STUDY ON EXTENDED SPECTRUM BETALACTAMASE (ESBL) PRODUCING GRAM NEGATIVE BACILLI ISOLATED FROM INTENSIVE CARE UNITS

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SEPTEMBER 2006

DECLARATION

I solemnly declare that this dissertation "**STUDY ON EXTENDED SPECTRUM BETALACTAMASE (ESBL) PRODUCING GRAM NEGATIVE BACILLI ISOLATED FROM INTENSIVE CARE UNITS"** is the bonafide work done by me at the Department of Microbiology, Govt. Stanley Medical College and Hospital, Chennai, under the guidance and supervision of **Prof. Dr. P. R. THENMOZHI VALLI, M.D.,** Professor of Microbiology, Govt. Stanley Medical College, Chennai-600 001.

This dissertation is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the University regulations for the award of degree of M.D. Branch IV Microbiology examinations to be held in September 2006.

Place : Chennai.

Date :

Dr. A. VASUMATHI

CERTIFICATE

This is to certify that this dissertation entitled "**STUDY ON EXTENDED SPECTRUM BETALACTAMASE (ESBL) PRODUCING GRAM NEGATIVE BACILLI ISOLATED FROM INTENSIVE CARE UNITS"** is the bonafide original work done by **Dr. A. VASUMATHI,** Post graduate in Microbiology, under my overall supervision and guidance in the department of Microbiology, Stanley Medical College, Chennai, in partial fulfillment of the regulations of The Tamil Nadu Dr. M.G.R. Medical University for the award of **M.D Degree in Microbiology (Branch IV).**

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CONTENTS

INTRODUCTION

Incidence of bacterial infection from Hospitals accounts for 5-10% in most developed nations while in India one in four patients admitted into hospitals acquire bacterial infection[59]. Around 30% of patients in Intensive Care Units were contracting infections during their stay in hospital[3], which leads to many complications in them.

Since 1940, Penicillin- the first beta lactam antibiotic remained the antibiotic of choice for many years. Bacteria were so inventive that they developed many mechanisms to escape the action of antibiotics and became resistant. Main mechanism was by the production of β-lactamases. The first plasmid mediated β-lactamase was TEM – 1 which was described in 1960s. Antibiotic resistance was high among organisms isolated from Intensive Care Units[3].

Over the last 25 years, many new β-lactam antibiotics have been developed, that were specifically designed to be resistant to the hydrolytic action of β-lactamases. However with each new class of antibiotic used to treat patients, because of inappropriate and extensive use new β-lactamases emerged that caused resistance to that particular group of drug. This was due to the continuous mutation of the enzymes.[22] Major effort to overcome this lead to introduction of the new class of β-lactam antibiotics, the third generation cephalosporin like

Cefotaxime, which became widely used for the treatment of serious infection due to gram negative bacilli, in 1980s.[49] Because of their expanded spectrum of action against bacteria, they were also called as Expanded Spectrum Antibiotics. Due to extensive use of third generation antibiotics, bacteria soon developed resistance, by means of a new β- lactamase. Because of their increased spectrum of activity against most of the β-lactam antibiotics including third generation cephalosporins, these enzymes were called Extended Spectrum Beta Lactamases (ESBL).

First ESBL was identified in Klebsiella pneumoniae in Germany in 1983, [49] it was found to be a derivative of SHV type Beta Lactamase. They were encoded by transferable plasmids and hence dissemination of resistance to other members of gram negative bacilli occurred.

Major factor contributing to the production of ESBL is prolonged stay in Intensive Care Units.[38]

The incidence and type of ESBL vary with geographical locations.[22]

Though Extended spectrum beta lactamases are responsible for nosocomial outbreaks of infections like urinary tract infections, pneumonia, septicemia etc, the real incidence and prevalence of ESBL producing bacteria at most hospitals remain unknown.[15] Failure to

control outbreaks has resulted in the appearance of new mutant type Extended spectrum beta lactamase in the same institution and the same patient source.

The appearance of eight different Extended Spectrum Beta Lactamases (TEM-3, TEM-5, CAZ-2, CAI-3, CAI-6, CAI-7, SHV-4 and SHV-5) at Clermont-Ferrand hospital, since the first one in 1984 is a classic example.[37]

Hence the present study was undertaken to study the Extended Spectrum Beta Lactamase producing gram negative bacilli isolated from Intensive Care Units of Government Stanley Hospital, Chennai.

REVIEW OF LITERATURE

HISTORY:

Antibiotic era started with discovery of penicillin by Alexander Flemming in 1928.[23] Use of Penicillin started in 1941. Emergence of resistance to β-lactam antibiotic began even before the introduction of penicillin.[18] First β-Lactamase was found in E-coli prior to introduction of penicillin. Next is the emergence of penicillin resistance in staphylococcus aureus due to plasmid encoded β-lactamase.

First plasmid mediated β-lactamase in gram negative organisms-TEM-1 was described in early 1960's. It was first isolated in Escherichia coli from a patient Temoniera in Greece. It spread to other genera soon.

Another common plasmid mediated β-lactamase found in Klebsiella pneumoniae and Escherichia coli are SHV-1 (for Sulph Hydryl Variable).

Over the last 20 years many new β-lactam antibiotics have been developed which were resistant to hydrolytic action of β-lactamases but because of over usage, these antibiotics also became resistant.

To overcome it, around 1980 3rd generation cephalosporins, also called broad spectrum cephalosporins were introduced. They were very resistant to already existing β-lactamases. Because of their extensive

use, they also became resistant. In 1983 in Germany, isolates of *Klebsiella pneumoniae* and other enterobacteriaceae were found to produce a plasmid-determined β-lactamase that hydrolyzed cefotaxime, as well as other newer cephalosporins. This new β-lactamase, called SHV-2 was derived from a mutation in the well-known SHV-1 β-lactamase commonly found in *Klebsiella,*[37] since they lead to resistance of extended spectrum cephalosporins they are called extended spectrum β-lactamases.

β- Lactam antibiotics:[23]

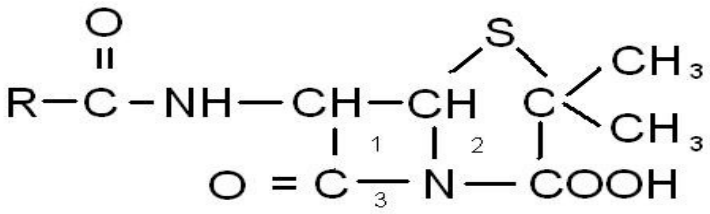
They are antibiotics having a β-lactam ring. It comprises of

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

All the β-lactam antibiotics have β-lactam ring in common which is made of 3 carbon atoms and one nitrogen atom. The other rings vary in respect of each group of antibiotics.

Penicillins:

The structure of Penicillin is



1. β-lactam ring
2. Thiazolidone ring
3. site where β-lactamase will act

Types of Penicillins

I. Natural Penicillins eg., Penicillin G

II. Semi synthetic Penicillins

1. Acid resistant Penicillins eg., Penicillin V
2. Penicillinase resistant Penicillins eg., Methicillin, Oxacillin, Cloxacillin
3. Extended spectrum Penicillins.
    a. Amino penicillins eg., Ampicillin, Amoxycillin
    b. Carboxy penicillins e.g., Carbenicillin
    c. Ureido penicillins e.g., Piperacillin, mezlocillin

Cephalosporins:

Have β- lactam ring attached to dihyro thiazine ring. By addition of different side chains to dihyro thiazine ring a large number of semi synthetic compounds have been produced. They are divided into four generations

I – Generation: Cephalothin, Cephalexin, Cefadroxil, Cefazolin.

II – Generation: Cefuroxime, Cefuroxime Axetil, Cefaclor, Cefoxitin.

III – Generation: Cefotaxim, Ceftazidime, Cefixim, Cefoperazone.

IV – Generation: Cefepime, Cefpirome.

Monobactams:

Monobactams have a monocyclic β-lactam ring and are resistant to β-lactamase. They are active against gram negative bacteria but not against gram positive bacteria. e.g., Aztreonam.

Carbapenems:

These drugs are structurally related to the β-lactam antibiotics. They are extremely potent and have very broad spectrum of activity. e.g., Imipenem, Carbapenem. They are indicated in infections due to organisms resistant to other drugs.[28]

Mechanism of Action of β-lactam antibiotics:

β-lactam antibiotics act by inhibiting cell wall synthesis of bacteria.

Bacteria synthesise UDP – N – Acetyl muramic acid pentapeptide and UDP – N – Acetyl glucosamine. Peptidoglycan residues are linked together and UDP is split off. Final step is cleavage of the terminal D-alanine of the peptide chains by Tran's peptidases and cross linking between peptide chains of the neighbouring strands. β-lactam antibiotics inhibits trans peptidases so that cross linking is not formed.[23]

Mechanism of resistance to β-lactam antibiotics:-

1) Enzymatic inhibition: plasmid mediated e.g., β-lactamase
2) Membrane impermeability - both chromosome and plasmid mediated
3) Alteration of target protein e.g., Penicillin binding protein.

