,two patients(9.5%) had received Norfloxacin who were diagnosed to have urinary tract infection. Similarly, Arindam Chakraborty *et al* suggested treatment of patients infected with AmpC producing isolates using Carbapenems and Aminoglycosides .[87]

Smitha O. Bagali *et al* concluded that Carbapenems are drug of choice for AmpC producing bacteria but resistance to Carbapenem may arise by mutation which diminish influx or augment efflux.[78]

In this study, 58% of cefoxitin resistant isolates were not positive for AmpC production by PCR and this warrants further investigation into the other mechanisms of resistance and their laboratory detection. Many factors may explain resistance to cefoxitin in the AmpC-negative isolates.

- In this study, twenty one isolates were positive for CMY-2 gene detected by RT PCR. The remaining 29 PCR negative isolates might contain other genes of AmpC(MOX, DHA, EBC, FOX and ACC-1).
- It may be due to porin channel alterations and mutations in *E. coli* and *Klebsiella* isolates.
- Cefoxitin resistant in *E. coli* may effect from over expression of the chromosomal mediated *ampC* gene which results in changes in the permeability of the cell to Cefoxitin.[90]

6.10. Prevention

Enhanced sanitary measures in the outpatient setting, restriction of patient transfer between healthcare facilities, active viewing of patients transferred from a high-risk institution and cohorting for already colonized patients are recommended for the prevention of AmpC colonization and infection. Increased

accurateness in the recognition of resistance mechanisms will be effective in planning infection control and treatment guidelines.[56]

Dissemination of AmpC producers within the hospital or between the different regions of our country may become significant public health issue. Hence, recognition of AmpC may enhance hospital infection control rate by making the physician to think about the selection of suitable antibiotics. [18] The sensitivity of the AmpC disc test was more (81%) when compared to MHT(71.4%).Hence, this AmpC disc test may be used for routine detection of the AmpC β lactamase in a clinical laboratory where the molecular methods are not available.

Findings of this study designate the necessity for sustained observation of mechanisms of resistance among nosocomial pathogens and evolving preventive measures aimed at reducing their spread.[13] The information from this study would be helpful for formulation of an antibiotic policy for its rational use.

7. SUMMARY

This study was undertaken at Tirunelveli Medical College, Tirunelveli for a period of one year from 50 cefoxitin resistant clinical isolates of *Enterobacteriaceae* . Modified Hodge test and AmpC disc test were done to detect AmpC enzyme production and isolates were also tested for *bla*AmpC gene by Real-Time PCR. The risk factors and antibiotic sensitivity patterns of the isolates were further analysed.

- A total of 24(48%) isolates were from males and the remaining 26(52%) were from females.
- Modified Hodge test detected 16 (32%)AmpC positive isolates indicated by clover leaf pattern.Among these ,eight isolates(34.8%) were *E.coli* and another eight isolates(38.1) were *Klebsiella pneumoniae*.
- AmpC disc test detected 18 AmpC positive isolates.Among these,ten isolates(43.5%) were *E.coli* and seven isolates(33.3%) were *Klebsiella pneumoniae* , one isolate(16.6%) was *Klebsiella oxytoca*.
- Real time PCR detected *bla* AmpC gene in twenty one (42%) of the 50 screen positive isolates .Among these,eleven isolates (47.8%)were *E.coli,* 10 isolates(47.6%) were *Klebsiella pneumoniae.*
- The sensitivity, specificity of Modified hodge test were 71.42%, 96.55%, and PPV and NPV were 93.75% and 83.53% respectively.

- The sensitivity, specificity of AmpC disc test were 80.95%, 96.55%, and PPV and NPV were 94.44% and 87.5% respectively.
- A total of 57.14% of the AmpC positive isolates were from females and 42.85% were from males.
- Majority of the AmpC producers are associated with urinary tract infections i.e. 14 (66.7%). One isolate was recovered from surgical site infection (4.8%), Six isolates(28.6) from obstructive uropathy cases.
- Duration of stay at hospital for more than fifteen days was statistically significant among corresponding AmpC positive patients.
- There was a statistically significant association between catheterization and AmpC production in urine samples.
- Exposure to Cephalosporins was statistically significant among corresponding AmpC positive patients..
- In AmpC-positive isolates, the resistance to third generation Cephalosporins was high, reaching 100% for Ceftriaxone and Ceftazidime and Ceftazidime+clavulanic acid and were resistant in 43%, 81%,75% and 80% respectively to Amikacin, Gentamicin, Nitrofurantoin and Norfloxacin .

8. CONCLUSION

- This study highlights the prevalence of AmpC enzyme production among clinical samples and also *bla* AmpC among AmpC producers.
- Modified Hodge test is simple to do and less costlier.
- AmpC disc test is to be considered as a diagnostic tool for AmpC detection in routine laboratory because of its high sensitivity, rapid and easy interpretation.
- In the present study, MDR among AmpC positive study isolates was high suggesting plasmid mediated spread .
- Carbapenems are superior to other antibiotics for the treatment of serious infections due to AmpC β lactamase-producing gram-negative bacteria.
- An approach to eradicate AmpC producers in the hospitals is to create awareness among health care workers and following effective barrier precautions and good hygienic practices to prevent further transmission. Dissemination of AmpC producers within the hospital or between the different regions of our country may become significant public health issue. Hence, recognition ofAmpC may enhance hospital infection control rate by making the physician to think about the selection of suitable antibiotics.

