การตรวจและจำแนกชนิดเอนไซม์ Extended-Spectrum β-Lactamases สร้างโดย *Escherichia coli* และ *Klebsiella* spp. ที่แยกได้จากสิ่งส่งตรวจของผู้ป่วยโรคติดเชื้อในโรงพยาบาลจุฬาลงกรณ์

นางสาวเมธีรา ทังสุบุตร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2545

ISBN 974-17-2732-1

DETECTION AND CHARACTERIZATION OF EXTENDED-SPECTRUM β-LACTAMASES PRODUCED BY *Escherichia coli* AND *Klebsiella* spp. FROM CLINICAL SPECIMENS OF PATIENTS WITH INFECTION IN KING CHULALONGKORN MEMORIAL HOSPITAL

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A Thesis Submitted in Partial Fulfillment of Requirements

for the Degree of Master of Science in Medical Microbiology

Inter-Department of Program in Medical Microbiology

Graduate School

Chulalongkorn University

Academic Year 2002

ISBN 974-17-2732-1

Thesis Title	Detection and Characterization of Extended-Spectrum β-Lactamases Produced by *Escherichia coli* and *Klebsiella* spp. from Clinical Specimens of Patients with Infection in King Chulalongkorn Memorial Hospital
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นางสาวเมธีรา ทังสุบุตร : การตรวจและจำแนกชนิดเอนไซม์ Extended-Spectrum β-Lactamases สร้างโดย *Escherichia coli* และ *Klebsiella* spp. ที่แยกได้จากสิ่งส่งตรวจของผู้ป่วยโรคติดเชื้อในโรงพยาบาลจุฬาลงกรณ์ (DETECTION AND CHARACTERIZATION OF EXTENDED-SPECTRUM β-LACTAMASES PRODUCED BY *Escherichia coli* AND *Klebsiella* spp. FROM CLINICAL SPECIMENS OF PATIENTS WITH INFECTION IN KING CHULALONGKORN MEMORIAL HOSPITAL).
อาจารย์ที่ปรึกษา: รองศาสตราจารย์ผ่องพรรณ นันทาภิสุทธิ์, อาจารย์ที่ปรึกษาร่วม: อาจารย์นิพนธ์ อุดมสันติสุข, ดร. ทวีศักดิ์ ตีระวัฒนพงศ์, 118 หน้า. ISBN 974-17-2732-1.

ปัจจุบันการดื้อยาในกลุ่ม β-lactams ที่ใช้ในการรักษาโรคติดเชื้อจาก *Escherichia coli* และ *Klebsiella spp.* มีอัตราการดื้อยาสูงขึ้นมาก กลไกการดื้อยาของเชื้อเหล่านี้ส่วนใหญ่มีสาเหตุเนื่องจากเชื้อสร้างเอนไซม์ extended-spectrum β-lactamases (ESBLs) เพื่อทำลายยาในกลุ่ม extended-spectrum cephalosporins การศึกษาครั้งนี้ได้ทำการตรวจหา ESBLs โดยวิธีdisk diffusion test, double disk diffusion test และ Etest ESBL โดยนำสายพันธุ์ที่สร้าง ESBLs มาตรวจหายีน bla_{TEM}, bla_{SHV}, bla_{CTX-M} และ bla_{VEB} ด้วยวิธี polymerase chain reaction (PCR) และหาลำดับเบสของยีน bla_{TEM}และ bla_{SHV} โดยวิธี Nucleotide sequencing เชื้อที่ใช้ในการศึกษาครั้งนี้แยกได้จากสิ่งส่งตรวจของผู้ป่วยที่เข้ารักษาในโรงพยาบาลจุฬาลงกรณ์ระหว่าง เดือนกุมภาพันธ์ ถึงเดือนพฤษภาคม พ.ศ. 2545 จำนวน 270 สายพันธุ์ เป็น *E.coli* 212 สายพันธุ์และ *K.pneumoniae* 58 สายพันธุ์ ผลการศึกษาพบ *E.coli* ที่สร้าง ESBL ร้อยละ 17 (36/212) และพบ *K.pneumoniae* ที่สร้าง ESBL ร้อยละ 34.5 (20/58) โดย *K.pneumoniae* 20 สายพันธุ์พบยีน bla_{SHV} ร้อยละ 90 (18/20), bla_{TEM} ร้อยละ 50 (10/20), $bla_{VEB-like}$ ร้อยละ 30 (6/20) and $bla_{CTX-M-like}$ร้อยละ 15 (3/20) ใน *E. coli* 36 สายพันธุ์พบยีน bla_{TEM}ร้อยละ 72.2(26/36), $bla_{CTX-M-like}$ ร้อยละ 52.8 (19/36) และ $bla_{VEB-like}$ร้อยละ 16.7 (6/36) ตรวจไม่พบ bla_{SHV} ใน *E.coli* ขณะที่ bla_{SHV} พบมากที่สุดใน *K.pneumoniae* เชื้อที่สร้าง ESBL ตรวจพบ *bla* gene อย่างน้อย 2 ชนิดในสายพันธุ์เดียวกันจำนวนร้อยละ 53.6 (30/56) bla_{TEM} ที่ตรวจพบทั้งหมดเป็น bla_{TEM-1B} ซึ่งไม่ใช่ ESBL CTX-M เป็น ESBL ที่พบบ่อยที่สุดใน *E.coli* การศึกษาครั้งนี้พบว่าเชื้อ *E.coli* และ*K.pneumoniae* ที่สร้าง ESBLs ในเมืองไทยส่วนใหญ่เป็นชนิด CTX-M และ VEB