The β- Lactamases:-

This is a heterogeneous group of penicillin recognizing proteins. They are members of a super family of active site serine proteases. They act by cleaving an amide bond of beta lactam ring to form an acyl-enzyme complex. Any β-lactam antibiotic may be inactivated by these enzymes. There are about > 170 enzymes of this kind.[38]

Classification of beta lactamases :-

1. Early classification scheme was developed by Richmond and Sykes based on substrate profile and the location of genes encoding the β-lactamases.
2. Modern scheme based on molecular structure was proposed by Ambler.

Most important is class A which is serine proteases that have either preference for penicillin or) broad spectrum activities.

They are found either on chromosomes (or) plasmids and are easily transferable from one bacteria to another.

They may be produced constitutively and may be induced. In this group are the staphylococcus and some Gram negative bacilli.

Class C: Primarily cephalosporinases, either constitutive (or) inducible. Found on chromosomes of Gram Negative Bacilli.

Class B & D are less important.

CLASSIFICATION OF β-LACTAMASES

Class	Examples	Location	Bacteria
Gram positive β- lactamases			
Enterococcal Staphylococcal Streptococcal	Serological types A-D	Plasmids Plasmid Plasmid	Staphylococcus aureus, Staphylococcus epidermidis Enterococcus faecalis Streptococcus uberis
Gram Negative β- Lactamase			
Class A	TEM-1&2	Plasmid; broad spectrum	Enterobacteriaceae, N.gonorrhoea, H.influenzae, V. cholerae
	SHV – 1	Plasmid; Broad spectrum	Enterobacteriaceae, also found in chromosomes of Klebsiella pneumoniae.
	OXA-1	Plasmid; oxacillinases	Escherichia coli
	PSE-1	Plasmid; carbenicillinase in pseudomonas	Enterobacteriaceae, most common type in Pseudomonas aeruginosa.
	BRO – 1	Plasmid; carbenicillinase base	Branhamella, moraxella
Class B		Cephalosporinase	Bacillus cereus
Class C	Broad spectrum	Chromosome	Klebsiella pneumoniae, Proteus vulgaris, Klebsiella oxytoca, K. aerogenes K-1
	Cephalosporinase	Chromosome	Escherichia coli, Bacteroides fragilis
	Inducible	Chromosome	Enterobacter aerogenes, E.cloacae, Providencia rettgeri, Pseudomonas. aeruginosa, Serratia marcescens, Acinetobacter baumannii
	Carbapenamase (Imipenem)	Chromosome	Xanthomonas maltophilia, B. fragilis, Serratia marcescens, Enterobacter cloacae.
Class D	Oxacillinase	-	-

Recent Classification of β-lactamases is by Bush-Jacoby – Medeiros scheme according to substrate profile and inhibition by clavulanic acid.[38]

The Bush Jacoby – Medeiros functional classification scheme for β-lactamames

Group	Enzyme Type	Inhibition by Clavulanate	Mole-cular Class	No. of Enzymes	Example
1	Cephalosporinase	No	C	53	E.cloacae P99,M
2a	Penicillinase	Yes	A	20	Staphylococcus aureus, Staphylococcus albus.
2b	Broad spectrum	Yes	A	16	TEM-1, SHV-1
2be	Extended spectrum	Yes	A	38	TEM-3, SHV-2, K.
2br	Inhibitor resistant	Diminished	A	9	TEM-30, TRC-1
2c	Carbenicillinase	Yes	A	15	PSE-1, CARB-3, BR
2d	Cloxacillinase	Yes	D or A	18	OXA-1, PSE-2, Str. Cacaoi
2e	Cephalosporinase	Yes	A	19	Proteus vulgaris, B. fragilis Cep. A
2f	Carbapenemase	Yes	A	3	E.cloacae IMI-1 N
3	Metalloenzyme	No	B	15	Xanthomonas maltophilia LI
4	Penicillinase	N		7	Pseudomonas cepacia.

Some β-lactamases like that produced by Staphylococcus aureus are stable over several decades. These enzymes have a rather narrow spectrum of activity aiming at penicillin molecule.

The broad spectrum, plasmid mediated β-lactamases of Gram negative bacilli (GNB) such as TEM-1 and SHV-1 were stable for many years.

From 1980, a series of enzymatic variants appeared that had a broadened spectrum of activity against the newly developed β-lactam antibiotics. These ESBLs were first found in Europe most commonly in Klebsiella species, less commonly in Escherichia coli. The number of enzymes continues to increase.

The new enzymes TEM-1 and SHV-1 are located on plasmids. But they might have been derived originally from a chromosomal enzyme. Many of the new β-lactamases differ from each other only in single aminoacid substitution but the changes have profound implications for clinical management of infectious diseases.

Key Dates Showing Emergence of B-Lactamases

Year	Enzyme	Organism	Place
1944	Penicillinase	Staphylococcus aureus	-
1963	TEM-1	Escherichia coli	Athens
1974	SHV-1	Escherichia coli	Switzerland
1978	OXA-10	Pseudomonas. aeruginosa	-
1982	SME-1	Serratia marcescens	London
1984	IMI-1	E. cloacae	California
1988	Metallo β-lactamase	Pseudomonas aeruginosa	Japan
1989	Inhibitor – resistant Penicillinase	Escherichia coli, Klebsiella pneumoniae	France, Spain
1990	NMcA	E. cloacae	Paris
1991	OXA-11 OXA-14	Pseudomonas aeruginosa	Turkey
1991	PER-1	Pseudomonas aeruginosa, Salmonella typhimurium	Turkey
1992	MEN-1	Escherichia coli, K. pneumoniae	France
1994	TOHO-1	Escherichia coli	Japan
1996	PER-2	Escherichia coli, K. pneumoniae, S. typhimurium, P. mirabilis	Germany
1997	VEB-1	Escherichia coli	Germany

Detection of β-lactamases [36]

Detection of β-lactamases was done by biochemical tests for the enzymes. This was by measuring penicilloic acids which was produced when β-lactamases hydrolyse benzyl penicillins. The acid production was detected by

1. Measuring the change in pH of an indicator dye (acidometric method)
2. By exploiting the ability of penicilloic acid to reduce iodine and reverse the formation of the blue colour when iodine complexes with starch. (Iodometric method)
3. Chromogenic cephalosporin method – usually nitrocephin was used. Nitrocephin was normally yellow but when the β-lactam ring was hydrolysed it turns red.

β -lactamase inhibitors :[16]

These compounds resemble β-lactam antibiotics. They can bind to β-lactam antibiotics either reversibly or irreversibly protecting the antibiotics from destruction. They serve as **suicide bombers** utilizing all available enzymes. These compounds have weak antibacterial activity but are potent inhibitors of many plasmid-encoded and some chromosomal β-lactamases.

Three important β-lactamase inhibitors are:

1) Clavulanic acid
2) Sulbactam
3) Tazobactam

Clavulanic acid show only low level of antibacterial action but when combined with β-lactam antibiotics inhibition of bacteria which are otherwise resistant to β-lactam antibiotics was noted.

Sulbactam has broader spectrum of inhibition but less potent.

Tazobactam is as potent as clavulanic acid

EXTENDED SPECTRUM BETA LACTAMASES :

Definition:

Enzymes capable of hydrolyzing major β – lactam antibiotics including third generation cephalosporins are called as extended spectrum beta lactamases.

CHARACTERISTICS OF ESBLs: [16]

1) They are mostly class- A cephalosporinases carried on plasmids
2) They are more common in Klebsiella species followed by Escherichia coli
3) Described first in Germany and France.
4) All enzymes active against Cephalothin.

5) Imipenem and Cefoxitin not hydrolysed.
6) Comparative activity against Cefotaxime and Ceftazidine varies with enzymes.
7) Some enzymes active against Astreonam.
8) Resistance may not be detected by standard susceptibility tests.
9) Inhibition of activity by β-lactamase inhibitors can be demonstrated.

Majority of ESBLs contain a serine at the active site and belong to Ambler's Molecule. Class A enzymes are characterized by an active site serine, a molecular mass of 29,000 Dalton and hydrolysis of penicillin.

ESBLs contain a number of mutations that allow them to hydrolyse ESBL antibiotics. While TEM and SHV type ESBLs retain their ability to hydrolyse penicillin, but they are not catalytically as efficient as the parent enzymes.

ESBLs are not active against cephamycin and most strains expressing ESBLs are susceptible to Cefoxitin and Cefotetan. But it has been reported that ESBL producing strains can become resistant to cephamycin due to the loss of an outer membrane porin protein.