9. BIBLIOGRAPHY

1. The Enterobacteriaceae: General Characteristics,.J.J. Farmer III, M.K. Farmer, AND Barry holmes, Topley &Wilson's Microbiology And Microbial Infection.10th Ed.Edward Arnold Ltd.Italy.2005.Volume-2, Part-3 Chapter -20 Pages 48-86.
2. Enterobacteriaceae: Fritz H. Kayser, Medical Microbiology © 2005 Thieme, Chapter 4,Page-278-296.
3. Enterobacteriaceae ,Kenneth .J.ryan,C.goerge ray,Sherris Medical microbiology, Fourth Edition, Chapter-21,-p 343-371
4. Akujobi, *C.O., Odu, N.N. And Okorondu, S.I. Detection Of Amp C Beta Lactamases In Clinical Isolates Of *Escherichia Coli* And *Klebsiella* African Journal Of Clinical And Experimental Microbiology January 2012, Vol 13(1): 51-55
5. Neelam Taneja, Gagandeep Singh, Meenakshi Singh, Surendra Madhup, Sapna Pahil &Meera Sharma, High Occurrence Of BlaCmy-1 Ampc Lactamase Producing *Escherichia Coli* .In Cases Of Complicated Urinary Tract Infection (UTI) From A Tertiary Health Care Centre In North India, Indian J Med Res 136, August 2012, Pp 289-291
6. Mechanisms Of Resistance to Antibacterial Agents, Louis B. Rice And Robert A. Bonomo,Murray Diagnostic Microbiology 9th Edition,Chapter-66, Page 1082-1105
7. Antimicrobial Susceptibility Testing, Washington Winn W, Jr Koneman's Color Atlas And Textbook Of Diagnostic Microbiology 6 Th Edition,Chapter 17, Page -945-1014
8. Varsha Gupta, Karthikeyan Kumarasamy*, Neelam Gulati, Ritu Garg, Padma Krishnan* &Jagdish Chander, Ampc B-Lactamases in

Nosocomial isolates Of *Klebsiella Pneumonia*e From India Indian J Med Res 136, August 2012, P 237-241

9. George A. Jacoby Ampc B-Lactamases ,Clin Microbiol Rev. 2009 January; 22(1): 161–182.
10. Alvarez M, Tran Jh, Chow N, Jacoby Ga. Epidemiology Of Conjugative Plasmid Mediated Ampc β-Lactamases in The United States. Antimicrob Agents Chemother 2004; 48 :533-7.
11. Thean Yen Tan,* Lily Siew Yong Ng, Jie He,Tse Hsien Koh, And Li Yang Hsu,Evaluation Of Screening Methods To Detect Plasmid-Mediated Ampc in *Escherichia Coli, Klebsiella Pneumoniae*, And *Proteus Mirabilis* Antimicrobial Agents And Chemotherapy, Jan. 2009, P. 146–149 Vol. 53, No. 1
12. Subha A, Renuka Devi V, Ananthan S. Ampc B-Lactamases Producing Multidrug Resistant Strains Of *Klebsiella* species &*Escherichia Coli* isolated From Children Under Five In Chennai.India. Indian J Med Res 2; 117 : 13-8.
13. Anand Manoharan, Madhan Sugumar, Anil Kumar, Hepzibah Jose, Dilip Mathai , Phenotypic & Molecular Characterization Of Ampc β-Lactamases Among *Escherichia Coli, Klebsiella* Species. & *Enterobacter* Species from Five Indian Medical Centers , Indian J Med Res, March 2012
14. Paul R. Ingram, Tim J. J. Inglis, Tessa R. Vanzetti, Barbara A. Henderson,Gerald B. Harnett And Ronan J. Murray , Comparison Of Methods for Ampc B-Lactamase detection in *Enterobacteriaceae* ,Indian J Med Res 135, March 2012, P 359-364
15. Chelsie N. Geyer, Mark D. Reisbig, and Nancy D. Hanson Development of a TaqMan Multiplex PCR Assay for Detection of Plasmid-Mediated AmpC β-Lactamase Genes J Clin Microbiol. 2012 November; 50(11): 3722–3725