สาขาวิชา จุลชีววิทยาทางการแพทย์ (สหสาขาวิชา) ลายมือชื่อนิสิต……..

ปีการศึกษา 2545 ลายมือชื่ออาจารย์ที่ปรึกษา…….

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……..

4389123720 : MAJOR : MEDICAL MICROBIOLOGY

KEYWORD : ESBLs / SHV / TEM / VEB/ CTX-M/ *Klebsiella* spp/ *Escherichia coli*

MAYTEERA DANSUPUTRA: DETECTION AND CHARACTERIZATION OF EXTENDED-SPECTRUM β-LACTAMASES PRODUCED BY *Escherichia coli* AND *Klebsiella* spp. FROM CLINICAL SPECIMENS OF PATIENTS WITH INFECTION IN KING CHULALONGKORN MEMORIAL HOSPITAL.

THESIS ADVISOR : ASSOC. PROF. PONGPUN NUNTHAPISUD. THESIS CO-ADVISOR NIBONDH UDOMSANTISUK., THAWEESAK TIRAWATNAPONG, Ph.D., 118 pp. ISBN 974-17-2732-1.

Resistance to β-lactams has been increasing in the treatment of infections caused by *Escherichia coli* and *Klebsiella* spp. The production of extended-spectrum β-lactamases (ESBLs), that hydrolyze extended-spectrum cephalosporins, is the major cause of β-lactams resistance. In this study, ESBLs were determined by disk diffusion test, double disk synergy test and Etest ESBLs. All ESBLs producing strains were investigated for the presence of bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{VEB} genes by polymerase chain reaction (PCR). Nucleotide sequencing of bla_{TEM} and bla_{SHV} were performed. *E.coli* and *K.pneumoniae* were isolated from clinical specimens of patients in Chulalongkorn Memorial Hospital during February to May 2002. Of the 270 isolates, 212 were *E.coli* and 58 were *K.pneumoniae.* ESBL production were detected in 17% (36/212) of *E.coli* and 34.5% (20/58) of *K.pneumoniae* isolates. Of the 20 *K.pneumoniae* strains, the β-lactamases gene were bla_{SHV} (18/20, 90%), bla_{TEM} (10/20, 50%), $bla_{VEB-like}$ (6/20, 30%) and $bla_{CTX-M-like}$ (3/20, 15%). Thirty-six *E.coli* strains carried bla_{TEM}, $bla_{CTX-M-like}$ and $bla_{VEB-like}$ genes in 72.2% (26/36), 52.8% (19/36) and 16.7% (6/36), respectively. Bla_{SHV} was not detected in ESBL-producing *E.coli*, whereas it predominated in *K.pneumoniae*. Of the 56 ESBL producing strains, 30 (53.6%) coharboured at least 2 different *bla* genes. All TEM identified, were TEM-1B, which is not ESBL. CTX-M ESBLs were the most common in *E.coli*. In this study, the presence of bla_{CTX-M} and bla_{VEB} in ESBL-producing *E.coli* and *K.pneumoniae* indicates the high prevalence of these genes in Thailand.

Field of study Medical microbiology Student's signature……..…..

Academic year 2002 Advisor's signature…….….

Co-Advisor's signature ……...…….

Co-Advisor's signature……...…..…

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to the following individuals who helped in making this thesis possible.

Associate Professor Pongpun Nuntapisud. Department of Microbiology, Faculty of Medicine, Chulalongkorn University, my advisor, for her kindness and indispensable help in supervising this thesis.

Nibondh Udomsantisuk. my co-advisor, for preliminary experiments to develop the bioassay and his invaluable advice.

Thaweesak Tirawatnapong, Ph.D., my co-advisor, for the help to set up the assays and his kindly advice the PCR, sequencing technique, equipments and encouragement through the period of this study.