Major risk factors for ESBL production

1) Prolonged stay in ICU

2) Long term use of antibiotics

3) Nursing home residency

4) Severe illness

5) High rate use of ceftazidime and other Third Generation Cephalosporins

6) Use of lifelines and catheters

Types of ESBLs:-

Most ESBLs are derivatives of TEM & SHV. Now > 90 TEM type and > 25 SHV types are there. A few point mutations at selected loci with in the gene give rise to the extended spectrum phenotype.

TEM & SHV type ESBLs are most often found in Escherichia coli and Klebsiella. pneumoniae but also found in Proteus species, Providentia species and other genera of Enterobacteriaceae.

TEM:-

TEM-2 was the 1st derivative of TEM which had a single aminoacid substitution from the original β-lactamase. This did not change the substrate profile. TEM-3 reported in 1989 was the first TEM type β-lactamase that displayed the ESBL phenotype.

Aminoacid residues important for ESBL production are

Glutamate → Lysine at position 104

Arginine →Serine (or) histidine at position 164

Glycine →Serine at Position 238

Glutamate →Lysine at position 240

TEM type is often found in Escherichia coli and Klebsiella. Pneumoniae, but also reported in other members of Enterobacteriaceae and some other gram negative bacilli.

TEM- 42 β-lactamase was found in p. aeruginosa

TEM-17 β-lactamase reported in a blood culture isolate of Capnocytophaga ochracia.

Characteristics of TEM-type β-lactamases

PI	**Enzyme type**		
	Broad spectrum	**ESBL**	**Inhibitor Resistant**
5.2		TEM-12, TEM-55, TEM-57, TEM-58	TEM-30, TEM-31, TEM-35, TEM-36, TEM-37, TEM-38, TEM-41, TEM-45, TEM-51, TEM-73, TEM-74
5.3		TEM-25	
5.4	TEM-1	TEM-7, TEM-19, TEM-20, TEM-65	TEM-32, TEM-33, TEM-34, TEM-39, TEM-40, TEM-44
5.42		TEM-29	

5.55		TEM-5, TEM-17	
5.59		TEM-9	
5.6	TEM-2	TEM-10, TEM-11, TEM-13, TEM-26, TEM-63, TEM-50	TEM-50, TEM-59
5.7		TEM-68	TEM-68
5.8		TEM-42	
5.9		TEM-4, TEM-6, TEM-8, TEM-27, TEM-72	
6.0		TEM-15, TEM-47, TEM-48, TEM-49, TEM-52, TEM-66, TEM-92	
6.1		TEM-28, TEM-43	
6.3		TEM-3, TEM-16, TEM-21, TEM-22	
6.4		TEM-56, TEM-60	
6.5		TEM-24, TEM-46, TEM-61	
Not determined		TEM-14, TEM-53, TEM-54	TEM-76, TEM-77, TEM-78, TEM-79, TEM-81, TEM-82, TEM-83, TEM-84

SHV :-

SHV β- lactamases are most commonly found in K. pneumoniae and responsible for plasmid mediated resistance.

There are only few derivatives of SHV-1 most of them have substitution of serine for glycine at position 238. Lysine for glutamate at position 240.

Serine residues responsible for efficient hydrolysis of ceftazidime and lysine residue critical for the efficient hydrolysis of cefotaxime.

Characteristics of SHV-type β-lactamases

pI	Enzyme type		
	Broad spectrum	**ESBL**	**Inhibitor resistant**
7.0	OHIO-1, LEN-1	SHV-3, SHV-14	
7.5		SHV-24	
7.6	SHV-1, SHV-11	SHV-2, SHV-2a, SHV-6, SHV-8, SHV-13, SHV-19, SHV-20,SHV-21, SHV-22	
7.8		SHV-4, SHV-7, SHV-18	
8.2		SHV-5, SHV-9, SHV-12	SHV-10

CTX-M :-

CTX M are plasmid mediated ESBLS which preferentially hydrolyse cefotaxime.

Found mainly in salmonella. enterica serovar typhimurium and Escherichia coli. But also found in other enterobacteriaceae.

It is suggested that it has originated from Amp-C β-lactamase enzymes, which are chromosomal mediated.

Major types are →CTX M1, CTX-M3

CTx-M2 Type includes CTX-M2, CTX-M4

CTx-M5, CTX M-6, CTX M-7, and Toho-1, Toho-2, and CTX M-8.

CTX-M types hydrolyse cefotaxime than ceftazidime.

Serine residue at position 237 plays an important role in their ESBL activity.

Another unique feature is that they are better inhibited by β-lactamase inhibitor tazobactam than by sulbactam and clavulanate.

Mostly expressed by salmonella.enterica serovar typhi

Characteristics of CTX-M-type ESBLs

β-Lactamase	Alternative name	pI	Country of origin	Bacterial species
CTX-M-1	MEN-1	8.9	Germany, Italy	*E. coli*
CTX-M-2		7.9	Argentina	*S. enterica*
CTX-M-3		8.4	Poland	*C. freundii, E. coli*
CTX-M-4		8.4	Russia	*S. enterica*
CTX-M-5	CTX-M-3	8.8	Latvia	*S. enterica*
CTX-M-6		8.4	Greece	*S. enterica*
CTX-M-7	CTX-M-5	8.4	Greece	*S. enterica*
CTX-M-8		7.6	Brazil	*P. mirabilis, E. cloacae, E. aerogenes, C. amalonaticus*
CTX-M-9		8.0	Spain	*E. coli*
CTX-M-10		8.1	Spain	*E. coli*
Toho-1		7.8	Japan	*E. coli*
Toho-2		7.7	Japan	*E. coli*

OXA

Belongs to Class d and functional group 2d. Characteristics feature is that they are able to hydrolyse ampicilin, cephalosporin, cloxacillin, oxacillin and are poorly inhibited by clavulanic acid.

OXA-type ESBLs are found mainly in pseudomonas aeruginosa.

Characteristics of OXA-type ESBLs

β-lactamase	Derivation	pI	Amino acid substitutions vs. OXA-10	Country of origin	Bacterial species
OXA-11	OXA-10	6.4	Asn143Ser, Gly157Asp	Turkey	*Pseudomonas aeruginosa*
OXA-13	OXA-10	8.0	Ile10Thr, Gly20Ser, Asp55N, Asn73Ser, Thr107Ser, Tyr174Phe, Glu229Gly, Ser245Asn, Glu259Ala	France	*Pseudomonas aeruginosa*
OXA-14	OXA-10	6.2	Gly157Asp	Turkey	*Pseudomonas aeruginosa*
OXA-15	OXA-2	8.7, 8.9 dou blet	NA	Turkey	*Pseudomonas aeruginosa*
OXA-16	OXA-10	6.2	Ala124Thr, Gly157Asp	Turkey	*Pseudomonas aeruginosa*
OXA-17	OXA-10	6.1	Asn73Ser	Turkey	*Pseudomonas aeruginosa*
OXA-18	OXA-9, OXA-12	5.5	NA	France	*Pseudomonas aeruginosa*
OXA-19	OXA-10	7.6	Ile10Thr, Gly20Ser, Asp55Asn, Thr107Ser, Gly157Asp,Tyr174Phe, Glu229Gly,Ser245Asn,Glu259Ala	France	*Pseudomonas aeruginosa*
OXA-28	OXA-10	7.6	Ile10Thr, ly20Ser, Thr107Ser, Trp154Gly, Gly157Asp,Tyr174Phe, Glu229Gly,Ser245Asn, Glu259Ala	France	*Pseudomonas aeruginosa*

OTHER ESBLs:-

1) PER-1 β-lactamase 18f isolated from Pseudomonas aeruginosa in Turkey.

2) PER-2 found in Salmonella. enterica from Argentina.

3) VEB-1 1st found in E-coli isolate from Vietnam.

4) CME-1

5) TLA-1 identified in Escherichia coli isolate from Mexico

6) SFO-1 cannot hydrolyse cephamycin and inhibited by clavulanic acid.

7) GES-1 resembles carbenicillinase

Characteristics of novel, unrelated ESBLs

β-Lactamase	Closest relative	pI	Preferred substrate	Country of origin	Bacterial species
BES-1	Penicllinase from *Yersinia enterocolitica*	7.5	CTX, CAZ, ATM	Brazil	*Serratia marcescens*
FEC-1		8.2	CTX	Japan	*Escherichia coli*
GES-1	Penicillinase from *P. mirabilis*	5.8	CAZ	French Guiana	*Klebsiella pneumoniae*
CME-1	VEB-1	>9.0	CAZ	Isolated from reference strain	*Chryseobacterium meningosepticum*
PER-1	PER-2	5.4	CAZ	France	*Pseudomonas aeruginosa*
PER-2	PER-1	5.4	CAZ	Argentina	*S. enterica* serovar Typhimurium
SFO-1	AmpA from *S. fonticola*	7.3	CTX	Japan	*E. cloacae*
TLA-1	CME-1	9.0	CAZ, CTX, ATM	Mexico	*Escherichia coli*
VEB-1	PER-1, PER-2	5.35	CAZ, ATM	Vietnam/ Thailand	*Escherichia coli*

DETECTION METHODS

1) Double-disk approximation test of Tarlier et al:

Organism was swabbed onto a Muller – Hinton agar plate. An antibiotic disk containing one of the oxyimino β-lactam antibiotics placed 30mm (centre to centre) from the amoxicillin – clavulanic acid disk. Enhancement of zone of inhibition of the oxyimino β-lactam caused by synergy of clavulanate present in Amoxy-clav disk was a positive result. This test is more reliable. Sensitivity is increased by reducing the distance between the disks to 20mm. Addition of clavulanate (4µg/ml) to the MHA increases the sensitivity.