16. Tanushree Barua1,*, Malini Shariff2, S.S. Thukral2 Detection and Characterization of AmpC B-Lactamases in Indian Clinical Isolates of Escherichia coli, Klebsiella pneumoniae and Klebsiella oxytoca Universal Journal of Microbiology Research 1(2): 15-21, 2013
17. Jennifer A. Black, Ellen Smith Moland, and Kenneth S. Thomson AmpC Disk Test for Detection of Plasmid-Mediated AmpC β-Lactamases in Enterobacteriaceae Lacking Chromosomal AmpC β-Lactamases J Clin Microbiol. 2005 July; 43(7): 3110–3113.
18. Kenneth H. Rand, MD,Bradley Turner, MD, Hilary Seifert, Christine Hansen, PharmD,Judith A. Johnson, PhD, and ,Clinical Laboratory Detection of AmpC **β**-Lactamase Does It Affect Patient Outcome? Microbiology and Infectious Disease Am J Clin Pathol 2011;135:572-576
19. B. Sasirekha Prevalence Of Esbl, Ampc B- Lactamases And Mrsa Among Uropathogens And Its Antibiogram. Excli Journal 2013;12:81-88
20. Thana Khawcharoenporn, Shawn Vasoo, And Kamaljit Singh,Urinary Tract Infections Due To Multidrug-Resistant Enterobacteriaceae: Prevalence And Risk Factors In A Chicagovolume 2013, Article Id 258517, 7 Pages
21. *Jb Sarma, Pk Bhattacharya, D Kalita, M Rajbangshi Multidrug-Resistant Enterobacteriaceae Including Metallo-B-Lactamase Producers Are Predominant Pathogens Of Healthcare-Associated Infections In An Indian Teaching Hospital Indian Journal Of Medical Microbiology, (2011) 29(1): 22-7 1
22. Michela Paolucci,Maria Paola Landini, and Vittorio Sambri ,How Can the Microbiologist Help in Diagnosing Neonatal Sepsis? Volume 2012, Article ID 120139, 14 pages

23. *Beena Antony, BPM Rajendra Prasad*,An Outbreak Of Neonatal Septicaemia By Enterobacter Cloacae ,Asian Pacific Journal Of Tropical Disease (2011)227-229
24. The Enterobacteriaceae , Washington Winn W, Jr Koneman's Color Atlas And Textbook Of Diagnostic Microbiology 6 Th Edition,Chapter 6 Page -211-302
25. Escherichia ,Tom Cheasty and Henry R. Smith , Topley &Wilson's Microbiology And Microbial Infection.10th Ed.Edward Arnold Ltd.Italy.2005.Volume-2, Chapter-21 Pages 91-112.
26. S Sharma, *Gk Bhat, S Shenoy Virulence Factors And Drug Resistance in *Escherichia Coli* Isolated From Extraintestinal Infections Indian Journal Of Medical Microbiology, (2007) 25(4): 369-73.
27. Ba Tantry, S Rahiman Antibacterial Resistance And Trend Of Urinary Tract Pathogens To Commonly Used Antibiotics In Kashmir Valley, West Indian Med J 2012; 61 (7): 704
28. *Marlieke E. A. De Kraker, Peter G. Davey, Hajo Grundmann,* Mortality And Hospital Stay Associated With Resistant Staphylococcus Aureus And Escherichia Coli Bacteremia: Estimating The Burden Of Antibiotic Resistance In Europe October 2011 | Volume 8 | Issue 10 | E1001104
29. *Asima Banu, Jyoti S Kabbin, Mridu Anand* Extraintestinal Infections Due To *Escherichia Coli*: An Emerging Issue,Key Journal Of Clinical And Diagnostic Research. 2011 June, Vol-5(3): 486-490.
30. Diseases Caused by Gram-Negative Enteric Bacilli Thomas A. Russo,Harrison's Principles Of Internal Medicine 16th Edition Chapter-134,Page-878-886
31. *Andrej Weintraub*, Enteroaggregative Escherichia Coli: Epidemiology, Virulence And Detection,Journal Of Medical Microbiology (2007), 56, 4–8.

32. Sanjucta Dutta, Sucharita Guin, Santanu Ghosh, Gururaja P. Pazhani1, Krishnan Rajendran,Mihir K. Bhattach ,Trends In The Prevalence Of Diarrheagenic Escherichia Coli Among Hospitalized Diarrheal Patients In Kolkata,India ,February 2013 | Volume 8 | Issue 2 |
33. Citrobacter, Enterobacter, Klebsiella,Plesiomonas, Serratia, And Other Members Of The Enterobacteriaceae,Patrick R. Murray, Barry Holmes, And Hazel M. Aucken,Topley &Wilson's Microbiology And Microbial Infection.10th Ed.Edward Arnold Ltd.Italy.2005.Volume-2, Part-3,Chapter-26, Pages 205-230.
34. Enterobacteriaceae,Michael's Donnenberg ,Mandell, Douglas, And Bennett's Principles And Practice Of Infectious Diseases Seventh Edition,Chapter 218,Page 2815-2834.
35. Dr.R.Sarathbabu, Dr.T.V.Ramani, Dr. K.Bhaskara Rao, Dr.Supriya Panda Antibiotic Susceptibility Pattern Of Klebsiella Pneumoniae Isolated From Sputum, Urine And Pus Samples ,Iosr Journal Of Pharmacy And Biological Sciences , 2278-3008 Volume 1, Issue 2, Pp 04-09
36. A Malik, Se Hasani, M Shahid, Hm Khan, Aj Ahmad Nosocomial Klebsiella Infection In Neonates In A Tertiary Care Hospital: Protein Profile By Sds-Page And Klebocin Typing As Epidemiological Markers ,Indian Journal Of Medical Microbiology, (2003) 21 (2):82-86
37. Francesco Casella, Luigi Finazzi, Valentina Repetti, Giampaolo Rubin,Maria Dimarco, Tiziana Mauro And Raffaello Furlan* Liver Abscess Caused By Klebsiella Pneumoniae: Two Case Reports Cases Journal 2009, 2:6879
38. B. N. Harish, G. A. Menezes,S. Shekatkar And S. C. Parija Extended-Spectrum B-Lactamase-Producing Klebsiella Pneumoniae From Blood Culture Journal Of Medical Microbiology ,10.1099/Jmm.0.47072-0 G 2007 Sgm.