Associate Professor Pintip Pongpech, Ph.D, the chairman of thesis committee, Department of Microbiology, Faculty of Pharmacy, Chulalongkorn University, and Assistant Professor Aroonwadee Chanawong, Ph.D, Department of Clinical Microbiology, Faculty of Associated Medical Sciences Khon Kaen University, the member of thesis committee for their constructive critisms.

We are grateful to Assistant Professor Aroonwadee Chanawong, Ph.D. for providing strains that produce the standard SHV and TEM β-lactamases.

Sincere thanks go to the staffs at the Department of Microbiology, for providing the sample specimens.

Finally, I am deeply indebted to my parents for their understanding and support during my study period.

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ABBREVIATIONS

A	Alanine
Bp	base pair
Bla_{CTX-M}	CTX-M betalactamase gene
Bla_{SHV}	SHV betalactamase gene
Bla_{TEM}	TEM betalactamase gene
Bla_{VEB}	VEB betalactamase gene
C	Cysteine
CTX-M	Cefotaximases
D	Aspartic acid
DNA	Deoxynucleotidetriphosphate
dNTPs	Deoxyribonucleotidetriphosphate
DW	Distilled water
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
i.e.	id est
K	Lysine
Kb	kilo base pair
L	Leucine
M	Methionine
MIC	Minimum Inhibitory Concentration
mg/L	Milligram per liter
$MgCl_2$	Magnesium chloride
min	Minute
ml	Milliliter
mM	Millimolar

ABBREVIATIONS (continued.)

mm	Millimeter
N	Asparagine
P	Proline
PCR	Polymerase Chain Reaction
pmole	Picomole
Q	Glutamine
R	Arginine
rpm	Round per minute
S	Serine
SHV	Sulphydryl variable
T	Threonine
TEM	Temoniera
TSR	Template suppressor reagent
U	Unit
UV	Ultraviolet
V	Valine
VEB	Vietnamese extended-spectrum β-lactamase
W	Tryptophan
Y	Tyrosine
β	Beta
ηg	Nanogram
ηm	Nanometer
μg	Microgram
μl	Microliter
μM	Micromolar
μm	Micrometer

CHAPTER I

INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) are enzymes that confer resistance to oxyimino-cephalosporins, such as cefotaxime, ceftazidime, and ceftriaxone, and to monobactams, such as aztreonam, resulting in resistance to these drugs. ESBLs predominantly derivatives of plasmid-mediated TEM or SHV β-lactamases, arise through a mutation or mutations that result in one or more amino acid substitutions. These mutations alter the configuration or binding properties of the active site, thereby expanding the hydrolytic spectrum of the enzyme. Though these enzymes, especially TEM and SHV-derived ESBLs, are most commonly detected in *Klebsiella pneumoniae* and *Escherichia coli* and have been reported worldwide (Jacoby and Bush, http://www.lahey.org/studies/webt.htm). ESBLs have also recently been found in other members of the family *Enterobacteriaceae*, such as *Citrobacter* spp., *Serratia* spp., *Proteus* spp., *Salmonella* spp. and *Enterobacter* spp.

The organisms that produce ESBLs are frequently associated with isolating from nosocomial infections. ESBLs caused treatment failure with extended-spectrum cephalosporins and cross-resistance to aminoglycosides, quinolones, and trimethoprim /sulfamethoxazole, leaves few options for alternative treatments. Infections from the organisms that produce ESBLs are related to higher morbidity and mortality. Bacterial resistance results in increase health care costs due to increased utilization of laboratory antimicrobial susceptibility tests, surveillance for resistant organisms, heightened isolation requirements for infected patients, and required more expensive, and possibly more toxic, antibiotics. Clinically, they need for accurate detection of ESBLs of individual therapy and efficient infection control to prevent outbreaks

ESBL-producing *Enterobacteriaceae*, which are being identified worldwide, is probably more prevalent than currently recognized because they are often undetected by routine susceptibility testing methods. Detection of ESBL producing organisms remains a challenge for

the microbiology laboratory. The problem is that routine methods for monitoring a decrease in susceptibility to oxyimino-cephalosporins and aztreonam have not been sensitive enough to detect ESBL-producing strains. Minimum inhibitory concentrations (MIC) may be raised only slightly and fail to reach the level of accepting breakpoint resistance. The current National Committee for Clinical Laboratory Standards (NCCLS; 2001) recommendations for detection of ESBLs in *Klebsiella pneumoniae* and *E. coli* includes an initial screening test with following β-lactam antibiotics: ceftazidime, aztreonam, cefotaxime, ceftriaxone, or cefpodoxime. A second test has been recommended for the detection of ESBL activity. The double-disk synergy test is most widely used due to its simplicity and ease of interpretation. The Etest ESBL screening test is another useful test for detection of ESBL activity by measuring the MIC scale. A 3 two-fold concentration decrease in a MIC for either antimicrobial agent tested in combination with clavulanic acid versus its MIC when tested alone should be considered as an ESBL producer.