Disc strength: - 3rd generation cephalosporins 30µg/ disc,

Coamoxyclav - 20µg amoxicillin + 10µg clavulanic acid.[36]

Ceftazidime is the best sentinel antibiotic for suspecting resistance to 3rd generation cephaloporins.[16]

2) Three Dimensional Test:

Advantage is simultaneous determination of antibiotic susceptibility and β-lactamase substrate profile.

2 types of inoculam are prepared.

1) Inoculum-1: contains $10^9 – 10^{10}$ CFU/ml of active ESBL producers.

2)Inoculum-2: Contains 0.5 Mc Farland Std. (150 million organisms/ml)

Plate was inoculated as for disc diffusion procedure with inoculam-2. A circular slit was cut on the agar 4mm inside the position at which the antibiotic discs was placed and 10^9-10^{10} inoculum was poured into it. Distortion or discontinuity in the circular inhibition zone is interpreted as positive for ESBL production.

3) Inhibitor Potentiated Disc Diffusion Test :

Cephalosporin disc is placed on clavulanate containing and without clavulanate MHA plates. More than 10mm increase in the zone of inhibition of the clavulanate containing MHA plate indicates ESBL production.

Disk Approximation test : Cefoxitin (inducer) disc in placed at a distance of 2.5cm from cephalosporin disc, production of inducible β-lactamase was indicated by flattening of the zone of inhibition of cephalosporin disk towards inducer disk by ≥ 1mm indicates ESBL production.

4) MIC Reduction test:

An eight fold reduction in the MIC of 3rd generation cephalosporins in the presence of clavulanic acid indicates production of ESBL.

5) Vitek ESBL test :

Four wells containing cards are inoculated. A predetermined reduction in growth of cephalosporin well containing clavulanic acid compared with level of growth in well with cephalosporin alone indicates the presence of ESBL.

6) E-test :

E test ESBL strips have 2 gradients, on one end ceftazidime and on the opposite end ceftazidime plus clavulanic acid. MIC is the point of intersection of the inhibition ellipse with the E-test strip edge.

Ratio of ceftazidime MIC and ceftazidime clavulanic acid MIC $\geq$ 8 indicates presence of ESBLs.

7) Phenotypic Confirmation Test

Antibiotic susceptibility testing done on Miller Hinton Agar with 0.5 Mc Farland's standard of the organism.[38]

Lawn culture of the organism was made and 3rd generation cephalosporin, Ceftazidime (30µg) disc was tested alone and along with their combination for 10mg of clavulanic acid. Organisms with 5mm

increase in zone of inhibition for ceftazidime / clavulanic acid (30µg/10µg) are confirmed as ESBLs.

(NCCLS recommends MIC ≥ 2µg/ml for cefotaxime, ceftazidime, astreonam, ceftriaxone (or) cefpodoxime as potential ESBL producers).

2 indicators of ESBLs are

i) 8 fold reduction in MIC when 3 GC are used with clavulanic acid.

ii) > 5mm increase in diameter of inhibition zone when using disc diffusion method with 3rd generation cephalosporin and clavulanic acid combined disc.

8) Molecular detection methods:

Tests already described only presumptively identify the presence of ESBL.

Earlier, determination of isoelectric point was sufficient for studying ESBL, But nowadays since there are >90 TEM type and >25 SHV type of β-lactamase and many of them have same isoelectric point, it has become impossible to detect the individual ESBLs.

So detection of β-lactamases using DNA probes that were specific for SHV was used but they were labour intensive.

Easiest and most reliable molecular method used to detect ESBLs is PCR with oligonucleotide primers that are specific for a β-lactamase gene Oligonucleotide primers can be chosen from sequence available in Gen Bank. Primers are usually chosen to anneal to regions where various point mutation are known to occur.

MEDICAL SIGNIFICANCE OF DETECTION OF ESBL

Patients having infections caused by ESBL – producing organisms are at increased risk of treatment failure with expanded spectrum β-lactam antibiotics. So it is recommended that if an organism was confirmed to produce ESBL it is considered as resistant to all 3rd Generation cephalosporins.

Many ESBL isolates will not be phenotypically resistant; even through their MIC is so high. ESBL producing strains have been established in many hospitals producing epidemic diseases especially in Intensive Care Units.[16] Failure to control outbreaks has resulted in new mutant types in some institution.

Problems in detection:-

1) Variable affinity of ESBLs for different substrate.

2) Inoculum effect: - MIC for 3rd generation cephalosporins increases if the inoculum is 10^7 CFU instead of 10^5 CFU. So we must be very careful.

TREATMENT:-

Carbapenems are most effective and reliable as they are highly resistant to the hydrolytic activity of all ESBLs due to the Trans 6 – hydroxy ethyl group.

Meropenem is the most active drug with MIC lower than that of Imipenem.[59]

Few β - lactam, 7α- methoxy cephalosporins such as Cefoxitin, cefotetan and latamoxcef are effective. Alternatively, fluoroquinolones and amino glycosides may be used if they show in vitro activity,[30] although clinical data for their use are absent, a Beta lactam-Beta lactamase inhibitor combination such as amoxicillin-clavulanate or Piperacillin Tazobactam may also be a further option to consider. All these agents should be used with caution, as their susceptibility varies among ESBL producers. Cephamycin, such as Cefoxitin and cefotetan, although active in vitro are not recommended for treating such infections, because of the relative ease with which these strains decrease the expression of outer membrane proteins, rendering them resistant[30]

In urinary tract infection combination with clavulanic acid can be used.

PREVENTION AND CONTROL MEASURES:-

Proper infection control practices and barrier methods are essential to prevent spreading and outbreaks of ESBL producing bacteria. The reservoir for these bacteria seems to be the gastrointestinal tract of patients. Alternative reservoirs could be the oropharynx, colonised wounds, and urine. The contaminated hands and stethoscopes of healthcare providers are important factors in spreading infection between patients. Essential infection control practices should include hand washing by hospital personnel, increased barrier precautions, and isolation of patients colonized or infected with ESBL producers. Other practices that have minimized the spread of such organisms include clinical and bacteriological surveillance of patients admitted to intensive care units and antibiotic cycling, as well as policies of restriction, especially on the empirical use of broad spectrum antimicrobial agents such as the third and fourth generation cephalosporins and imipenem

AIM AND OBJECTIVES OF THE STUDY

- To find the incidence of ESBL producing organisms in Intensive care units of Government Stanley Hospital.

- To prove association between prolonged stay in intensive care units and production of ESBL.

- To assess the outcome of treatment in patients with infections due to ESBL producing bacteria.

- To formulate treatment policy for those patients who are having severe infection resistant to treatment with routine antibiotics for avoiding further emergence of Extended Spectrum β- Lactamases

MATERIALS AND METHODS

This study was a 'descriptive study' done among patients in Intensive Care Units from December 2004 to February, 2006 at Government Stanley Hospital, Chennai.

A total of 100 patients from both sexes in age group between 20 to 60 years admitted in Intensive Care Units and having infection for more than a week were included in the study. Isolates obtained from various clinical samples like blood, urine, pus, sputum, tracheobronchial aspirates, drainage tube tips taken from Intensive medical and intensive surgical care units were included in the study.

Inclusion criteria:

Presence of obvious prolonged infections not responding to treatment with routine drugs used in intensive care units, like ciprofloxacin, Gentamycin, Amikacin and Cefotaxim.

METHODOLOGY:-

Methodology includes

1. Collection of samples
2. Identification of the organisms
3. Selection of resistant strains
4. Detection of ESBL production
5. Identification of SHV, CTX-M-3 and CTX-M-14 genes

SPECIMEN COLLECTION :[9,16]

Urine – Clean-catch midstream urine was collected in non-catheterized patients

In case of indwelling catheter, tubing was clamped off above the port to allow collection of freshly voided urine. The catheter port or wall of the tubing was then cleaned vigorously with 70 % ethanol, and urine was aspirated via a needle and syringe; the integrity of the closed drainage system was maintained to prevent the introduction of micro-organisms into the bladder.