39. Varsha Chaudhary, Ved Prakesh, Kiran Agarwal,Vijender Kumar Agrawal, Arun Singh, Sachin Pandey,Clinico-Microbiological Profile Of Women With Vaginal Discharge In A Tertiary Care Hospital Of Northern India, International Journal Of Medical Science And Public Health | 2012 | Vol 1 | Issue 2.
40. Antibacterial Agents**,**Joseph D. C. Yao And Robert C.Moellering,Jr.,Murray Diagnostic Microbiology,9th Edition,Chapter-65 Page 1043-1081
41. Principles Of Antimicrobial Action And Resistance ,Bailey&Scott's Diagnostic Microbiology,12 Th Edition,Chapter11 Pages 172-187.
42. Bacterial Resistance: Origins, Epidemiology, and Impact. Clinical Infectious Diseases, Livermore, D.M 2003. 36(Suppl 1): P. S11-23.
43. Brock Biology of Microorganisms, Madigan, M.T. and J.M. Martinko,. Eleventh ed. 2006: Pearson Prentice Hall.
44. Molecular genetics of bacteria ,Snyder, L. and W. Champness, 3rd ed. 2007, Washington D.C.: ASM Press. XVII, 735 s
45. Mark E. Rupp and Paul D. Fey ,Extended Spectrum β-Lactamase (ESBL)-Producing Enterobacteriaceae Considerations for Diagnosis, Prevention and Drug Treatment. Drugs 2003; 63 (4): 353-365.
46. Arne Søraas , Arnfinn Sundsfjord, Irene Sandven, Cathrine Brunborg, Pål A. Jenum ,Risk Factors for Community-Acquired Urinary Tract Infections Caused by ESBL-Producing Enterobacteriaceae , July 23, 2013 DOI: 10.1371.
47. Arif Hussain, Christa Ewers, Nishant Nandanwar, Sebastian Multiresistant Uropathogenic Escherichia coli from a Region in India Where Urinary Tract Infections are Endemic: Genotypic and Phenotypic Characteristics of Sequence Type 131 Isolates of the CTX-M-15 Extended-Spectrum- b -Lactamase-Producing Lineage, Antimicrob. Agents Chemother. 2012, 56(12):6358.

48. Extended spectrum β lactamase,AmpC, and Carbapenemase Issues Kenneth S. Thomson ,J. Clin. Microbiol. 2010, 48(4):1019.
49. Mohammad Faheem., Md Tabish Rehman., Mohd Danishuddin, Asad U. Khan,Biochemical Characterization of CTX-M-15 from Enterobacter cloacae and Designing a Novel Non-b-Lactam-b-Lactamase Inhibitor. Volume 8 | Issue 2
50. Mohammed Shahid*, Anuradha Singh, Farrukh Sobia, Mohammad Rashid, Abida Malik, Indu Shukla, Haris Manzoor Khan,blaCTX-M, blaTEM, and blaSHV in Enterobacteriaceae from North-Indian tertiary hospital: high occurrence of combination genes , Asian Pacific Journal of Tropical Medicine (2011)101-105.
51. Neil Gupta,Brandi M. Limbago, Jean B. Patel, and Alexander J. Kallen, Carbapenem-Resistant Enterobacteriaceae: Epidemiology and Prevention, Clinical Infectious Diseases 2011;53(1):60–67
52. Prasanta Raghab Mohapatra, Metallo-β-lactamase 1 - why blame New Delhi & India, Indian J Med Res. Jan 2013; 137(1): 213–215.
53. S Nagaraj, SP Chandran, P Shamanna, *R Macaden Carbapenem resistance among Escherichia coli and Klebsiella pneumoniae in a tertiary care hospital in south India, Indian Journal of Medical Microbiology, (2012) 30(1): 93-5
54. *Ms Kumar, V Lakshmi, R Rajagopalan ,Occurrence Of Extended Spectrum ß-Lactamases Amongenterobacteriaceae , Indian Journal Of Medical Microbiology, (2006) 24 (3):208 11 .
55. Mulvey Mr, Bryce E, Boyd Da, Ofner-Agostini M, Land Am. Molecular Characterization Of Cefoxitin Resistant Escherichia Coli From Canadian Hospitals. Antimicrob Agents Chemotherapy, 2005; 49 : 358-65.