Blood - Venipuncture site was cleaned with betadine and 70% alcohol. Blood was drawn at time of febrile episodes. 5 ml of blood was collected in 50 ml of Brain Heart Infusion broth to give a dilution of one in ten and incubated at 37^{o} C.

Wound Swab : Wound area was wiped with sterile saline or 70% alcohol. Swab was rolled along leading edge of the wound.

Endotracheal Aspirate collected in a sterile container under a septic precaution.

Drainage Tube Tips - Collected in a sterile container

IDENTIFICATION OF THE ORGANISM :-

Organisms were identified by colony morphology Gram's staining and biochemical reactions such as oxidase test, catalase test, indole test, citrate utilisation test, triple sugar iron (TSI) test, carbohydrate fermentation tests, oxidation- fermentation (OF) test and motility after inoculating them on routine media used in our microbiology laboratory namely nutrient agar, blood agar and Macconkey's agar. Main isolates included Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa, and few other species. .

Differentiating Characters of some isolates commonly producing ESBL

Organism	TSI	**Citrate**	**Indole**	**Oxidase**	**Catalase**	**Glucose**	**Lactose**	**Sucrose**	**Maltose**	**Mannose**	**Motility**
Escherichia coli	A/A	Not utilised	+	-	+	+	+	+	+	+	+
Klebsiella Pneumonia	A/A	utilised	-	-	+	+	+	+	+	+	-
Klebsiella oxytoca	A/A	utilised	+	-	+	+	+	+	+	+	-
Proteus. species	A/A with H2S	+	±	-	+	+	-	+	-	-	+
Pseudomonas .aeruginosa	K/No change	utilised	+	+	+	-	-	-	±	-	+
Acinetobacter baumannii	K/No Change	Not utilised	-	-	+	-	-	-	-	-	-

Note: A/A = Acid slant / Acid butt, + = Positive, - = Negative

SELECTION OF RESISTANT STRAINS

Routine disc diffusion susceptibility testing of the strains was performed by modified Kirby Bauer Method[46] in Mueller-Hinton agar medium.

Storage of Antimicrobial Disks

The container was refrigerated at 4 – 8° C or kept frozen at minus 14° C. β- Lactam antibiotics were stored frozen. Some labile agents like imipenem and clavulanic acid retained greater stability when stored frozen until the day of use.

Disk container was removed from refrigerator one or two hours before use and brought to room temperature.

Once a cartridge of disks has been removed from its sealed package, it was placed in a tightly sealed, desiccated container.

Turbidity Standard for Inoculam Preparation

To standardize the inoculam density for a susceptibility test, a BaSO4 turbidity standard equivalent to a 0.5 McFarland standard was used.

-

Preparation of Inoculum :

Tip of 3-5 representative colonies were picked up and put in 4-5ml of broth and incubated at 37°C for 2 – 6 hrs to attain 0.5 Mc Farland's standard which corresponds to 150 million organisms/ml.

If it was more turbid then some more broth was added and adjusted to 0.5 Mc Farland's standard by comparing against a card with white background and contrasting black lines.

Inoculation of MHA Plates:

Within 15 minutes of adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into it. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess broth from the swab.

Dried surface of Muller Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed.

The lid was let ajar for 3 to 5 minutes but not more than 15 minutes to allow any excess surface moisture to be absorbed before applying drug impregnated discs.

Application of discs to Inoculated agar plates.

The predetermined battery of antimicrobial discs which included Co-trimoxazole, Amoxycillin, Gentamycin, Amikacin, Ciprofloxacin, Cefotaxime, Ceftazidime, Ceftriaxone and meropenem were dispensed on agar plates and pressed down to ensure completes contact with the agar surface. Discs were distributed evenly so that they were not closer than 24mm from centre to centre. Not more than 5 discs for a 100mm plate.[46] Because some of the drug diffuses almost instantaneously, 'a disc was not relocated once it has come into contact with the agar surface. Instead, a new disc was placed in another location on the agar'. Plates were inverted and incubated at 37^{o}C for 16-18 hrs.

Reading plates and interpretation of results :

After 16-18 hrs of incubation each plate was examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies were apparent, then the inoculum was too light and the test was repeated. The diameter of the zones of complete inhibition was measured, including the diameter of the disc. Zones were measured to the nearest whole millimeter using a ruler which was held on the back of the inverted Petri plate.

The Petri plate was held a few inches above a black, non reflecting background and illuminated with reflected light.

The zone margin was taken as area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which could be detected only with a magnifying lens at the edge of the zone of inhibited growth, was ignored. However, discrete colonies growing within a clear zone of inhibition was sub-cultured, re-identified and retested.

The sizes of the zones of inhibition were interpreted by referring to the NCCLS standards and reported as susceptible, intermediate, or resistant to the agents that have been tested.

Controls used with each batch:-

1. Escherichia coli ATCC 25922

2. Pseudomonas aeruginosa ATCC 27853

3. Staphylococcus aureus ATCC 25923

ZONE SIZE INTERPRETATIVE CHART IN ACCORDING TO NCCLS Kirby-Bauer Chart

Sl. No.	Drug	Disk Content mcg	Resistant mm or less	Intermediate mm	Sensitive mm or more
1	Amoxycillin	10 mcg	14 mm	15-16 mm	17 mm
2	Co-trimoxazole	1.25+3.75	10	11-15	16
3	Cephalexin	30	14	15-17	18
4	Norfloxacin	10	12	13-16	17
5	Ciprofloxacin	5	15	16-20	21
6	Gentamycin	10	12	13-14	15
7	Cefotaxime	30	14	15-22	23
8	Ceftriaxone	30	13	14-20	21
9	Ceftazidime	30	14	15-17	18
10	Amikacin	30	14	15-16	17
11	Amoxycillin – Clavulanic acid	20/10	19	-	20
12.	Meropenem	10µg	≤ 13	14-15	≥ 16

ESBL DETECTION

Isolates found to be resistant (or) with decreased susceptibility to cefotaxime and ceftazidime were tested for the presence of ESBLs.

1) Double disc synergy test :

To demonstrate a synergistic action between a 3rd generation cephalosporin and clavulanic acid.

Isolates were grown to 0.5 Mc Farland's standard and lawn culture of it was made on a Muller Hinton agar plate.

Discs of 3rd generation cephalosporin cefotaxime (30µg) and ceftazidime (30µg) were placed 20mm apart from an amoxicillin (20µg) and clavulanic acid (10µg) combined disc (augmentin) centre to centre.[10] Incubated at 37°C for 16-18 hrs. Organisms were said to be ESBL producing.

If inhibition around the 3rd generation cephalosporins showed a clear extension towards augmentin disc.

If neither disc were inhibitory alone but bacterial grown was inhibited between the 2 discs.

2)MIC determination : Done by agar dilution method.

Preparation of media: Muller Hinton agar was prepared in tubes and autoclaved. It is then allowed to cool in a 50°C water bath.

Serial dilution of the 3 GCs ceftazidime and cefotaxime were prepared in sterile distilled water to give a final concentration ranging from 2µg – 2048µg/ml of agar.

After adding the drugs to the medium at 50° C it was mixed well and poured into sterile petridishes. (The media was used immediately otherwise potency of drugs will be affected. We can inoculate upto 12 different organisms in a single plate).

A control plate containing the test medium without the antibiotic was prepared for each series of test.

Inoculum Preparation:

At least 3-5 well isolated colonies of the same morphological type were selected from an agar culture plate. Top of each colony was touched with a loop and the growth was transferred into a tube containing 4-5ml of broth. The broth culture was incubated at 37°C until it reaches 0.5 Mc Farland's standard (usually 2-6 hrs). This result in growth corresponding to 150 million organisms/ml.

Inoculation of test plates:

Plates of various concentrations were divided into required number (9-12 divisions / plate) 0.003 ml of inoculum was put into the appropriate quadrant and incubated at 37°C for 16-20 hrs.

Minimum inhibitory concentration was the lowest concentration at which no visible growth occurs.

Isolates were tested for various concentrations of cephalosporin combined with 2µg/ml of clavulanic acid from 0.5µg to 2048µg / ml of agar and the MIC determined.

<u>Quality Control used for ESBL detection :</u>
Klebsiella pneumoniae ATCC 700603 [as positive control]

Escherichia coli ATCC 25922 as negative control.

IDENTIFICATION OF SHV, CTX-M-3 AND CTX-M-14 GENES

1. DNA EXTRACTION was done by alcohol extraction method from young broth culture of the organisms.

2 .POLYMERASE CHAIN REACTION

PCR was carried out in a Peltier Thermal cycler (PTC 200) MJ Research PCR which had

Inital denaturation for about 5 minutes at 94 °C. Each cycle had a 2nd denaturation for about 1 minute.

Annealing at 58 ° C for 1 minute.

Thirty five cycles were performed and a final extension was done for 10 minutes at 72 ° C

3. GEL DOCUMENTATION:

After the reaction, 25 µl. of the amplified samples were run on a 1.5 % agarose gel and electrophoresis done at 50 V with 1 x Tris Accetate EDTA buffer. Amplicons were visualised using Ethidium bromide staining and scored using 100 bp DNA ladder as reference.
Gels were viewed in a U - V gel documentation Unit and Photographed.

RESULTS

Bacterial isolates obtained from One hundred patients admitted in various intensive care units of Government Stanley Hospital were studied during a period of about 1 year from December, 2004 to February, 2006 to find the incidence of Extended Spectrum Beta Lactamases (ESBL) producing gram negative bacilli among them. Study included patients of both sexes between 20 to 60 years of age. Specimen included were Urine, Blood, Trachebronchial aspirate, wound swab and drainage tube tips.

Out of one hundred and fifteen specimens collected, one hundred and ten organisms were isolated. Ninety five isolates of gram negative bacilli were identified as Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa and Acinetobacter baumannii by biochemical reactions. Fifteen out of them were found to be gram positive cocci.

The Results were analysed as follows.

Table 1

Age And Sex Distribution Of Cases (n = 100)

Total numbers of cases taken for study were 100, which included total of 58 male (58 %) and 42 female (42 %). Among them between 20-30 years of age there were 11 male and 8 female, between 31-40 years 15 male and 7 female, between 41-50 years there were 17 male and 11 female and between 51-60 years there were 15 male and 16 female.

Table – 2 A

Isolates obtained from various specimens From intensive care units (n=115)

Total	Culture positives	Culture negatives
115	110 (95.65 %)	5 (4.35 %)

Out of the 115 specimens obtained, 110 were found to be culture positive and 5 were culture negative

Table – 2 B

Organisms Isolated from Culture Positive Samples (n = 110)

Specimen	Total Isolates	Escherichia coli	Klebsiella pneumoniae	Klebsiella oxytoca	Proteus mirabilis	Proteus vulgaris	aeruginosaPseudomonas	baumanniiAcinetobacter	Staphylococcus aureus.	Coagulase negative Staphylococcus
Urine	50	22	10	2	6	4	3	1	0	2
Blood	16	2	6	0	0	0	0	1	6	1
Tracheo bronchial Aspirate	11	2	4	2	0	1	0	0	1	1
Wound Swab	16	3	2	0	3	0	5	0	2	1
Drainage Tube Tips	17	7	2	1	3	0	3	0	0	1
Total	110	36 (32.7%)	24 (21.8%)	5 (4.5%)	12 (10.9%)	5 (4.5%)	11 (10.0%)	2 (1.8%)	9 (8.2%)	6 (5.5%)

Out of 110 isolates 36 (32.7%) were Escherichia coli, 24 (21.8%) were Klebsiella pneumoniae, 5 (4.5%) were Klebsiella oxytoca 12 (10.9%) were proteus mirabilis, 5 (4.5%) were proteus vulgaris, 11 (10%) were pseudomonas aeruginosa 2 (1.8%) were Acinetobacter, 9(8.2%) were Staphylococcus aureus and 6 (5.5%) were Coagulase negative Staphylococcus.

Table –3 A

Resistant Strains Among GNB

Number of Isolates	Resistant to 3rd generation cephalosporins
95	49

Table –3 B

ESBL Positive Isolates Among Gram Negative Bacilli (n=95)

Methods	DDST	MIC	Meropenem sensitivity
Positive Isolates	37 (38.95%)	37 (38.95%)	37 (38.95%)

Out of ninety five gram negative bacilli 37 (38.95%) were found to be ESBL producers by Double disc synergy test and MIC test. All the 37 were sensitive to Meropenem.

Table – 4

ESBL Producing Gram Nagative Organisms
Obtained from various specimens (n=95)

Specimen	Total Isolates	ESBL Producers	Percentage
Urine	48	19	39.58%
Blood	9	3	33.33%
TBA	9	4	44.44%
Wound Swab	13	5	38.46%
DTT	16	6	37.50%
Total	95	37	38.95%

Out of 95 gram negative bacilli 37 (38.95 %) were ESBL producers. Out of 48 urinary isolates 19 (39.58%) were ESBL producers. .From blood total numbers of isolates were 9, out of which 3 (33.33%) were ESBL producers. Out of 9 isolates from Trachebronchial aspirate 4(44.44%) were ESBL producers. Total numbers of isolates from wound swab were 13, out of which 5(38.46%) were found to be ESBL producers. Out of 16 isolates from drainage tube tips 6(37.50%) were ESBL producers.

Table – 5

ESBL Among Gram Negative Bacilli

From various specimens (n=95)

Organism	Total	ESBL	Percentage
Escherichia.coli	36	16	44.44%
Klebsiella pneumoniae	24	11	45.83%
Klebsiella oxytoca	5	2	40.00%
Proteus mirabilis	12	3	25.00%
Proteus vulgaris	5	1	20.00%
Pseudomonas aeruginosa	11	4	27.27%
Acinetobacter baumannii	2	0	0.00 %
TOTAL	95	37	38.95%

Out of 36 Escherichia.coli isolates 16 (44.44%) were ESBL producers. From 24 Klebsiella pneumoniae isolates 11(45.83%) were ESBL producers. 2 (40%) Out of 5 Klebsiella oxytoca isolates were ESBL producers. Out of 12 Proteus mirabilis isolates 3 (25%) were ESBL producers. Out of 5 Proteus vulgaris isolates 1 (20%) was an ESBL producer. From 11 Pseudomonas aeruginosa isolates 4 (27.27%) were ESBL producers. Acinetobacter baumannii isolates were 2, but they were not ESBL producer.

Table – 6

ESBL Producing Organisms among Urine Isolates (n=48)

Out of 48 isolates of gram negative bacilli 19 (39.58 %) were ESBL producers. Out of 22 Escherichia.coli isolates 10 (45.45 %) were ESBL producers. Out of 10 Klebsiella pneumoniae isolates 5(50%) were ESBL producers. Out of 2 Klebsiella oxytoca isolates 1 (50%) was ESBL producer. From 6 Proteus mirabilis isolates 1 (16.67%) was ESBL producer. Out of 4 Proteus vulgaris isolates 1 (25%) was an ESBL producer. From 3 Pseudomonas aeruginosa isolates 1 (33.33%) was ESBL producer. Acinetobacter baumannii isolates was 1, but it was not an ESBL producer.

Table – 7

ESBL Producers among Blood Isolates (n=9)

Isolates	Total	ESBL PRODUCERS	Percentage
Escherichia.coli	2	1	50.00%
Klebsiella pneumoniae	6	2	33.33%
Klebsiella oxytoca	0	0	0
Proteus mirabilis	0	0	0
Proteus vulgaris	0	0	0
Pseudomonas aeruginosa	0	0	0
Acinetobacter	1	0	0
Total	9	3	33.33 %

Out of 9 gram negative bacilli 3 (33.33 %) were ESBL producer. Out of 2 Escherichia.coli isolates 1 (50 %) was ESBL producer. Out of 6 Klebsiella pneumoniae isolates 2(33.33%) were ESBL producers. Acinetobacter baumannii isolate was 1, but it was not an ESBL producer.

Table – 8

ESBL Producing Organisms from Tracheobronchial Aspirate (TBA) (n=9)

Isolates	Total	ESBL Producers	Percentage
Escherichia.coli	2	1	50.00%
Klebsiella pneumoniae	4	2	50.00%
Klebsiella oxytoca	2	1	50.00%
Proteus mirabilis	-	-	-
Proteus vulgaris	1	-	-
Pseudomonas aeruginosa	-	-	-
Total	9	4	44.44 %

Out of 9 isolates of gram negative bacilli 4 (44.44 %) were ESBL producers. Out of 2 Escherichia.coli isolates 1 (50 %) was ESBL producer. Out of 4 Klebsiella pneumoniae isolates 2(50%) were ESBL producers. Out of 2 Klebsiella oxytoca isolates 1 (50%) was ESBL producer.

Table – 9

ESBL Producing Organisms Isolated From Wound Swab (n=13)

Out of 13 isolates of gram negative bacilli 5 (38.46 %) were ESBL producers. Out of 3 Escherichia.coli isolates 1 (33.33 %) were ESBL producers. 1(50%) out of 2 Klebsiella pneumoniae isolates was ESBL producer. Out of 3 Proteus mirabilis isolates 1 (33.33%) was ESBL producer. Out of 5 Pseudomonas aeruginosa isolates 2 (40 %) were ESBL producers.