56. F. Javier Pérez-Pérez And Nancy D. Hanson, Detecton Of Plasmid Mediated Ampc B -Lactamase Genes In Clinical Isolates By Using Multiplex Pcr, J. Clin. Microbiol. 2002, 40(6):2153.
57. Supriya Upadhyay, Malay Ranjan Sen, Amitabha Bhattacharjee, Presence Of Different Beta-Lactamase Classes Among Clinical Isolates Of Pseudomonas Aeruginosa Expressing Ampc Beta-Lactamase Enzyme J Infect Dev Ctries 2010; 4(4):239-242
58. Şerife Altun, Zeliha Kocak Tufan*, Server Yağcı, Extended Spectrum Beta-Lactamases, Ampc And Metallo Beta-Lactamases In Emerging Multi-Drug Resistant Gram-Negative Bacteria In Intensive Care Units, Volume 2 • Issue 4 • 2013.
59. A Bauernfeind, I Stemplinger, R Jungwirth, R Wilhelm And Y Comparative Characterization Of The Cephamycinase Blacmy-1 Gene And Its Relationship With Other Beta-Lactamase Genes. Antimicrob. Agents Chemother. 1996, 40(8):1926.
60. Kwong, Jennifer R. Stapp, Luis G. Tulloch, Prevalence And Mechanism Of Broad-Spectrum B-Lactam Resistance In *Enterobacteriaceae*: A Children's Hospital Experience. Justin,Antimicrob. Agents Chemother. 2008, 52(11):3909
61. Silke Polsfuss,† Guido V. Bloemberg,† Jacqueline Giger, Vera Meyer,Erik C,Practical Approach For Reliable Detection Of Ampc Beta-Lactamase-Producing *Enterobacteriaceae*, Journal Of Clinical Microbiology, Aug. 2011, P. 2798–2803 Vol. 49, No. 8
62. Azza A. Elsharkawy, Laila M. Elkady, Shymaa A. Mansour, Noura E. Esmaeel, Detection Of B-Lactasmases In Nosocomial Klebsiella Isolates, Journal Of Microbiology And Infectious Diseases 2013, 3 (1): 24-30.
63. Nevine Fam, Doaa Gamal, Manal El Said, Inas El Defrawy ,Prevalence Of Plasmid-Mediated Ampc Genes In Clinical Isolates Of

Enterobacteriaceae From Cairo, Egypt, British Microbiology Research Journal 3(4): 525-537, 2013.

64. Hai-Fei Yang, Jun Cheng, Li-Fen Hu, Ying Ye, Plasmid-Mediated Quinolone Resistance In Extended-Spectrum BLactamase- And Ampc B-Lactamase-Producing *Serratia Marcescens* InChina, Antimicrobial Agents And Chemotherapy P. 4529–4531.
65. Shilan S. Ahmad, Detection Of Esbl, Ampc, And Metallo Beta-Lactamase Mediated Resistance In Gram- Negative Bacteria Isolated From Women With Genital Tract Infection,European Scientific Journal March 2014 Edition Vol.10.
66. Yusuf,., Haruna Detection Of Ampc And Esbl Producers Among Enterobacteriaceae In A Tertiary Health Care In, Kano- Nigeria, International Journal Of Science And Technology ,Volume 3 No.4.
67. Vikas Manchanda* And Narendra P. Singh, Occurrence And Detection Of Ampc B-Lactamases Among Gram-Negative Clinical Isolates Using A Modified Three-Dimensional Test At Guru Tegh Bahadur Hospital, Delhi, India,Journal Of Antimicrobial Chemotherapy (2003) 51, 415–418
68. S.Singhal,T.Mathur,Evaluation Of Methods For Ampc B Lactamase In Gram Negative Clinical Isolates From Tertiary Care Hospitals,Indian Journal Of Medical Microbiology ,23(2):120-124.
69. V. Hemalatha, M. Padma, Uma Sekar, T. M. Vinodh* & A.S. Arunkumar* Detection Of Amp C Beta Lactamases Production In *Escherichia Coli*& *Klebsiella* By An Inhibitor Based Method, Indian J Med Res 126, September 2007, Pp 220-223.
70. Deepika Handa, Anita Pandey, Ashish Kumar Asthana, Evaluation Of Phenotypic Tests For The Detection Of Ampc Beta-Lactamase In Clinical Isolates Of *Escherichia Coli,*Indian Journal Of Pathology And Microbiology - 5 6 (2) .

71. Parveen R. Mohamudha, B.N. Harish, And S.C. Parija Molecular Description Of Plasmid-Mediated Ampc B-Lactamases Among Nosocomial Isolates Of Escherichia Coli & Klebsiella Pneumoniae From Six Different Hospitals In India, Indian J Med Res . 2012 January; 135(1): 114–119.
72. Mohamudha Parveen R.; Harish B.N.; Parija S.C, Ampc Beta Lactamases Among Gram Negative Clinical Isolates From A Tertiary Hospital, South India, Brazilian Journal Of Microbiology , Vol.41 No.3.
73. S. Peter-Getzlaff, S. Polsfuss, M. Poledica, M. Hombach, J. Giger, E. C. Bo Ttger, Detection Of Ampc Beta-Lactamase In *Escherichia Coli*: Comparison Of Three Phenotypic Confirmation Assays And Genetic Analysis, Journal Of Clinical Microbiology, Aug. 2011, P. 2924–2932 Vol. 49, No. 8.
74. S. Peter-Getzlaff, S. Polsfuss, M. Poledica,‡ M. Hombach, J. Giger, E. C., Detection Of Ampc Beta-Lactamase In *Escherichia Coli*: Comparison Of Three Phenotypic Confirmation Assays And Genetic Analysis,Journal Of Clinical Microbiology, Aug. 2011, P. 2924–2932 Vol. 49, No 8.
75. B. L. Chaudhary ,Dr. Anahita,V. Bhesania,Hodiwala detection Of Extended Spectrum B - Lactamase & Amp C In Clinical Isolates Of *Klebsiella* Species, Research paper,Volume -2,issue-8.
76. Rajesh Bareja, Shinu Pottathil, Rajesh Kumar Shah, Prem Singh Grover,Varsha Ashok, Simultaneous Detection Of Extended-Spectrum-B-Lactamase,Ampc-B-Lactamase And Metallo-B-Lactamase In Gram Negative Clinical Isolates On A Single Plate, Journal Of Dental And Medical Sciences ,Pp 74-77
77. Sridhar Rao Pn, Leelakrishna G Co-Production Of Esbl And Ampc Beta-Lactamases In Clinical Isolates Of Davanger Department Of

Microbiology,Jjm Medical College,Davangere 577004,Karnataka, India.