Table – 10

ESBL Producing Organisms Isolated From Drainage Tube Tips (n=16)

Isolates	Total	ESBL Producers	Percentage
Escherichia.coli	7	3	42.86 %
Klebsiella pneumoniae	2	1	50.00 %
Klebsiella oxytoca	1	-	-
Proteus mirabilis	3	1	33.33 %
Proteus vulgaris	-	-	-
Pseudomonas aeruginosa	3	1	33.33 %
Total	16	6	37.50 %

Out of 16 isolates of gram negative bacilli 6 (37.50 %) were ESBL producers. From 7 Escherichia.coli isolates 3 (42.86 %) were ESBL producers. 1(50%) out of 2 Klebsiella pneumoniae isolates was ESBL producer. Out of 3 Proteus mirabilis isolates 1 (33.33%) was ESBL producer. Out of 3 Pseudomonas aeruginosa isolates 1 (33.33%) was ESBL producer.

Table – 11

MIC of Cefotaxime For ESBL Producing Organisms (n = 37)

Minimum inhibitory concentration of Cefotaxime for the ESBL producing organisms in the study was between 64 µg/ml of agar to 2048 µg/ml of agar

Table – 12

MIC OF CEFOTAXIME FOR ESBL PRODUCING ORGANISMS IN PRESENCE OF 2 µg/ml OF CLAVULANIC ACID (n = 37)

Isolates	Total	Concentration of Cefotaxime in µg/ml of Agar										
		0.5	1	2	4	8	16	32	64	128	256	512
Escherichia.coli	16	1	1	10	14	16	16	16	16	16	16	16
Klebsiella pneumoniae	11	2	3	9	10	10	10	10	11	11	11	11
Klebsiella oxytoca	2	-	2	2	2	2	2	2	2	2	2	2
Proteus mirabilis	3	-	1	2	3	3	3	3	3	3	3	3
Proteus vulgaris	1	-	-	-	1	1	1	1	1	1	1	1
Pseudomonas aeruginosa	4	-	-	2	4	4	4	4	4	4	4	4

Minimum inhibitory concentration of Cefotaxime for the ESBL producing organisms in the study was between 0.5 µg/ml of agar to 64 µg/ml of agar in the presence of clavulanic acid at a concentration of 2 µg/ml of agar showing 8 fold reductions in MIC.

Table – 13

MIC of Ceftazidime For ESBL Producing Organisms (n =37)

Isolates	Total	Concentration of ceftazidime in µg/ml of agar										
		2	4	8	16	32	64	128	256	512	1024	2048
Escherichia.coli	16	-	-	-	-	-	2	5	12	14	16	16
Klebsiella pneumoniae	11	-	-	-	-	-	1	2	3	6	10	11
Klebsiella oxytoca	2	-	-	-	-	-	1	2	2	2	2	2
Proteus mirabilis	3	-	-	-	-	-	-	1	2	2	3	3
Proteus vulgaris	1	-	-	-	-	-	-	-	-	1	1	1
Pseudomonas aeruginosa	4	-	-	-	-	-	-	1	1	2	4	4

Minimum inhibitory concentration of Ceftazidime for the ESBL producing organisms in the study was between 64 µg/ml of agar to 2048 µg/ml of agar

Table – 14

MIC of Ceftazidime for ESBL Producing Organisms in presence of 2 µ g/ml of Clavulanic acid (n = 37)

Isolates	Total	Concentration of Ceftazidime in µg/ml of agar										
		0.5	1	2	4	8	16	32	64	128	256	512
Escherichia.coli	16	1	12	14	16	16	16	16	16	16	16	16
Klebsiella pneumoniae	11	2	4	11	11	11	11	11	11	11	11	11
Klebsiella oxytoca	2	2	2	2	2	2	2	2	2	2	2	2
Proteus mirabilis	3	1	3	3	3	3	3	3	3	3	3	3
Proteus vulgaris	1	-	-	1	1	1	1	1	1	1	1	1
Pseudomonas aeruginosa	4	-	2	4	4	4	4	4	4	4	4	4

Minimum inhibitory concentration of Ceftazidime for the ESBL producing organisms in the study was between 0.5 µg/ml of agar to 4 µg/ml of agar in the presence of clavulanic acid at a concentration of 2 µg/ml of agar showing 8 fold reductions in MIC

Table – 15

Gene Identification by Polymerase Chain Reaction (n = 37)

Isolates	Total	SHV	CTX-M3	CTX-M14
Escherichia.coli	16	-	9	2
Klebsiella pneumoniae	11	11	2	-
Klebsiella oxytoca	2	1	1	-
Proteus mirabilis	3	-	-	-
Proteus vulgaris	1	-	-	-
Pseudomonas aeruginosa	4	-	-	-

PCR for determination of SHV, CTX-M-3, and CTX-M-14 identified. SHV gene in all the Klebsiella pneumoniae and one Klebsiella oxytoca isolates, CTX-M-3 was found in 9 Escherichia.coli, 2 Klebsiella pneumoniae and one Klebsiella oxytoca isolates. CTX-M-14 was found in 2 Escherichia.coli isolates. 2 Klebsiella pneumoniae and one Klebsiella oxytoca isolates showed both SHV and CTX-M-3.

Table – 16

Antibiotic Susceptibility of ESBL producing and non ESBL producing GNB To Various non – β –Lactam Antibiotics (n = 95)

Antibiotics	Resistant Strains n = 49		Sensitive Strains n=46
	ESBL Producers n = 37	Non ESBL Producers n= 12	
Ampicillin	-	-	15 (32.60%)
Amoxycillin	-	-	20 (43.48%)
Co-trimoxazole	5 (13.51%)	-	28 (60.87%)
Gentamycin	-	-	23 (50.00%)
Amikacin	18 (48.65%)	5 (41.67%)	46 (100 %)
Ciprofloxacin	9 (24.32%)	3 (25.00%)	46 (100 %)

Out of 37 ESBL producers, 5 (13.51%) were sensitive to Co-trimoxazole, 18 (48.65%) were sensitive to Amikacin and 9 (24.32%) were sensitive to Ciprofloxacin.

DISCUSSION

Emergence of ESBL due to the extensive use of extended spectrum Cephalosporins since 1980s were a significant evolution in antimicrobial resistance.

ESBLs are now a problem in hospitalised patients throughout the world. Incidence among clinical isolates vary greatly world wide and in Geographic areas and are rapidly changing overtime. Only few studies have been conducted to find the incidence and prevalence of ESBL producers in Indian hospitals but ESBL producing Gram negative bacilli may have evolved in several hospitals all over the country. Since the incidence from ICU was found to be high from various studies, the present study was conducted to find the incidence of ESBL producing Gram negative bacilli from ICU of Stanley medical college hospital from Urine, Blood, Tracheobronchial aspirate, wound swab and drainage tube tips.

Double Disc Synergy Test (DDST) being a simple and cost effective procedure was taken as the method of choice for screening of ESBL producing organisms in this study.

100 cases of both male and female between 20-60 years of age were included in this study which gives a ratio of 1: 0.72. (58/42)

Among the 115 specimen obtained 110 organisms were isolated and 95 out of them were Gram negative bacilli.

Among the 95 Gram negative bacilli 49 were resistant to 3rd Generation cephalosporins (51.58 %) and 37 out of them were positive by DDST for ESBL (38.95 %) Correlating with study of Shukla et al[55] (30.18%), Baby Padmini[53] (40 %), Sumeeta et al[56] (30.2%) and Ashwin et al[8] (33.3%).

In the present study ESBLs from urine was 39.58% which is correlating with study by Baby Padmini[53] (40%). 45.45% (10/12) of Escherichia.coli isolated from urine were ESBL producers, which is correlating with study by Bithika Duttaroy[10] (46.5%)

ESBLs among Klebsiella pneumoniae from urine was 50% in the present study, which is correlated with the study of Bithika[10] (58%) and Baby Padmini[53] (40%).

In the present study ESBL producing Klebsiella oxytoca from all specimen was40% (2/5) and from urine it was 50% (1/2). In a study by Christopher et al[15] from Virginia in 1997 it was 4.4%. This difference may be due to change in Geographical area and change in period of study.

In the present study total ESBL of proteus mirabilis was 25% (3/12) and from urine it was 16.67% (1/6). From the study of C.Rodrigues et al[12] it was 33.33% (1/3). This disparity may be due to the less number of isolates in their study than the present study.

Total ESBL among Proteus vulgaris in the present study was 20% (1/5) which is correlating with the study of C. Rodrigues et al[12] and from urine it was 25%(1/4) This also correlated with the study of C.Rodrigues et al [12]

Total ESBL among Pseudomonas aeruginosa in the present study was 36.36% (4/11) but in the study by C. Rodrigues et al[12] it was only 5.9% (4/68). This disparity may be due to extensive use of 3rd generation cephalosporins in the Intensive Care Units of Stanley Medical College Hospital

Number of ESBLs producing Pseudomonas aeruginosa from urine was 33.33% (1/3) which was high when compared to study by Rodrigues et al[12] which may be due to increased use of third generation cephalosporins and problem with urinary catheterisation.