78. Smitha O. Bagali* And B.V. Peerapur, Detection Of Ampc Beta-Lactamases Among Escherichia Coli Isolates At A Tertiary Care Hospital In Karnataka,Al Ameen J Med Sc I 2013; 6(1) :85-87
79. Yong D , Park R, Yum Jh, Lee K, Choi Ec, Chong Y ,Further Modification Of The Hodge Test To Screen Ampc Beta-Lactamase(Cmy-1)-Producing Strains Of *Escherichia Coli* And *Klebsiella Pneumoniae*J Microbiol Methods. 2002 Nov;51(3):407-10.
80. Black Ja, Moland Es, Thomson Ks ,Ampc Disk Test For Detection Of Plasmid-Mediated Ampc Beta-Lactamases In Enterobacteriaceae Lacking Chromosomal Ampc Beta-Lactamases. J Clin Microbiol. 2005 Jul;43(7):3110-3.
81. Justin Ellem; Lee Thomas; Tom Olma; Jon Iredell, Comparison & Evaluation Of A Newly Developed Mast 4-Disc Test For The 4 Detection Of Plasmid-Mediated Ampc B-Lactamases,Centre For Infectious Diseases And Microbiology Laboratory Services, Icpmr, Westmead Hospital Nsw 2145 Australia.
82. Philip E. Coudron ,Inhibitor-Based Methods For Detection Of Plasmid-Mediated Ampc β Lactamases In *Klebsiella* Spp., *Escherichia Coli*, And *Proteus Mirabilis* Journal Of Clinical Microbiology, Aug. 2005, P. 4163–4167 Vol. 43, No. 8.
83. Noyal Mariya Joseph and Shanthi Mathias,Challenges in detection of AmpC β-lactamases among Enterobacteriaceae, Indian J Med Res. Jan 2013; 137(1): 216–217.
84. Hanna E. Sidjabat,1 David L. Paterson,1,2 Zubair A Clinical Features and Molecular Epidemiology of CMY-Type b-Lactamase–Producing *Escherichia coli,* Clinical Infectious Diseases 2009; 48:739–44.

85. Fred C. Tenover, Shannon L. Emery, Carol A. Spiegel,Plasmid mediated AmpC β -Lactamases in *Escherichia coli*, *Klebsiella* spp., and *Proteus* Species Can Potentially Improve Reporting of Cephalosporin Susceptibility Testing Results ,*J. Clin. Microbiol.* 2009, 47(2):294.
86. Smith L,Mather B,Evaluation of a Novel AmpC Detection Method to Detect All Types of AmpC Production, Microbiology Department, Whiston Hospital, Warrington Road, Prescot, L35 5DR.
87. Arindam Chakraborty, Prabha Adhikari, Shalini Shenoy, Vishwas Saralaya,Characterization of plasmid mediated *AmpC* producing *Escherichia coli* clinical isolates from a tertiary care hospital in South India,Indian journal of pathology and Microbiology-57(2)
88. R.K. Manojkumar Singh1, Nishith Kumar Pal2A Simplified method of Three Dimensional Technique For The Detection Of AmpC Beta-Lactamases ,Volume-4 No 3-1.
89. MaiM. Helmy, and Reham Wasfi,Phenotypic and Molecular Characterization of Plasmid Mediated AmpC β-Lactamases among *Escherichia coli*,*Klebsiella* spp., and *Proteus mirabilis* Isolated from Urinary Tract Infections in Egyptian Hospitals ,Volume 2014, 8 pages.
90. Nevine Fam, Doaa Gamal, Manal E Said, Detection of Plasmid-Mediated AmpC Beta-Lactamases in Clinically Significant Bacterial Isolates in a Research Institute Hospital in Egypt, Life Science Journal 2013;10(2)
91. Mohammad Soltan Dallal , Ailar Sabbaghi , Hedrosha Molla Aghamirzaeie, Prevalence of AmpC and SHV β-Lactamases in Clinical Isolates of *Escherichia coli* From Tehran Hospitals, *Jundishapur J Microbiol*. 2013;**6**(2):176-180.

10.ANNEXURE-1

1.Preparation of Media

Nutrient agar medium

Composition

Ingredients gram/liter

Peptone - 5.00

Sodium Chloride- 5.00

Meat Extract - 10

Agar -15.00

Twenty-eight grams of dehydrated nutrient agar medium was added to 1000 ml of cold distilled water in a flask and boiled to dissolve the medium completely. The medium was then sterilized in an autoclave at 1210C and 15 lbs pressure for 15 minutes. The sterile media were stored in a refrigerator at 40C for future use.

Blood agar medium

Composition

Ingredients gram/liter

Heart infusion 500.00

Tryptose 10.00

Sodium chloride 5.00

Agar 15.00

Forty grams of the dehydrated blood agar medium was suspended in 1000 ml cold distilled water in a flask and boiled to dissolve the medium completely. It was then sterilized by autoclaving at 1210C and 15 lbs pressure for 15 minutes. The autoclaved materials were allowed to cool to a temperature of 450C in a water bath. Defibrinated 5-10% sheep blood was then added to the medium aseptically and distributed to sterile petridishes. Sterile media was stored in refrigerator at 40C for future use.

Muller Hinton agar medium

Composition

Ingredients gram/liter

Beef dehytrated infusion- 300

Casein hydrolysate - 17.50

Starch agar -1.5

Agar - 10.00

Thirty-eight grams of dehydrated Mueller Hinton agar medium was suspended in 1000 ml cold distilled water and boiled to dissolve the medium completely. The solution was then sterilized by autoclaving at 1210C and 15 lbs pressure for 15 minutes. The autoclaved media was stored at 4^0C.

MacConkey agar medium

Composition

Ingredients gram/liter

Peptone 20.00

Lactose 100ml(10% aqueous solution)

NaCl 5.00

Na-taurocholate 5.00

Neutral Red 3.5ml(2% in 50%ethanol)

Agar 20.00

Fifty-two grams of dehydrated MacConkey agar medium was suspended in 1000 ml cold distilled water and boiled to dissolve the medium completely. The solution was then sterilized by autoclaving at 1210C and 15 lbs pressure for 15 minutes.

5. McFarland Standard (0.5):

Reagents:

Sulphuric acid,1%: To 100 ml of distilled water,1 ml of conc.sulphuric acid is added.Barium chloride, 1.175%: To 100 ml of distilled water, 1.175gm of barium chloride is added and mixed well.

To prepare McFarland 0.5 standards:

To 85 ml of 1% conc.sulphuric acid, 0.5 ml of Barium chloride is added in a flask while constantly swirling the flask. Bring to 100 ml with 1% conc.sulphuric acid. Aliquot in test tubes and cap tubes tightly. Store in the dark at room temperature for 3 months or longer.

Physiological saline solution

To make 1000 ml of Physiological saline solution, 0.9 gm of chemically pure sodium chloride was added in 1000 ml of distilled water in a sterile conical flask. The solution was then sterilized by autoclave at 121^0C maintaing a pressure of 15 lbs per square inch for 15 minutes. After sterilization, the sterile physiological saline solution was cooled and stored in a refrigerator at 4^0C for future use.

ANNEXURE -2

PROFORMA

Name :

Age :

Sex :

OP/IP No :

Lab No :

Ward :

Complaints :

Clinical diagnosis :

Nature of Specimen : Urine,Pus

Duration of hospital stay :

Antibiotics administered :

Investigation :

Biochemical tests :Indole,Citrate,Urease,Triple sugar iron, Catalase,Oxidase,Disc Diffusion test with Cefoxitin

Modified hodge test

AmpC Disc Test

PCR

Antibiogram :

Ceftriaxone,Ceftazidime

Ceftazidime with Clavulanicacid,Cefoxitin ,

Norfloxacin,Nitrofurantoin,Amikacin,Gentamicin

Imipenem

MASTER CHART

S.no	Lab no	Age	sex	Diagnosis	HOSP. STAY DURATION	Samples	Ceftazidime(30µg)	Ceftriaxone(30µg)	Imipenem(10µg)	ceftazidime+clavulanic acid	Amikacin(30µg)	Nitrofurantoin	Gentamycin	Norfloxacin(10µg)	cefoxitin	MHT	AmpC disc test	PCR	MISCELLANEOUS (mortality, Ab. admm.)	E.COLI	K.P	K.O	Ward
1	109	35	M	Ob.uro	20	U	R	R	S	R	S	R	R	R	R	p	p	P	MERO		P		uro
2	136	26	M	UTI	15	U	R	R	S	R	R	R	S	R	R	P	P	P	ceftriaxone		P		Medicine
3	1087	57	F	SSI	15	P	R	R	S	R	S	S	R	S	R	P	P	P	ceftriaxone	P			childmed
4	134	24	F	UTI	20	U	R	R	S	R	R	R	R	S	R	P	P	p	Ceftriaxone		P		labour ward
5	7641	53	M	UTI	15	U	R	R	S	S	S	R	S	R	R	P	P	P	ceftriaxone	P			Medicine
6	1531	58	F	UTI	17	U	R	R	S	R	S	R	R	R	R	N	P	p	ceftriaxone	P			medicine
7	7939	65	F	OB.uro	20	U	R	R	S	R	S	R	R	R	R	P	P	P	Ceftriaxone	P			uro
8	8140	70	F	UTI	10	U	R	R	S	R	R	S	R	R	R	N	N	N	NOR	P			Medicine
9	8160	65	M	UTI	10	U	R	R	S	R	R	R	R	S	R	N	N	N	AMI		P		Medicine
10	8142	69	M	UTI	15	U	R	R	S	R	R	S	R	R	R	N	P	P	AMI		P		medicine
11	1085	66	M	UTI	15	U	R	R	S	R	R	S	R	S	R	N	P	p	NOR	P			Medicine
12	502	65	M	Ob uro	15	U	R	R	S	R	S	S	S	S	R	N	P	N	NOR			p	uro
13	733	57	M	UTI	15	U	R	R	S	R	S	S	R	R	R	P	P	P	ceftriaxone	P			Medicine
14	3	1	F	UTI	15	U	R	R	S	R	S	R	R	R	R	N	p	P	MERO	P			CHildmed
15	1310	52	F	UTI	18	U	R	R	S	R	R	S	R	R	R	N	p	P	CEFTZ	P			Medicine
16	1202	63	F	Ob.uro	15	U	R	R	S	R	R	R	R	S	R	N	p	P	NOR		P		Medicine
17	1228	80	F	UTI	17	U	R	R	S	R	R	R	S	R	R	P	P	p	ceftriaxone	P			SURGERY
18	1046	49	M	UTI	7	U	R	R	S	R	S	R	R	S	R	N	N	N	NOR	P			Medicine
19	8540	75	M	UTI	10	U	R	R	S	R	R	R	R	R	R	P	p	P	Ceftriaxone		P		Medicine
20	8382	23	F	UTI	15	U	R	R	S	R	S	S	R	R	R	P	N	P	CEFTZ		P		Labour ward
21	100	55	F	UTI	7	U	R	R	S	R	S	R	S	S	R	N	N	N	NOR			P	Medicine
22	101	22	F	UTI	7	U	R	R	S	R	S	R	S	R	R	N	N	N	MERO	P			Labour ward
23	77	38	M	SSI	20	P	R	R	S	R	S	S	R	R	R	N	N	N	AMI	P			SURGERY
24	18	2	M	UTI	7	U	R	R	S	R	S	S	R	S	R	N	N	N	NOR		P		childmed
25	111	65	F	UTI	7	U	R	R	S	R	S	S	S	S	R	N	N	N	AMI		P		Medicine