In the present study only 2 isolates were Acinetobacter baumannii, among which one from urine and another one from blood. One from urine was found to be resistant to third generation cephalosporin but not found to be an ESBL producer. One

Acinetobacter baumannii isolated from blood was found to be sensitive to 3rd generation cephalosporin. This correlated with the study of Rodgrigues et al in which number of ESBL among 16 Acinetobacter baumannii isolates was none but they have proved the association of depressed mutants' with ESBLs is 33.3% (5/16)

From blood in the present study there were totally 33.33% ESBL producing organisms (3/9) but from a study by Bithika et al[10] it was 11.32% (6/53) and in a study from Belgium by Eddy vercauteran et al[19] in 1997 it was 5.8% (5/86). The increase of ESBL in the present study may be due to the prolonged stay of the patients in the Intensive care units in our study.

From blood, in the present study ESBL producing Escherichia coli was 50 %(1/2). But in the study by Bithika et al[10] from Baroda it was 18.18% (6/33). This difference may be because of the number of isolates

From blood, in the present study ESBL producing Klebsiella pneumoniae was 33.33% (2/16) which correlated with study by Vercauteran et al[19] from Belgium

In the present study ESBL producing organisms from Tracheobronchial aspirate was 44.44% (4/9). In a study by A. Subha[1] from Chennai there were no ESBL from the respiratory specimen. This

may be due to the low number of isolates tested by them, or may be due to the difference in age group studied or may be due to the increased use of third generation cephalosporin in our Intensive Care Units.

In the present study ESBL producing Escherichia coli, Klebsiella pneumoniae and Klebsiella oxytoca from Tracheobronchial aspirate were 50%(1/2), 50% (2/4) and 50%(1/2) in which Klebsiella oxytoca equals Escherichia coli and Klebsiella pneumoniae which correlates with the study of Christopher et al[15] from Virginia in 1997.

In the present study ESBLs from wound swab was 30.77% (4/13). This correlated with the study by Shukla et al[55] 36.1%, study by Bithika et al[10] 30.18% (16/54) and study from JIPMER by Ashwin et al[8] 22.14% (11/49)

In the present study ESBL producing Escherichia coli from wound swab was 33.33 %(1/3). This varies from the study of Ashwin et al[8]from JIPMER in 2000 was 58.06 %(18/31). This may be due to the low number of isolates in the present study.

ESBL producing Klebsiella pneumoniae from wound swab in the present study was 50.00 %(1/2) which correlated with study by Ashwin et al[8] was 43.75% (7/16) and Shukla et al[55] was 36.1%

ESBL producing Proteus mirabilis from wound swab in the present study was 33.33% (1/3).

ESBL producing Pseudomonas aeruginosa in the present study was 40% (2/5) from wound swab which correlates with the study of Ashwin et al [8] (15 %.)

ESBL producing organisms from various other specimen like catheter tips wound drainage tube tips and endotracheal tube tips was 37.5%(6/16) constituting Escherichia coli 42.86% (3/7), Klebsiella pneumoniae 50% (1/2), Proteus mirabilis 33.33% (1/3), Pseudomonas aeruginosa 33.33% (1/3). This correlated with the study of Christopher et al[15] from Virginia29.41% (5/17)

Minimum inhibitory concentration for Cefotaxime by agar dilution method for the 37 ESBL producing organisms varied from 64 µg/ml to 2048 µg/ml. This showed MIC 50s at 256 µg/ml and MIC 90s at 1024 µg/ml for Escherichia coli. MIC 50s at 512 µg/ml and MIC 90s at 1024 µg/ml for Klebsiella pneumoniae.

MIC 50s at 64µg/ml and MIC 90s at 256 µg/ml for Klebsiella oxytoca.

MIC 50s was 512 µg/ml and MIC 90s was 1024 µg/ml for Proteus mirabilis.

ESBL producing Proteus vulgaris and Pseudomonas aeruginosa were inhibited at 1024 µg/ml.

When cefotaxime was combined with 2 µg/ml. of clavulanic acid the MIC was reduced to 0.5 µg/ml - 8 µg/ml, except one organisms which showed MIC of 64 µg/ml.

MIC for ceftazidime was between 64 µg/ml - 2048 µg/ml, with MIC 50s for Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis and Pseudomonas aeruginosa as 256 µg/ml, 512 µg/ml, 64 µg/ml, 256 µg/ml respectively.

MIC was reduced to 0.5 µg/ml - 8 µg/ml of ceftazidime, when combined with 2 µg/ml of clavulanic acid. From a study by Bithika et al[10] from Baroda MIC for third generation cephalosporins was between 2- 1024 µg/ml and it was reduced to 0.25-128 µg/ml when clavulanic acid was added at a concentration of 2 µg/ml. So the present study correlates well with the study of Bithika et al.

Polymerase Chain Reaction for identification of SHV, CTX – M-3 and CTX-M-14 showed presence of SHV genes in all the Klebsiella pneumoniae and one Klebsiella oxytoca isolate, CTX-M-3 was identified in 9 Escherichia coli, 2 Klebsiella pneumoniae and 1 Klebsiella oxytoca isolate. CTX-M-14 was identified in 2 Escherichia coli isolates.

This correlates with the study of Ju-Hsin Chia et al[31] from Taiwan, where most of the Escherichia coli isolates showed CTX-M-3 and Klebsiella pneumoniae isolates showed SHV.

SHV, CTX-M-3, CTX-M-14 genes were not identified in Proteus mirabilis and Pseudomonas aeruginosa; this may be due to presence of

some other genes like OXA responsible for resistance in these organisms.

In the present study the ESBL producing organisms found to be sensitive to co-trimoxazole were 13.51% (5/37), sensitive to amikacin were 48.65% (18/37), sensitive to ciprofloxacin were 16.22%(6/37).which correlated with a study by S. Baby padmini[53] from Coimbatore in which the sensitivity were 26%, 89%,9% respectively for co-trimoxazole, amikacin and ciprofloxacin.

All the ESBLs were sensitive to meropenem (100%) which correlated with the study by Shukla et al[55] from Haryana.

SUMMARY

Hundred cases with prolonged infection (more than a week) from Intensive care units of Government Stanley Hospital, Chennai, between "December 2004 to February 2006" formed the study group.

Specimens like urine, blood, Tracheobronchial aspirate, wound swabs, catheter tips and endotracheal tube tips were cultured and organisms were identified by Gram's staining and various biochemical reactions.

Antibiotic susceptibility testing was done including third generation cephalosporins and the gram negative bacilli which included Escherichia coli , Klebsiella pneumoniae , Klebsiella oxytoca ,Proteus mirabilis , proteus vulgaris , Pseudomonas aeruginosa and Acinetobacter baumannii found to be resistant to third generation cephalosporin were screened for ESBL production by Double disc synergy test (DDST) .

Total of 37 out of 95 Gram negative bacilli (38.95%) were found to be ESBL producers . presence of ESBL among urine , blood , Tracheobronchial aspirate ,wound swabs were 39.58%,33.33%, 44.44%, 37.5% and 38.46 % respectively.

Among the Gram negative bacilli presence of ESBL was high among Klebsiella pneumoniae (45.33%) followed by Escherichia coli (44.44%).

MIC for the isolates was between 64- 2048 µg/ml of agar for cefotaxime and ceftazidime which was reduced to 0.5 - 64 µg/ml and 0.5- 4 µg/ml of agar respectively in the presence of 2 µg/ml of agar.

All the 37 organisms (100%) were found to be sensitive to meropenem which is expensive. Some of the ESBL producing organisms were sensitive to cheaper drugs like Co-trimoxazole (13.51%), Amikacin (48.65%) and to Ciprofloxacin (16.22%).

By Polymerase Chain Reaction SHV gene was identified in all the Klebsiella pneumoniae, and CTX-M-3 identified in most of the Escherichia coli isolates.

CONCLUSION

Isolation of ESBL producing organisms from Intensive Care Units of Govt. Stanley Medical College Hospital was 38.95% by DDST and MIC which was high when compared to other studies from south India. Isolation of ESBL producing organisms were more in tracheobronchial aspirate of patients put on ventilator (44.44%) followed by urine (39.46%) and wound swab (38.46%).

1. In Intensive care units widespread use of third generation Cephalosporins has been cited as a contributing factor in the development of ESBL mediated resistance along with prolonged stay and intubations. So by following proper infection control and giving correct antibiotics after proper screening ESBL production can be minimised

2. DDST being simple and cheaper should be included in the microbiology laboratories as a routine test for early deduction of ESBL producing organisms in specimen from critically ill patients.

3. Though meropenem was 100% sensitive, ESBL producing organisms were also sensitive to cheaper drugs like, Co-trimoxazole (13.51%), Amikacin (48.65%) and Ciprofloxacin (16.22%). Hence if we screen carefully we can minimise the cost of treatment for the patients with infections due to ESBL producing organisms.

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