26	463	58	M	UTI	5	U	R	R	S	R	S	S	R	S	R	N	N	N	AMI	P			Medicine
27	131	47	F	UTI	7	U	R	R	S	R	S	S	S	S	R	N	N	N	AMI			P	uro
28	1131	36	M	UTI	10	U	R	R	S	R	S	R	R	S	R	P	N	p	ceftriaxone		P		Medicine
29	1832	2	F	UTI	7	U	R	R	S	R	S	R	R	R	R	P	N	p	ceftriaxone	P			childmed
30	7989	70	F	UTI	5	U	R	R	S	R	S	R	R	R	R	P	N	p	ceftriaxone		P		uro
31	8153	63	M	OB,uro	7	U	R	R	S	R	S	S	S	S	R	P	N	N	NOR	P			uro
32	7950	67	M	UTI	15	U	R	R	S	R	S	R	S	R	R	P	p	P	ceftriaxone	p			uro
33	1263	60	F	UTI	15	U	R	R	S	R	R	R	R	R	R	P	p	P	ceftriaxone		p		labour ward
34	5802	28	M	UTI	7	U	R	R	S	R	R	S	R	R	R	N	N	N	AMI		P		Medicine
35	31	43	M	UTI	7	U	R	R	S	R	S	S	S	S	R	N	N	N	NOR	P			Medicine
36	46	55	F	Ob uro	5	U	R	R	S	R	S	S	S	S	R	N	N	N	AMI			P	SURGERY
37	1272	37	F	UTI	15	U	R	R	S	R	S	S	R	S	R	N	N	N	ceftriaxone	P			SURGERY
38	1534	75	M	UTI	10	U	R	R	S	R	S	R	R	S	R	N	N	N	CEFTZ	P			SURGERY
39	7927	67	M	.OB.uro	7	U	R	R	S	R	R	S	R	R	R	N	N	N	AMI	P			uro
40	8002	60	F	UTI	7	U	R	R	S	R	S	R	R	S	R	N	N	N	ceftriaxone		p		Medicine
41	1140	37	M	SSI	20	P	R	R	S	R	S	S	R	R	R	N	N	N	ceftriaxone		p		SURGERY
42	8143	12	F	wound inf	10	P	R	R	S	R	S	S	R	S	R	N	N	N	AMI		P		childmed
43	717	50	M	UTI	7	U	R	R	S	R	S	R	R	S	R	N	N	N	AMI	P			Medicine
44	64	55	F	Ob.uro	15	U	R	R	S	R	S	S	S	R	R	N	N	N	CEFTZ			P	SURGERY
45	57	50	F	UTI	7	U	R	R	S	R	S	R	R	S	R	N	N	N	ceftriaxone		p		Medicine
46	2073	55	M	UTI	15	U	R	R	S	R	S	S	S	R	R	N	N	N	AMI		P		uro
47	1939	66	F	wound inf	10	p	R	R	S	R	S	R	S	S	R	N	N	N	AMI		P		SURGERY
48	1950	22	F	UTI	10	U	R	R	S	R	S	S	R	R	R	N	N	N	AMI		P		labour ward
49	2074	65	M	UTI	7	U	R	R	S	R	S	R	R	S	R	N	N	N	AMI			P	uro
50	1910	22	F	UTI	10	U	R	R	S	R	S	R	R	R	R	N	N	N	NOR	P			Medicine