The prevalence of ESBL production varies greatly from country to country, institution to institution. Until now published information on the characterization of ESBL-producing stains in Thailand remains limited. In the present study, we determined the prevalence of strains producing ESBLs among *Klebsiella* spp. and *E.coli* in King Chulalongkorn Memorial Hospital. We also compared the reliability of the double-disk synergy test and the Etest ESBL screening test for the detection of ESBL expression. We also described the molecular epidemiology of genes associated with TEM-type and SHV-type β-lactamases and to detect the possible existence of new TEM and SHV enzymes, which were produced by *Klebsiella* spp. and *E.coli* in King Chulalongkorn Memorial Hospital. These data will provide information of the prevalence of various types of TEM and SHV derivatives, which might also be apply the techniques to detect ESBL producing strains in our laboratory.

CHAPTER II

OBJECTIVES

1. Detect the prevalence of ESBLs by disk diffusion test, double-disk synergy test and Etest in *E.coli* and *Klebsiella* spp. isolates from clinical specimens in King Chulalongkorn Memorial Hospital.

2. Typing ESBLs, which is produced by *E.coli* and *Klebsiella* spp. by sequencing.

3. Apply the techniques to detect ESBLs SHV and TEM type, which is produced by *E.coli* and *Klebsiella* spp.

CHAPTER III

LITERATURE REVIEW

1. β-Lactam Antibiotics

The β-lactam antibiotics are the most varied and widely used of all the groups of antimicrobials. ß-Lactams belong to a family of antibiotics, which is characterized by a β-lactam ring. Penicillins, cephalosporins, clavams (or oxapenams), cephamycins, monobactams and carbapenems (Figure 1) are members of this family. The major antibacterial action, β-lactams act by binding to inhibit a number of bacterial enzymes, namely, penicillin-binding proteins (PBPs) of susceptible organisms, which are essential for synthesis of peptidoglycan of bacterial cell wall. In addition, these β-lactam agents may produce bactericidal effects by triggering autolytic enzymes in the cell envelope (YAO *et al*., 1999).

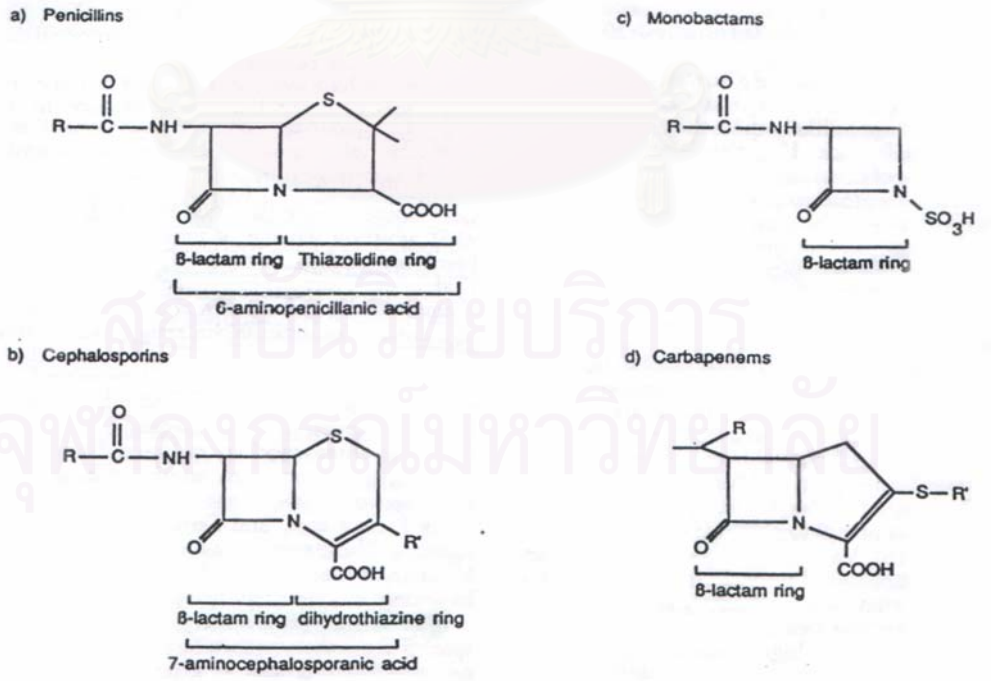


Figure 1: Chemical structures of β-lactam antibiotics (YAO *et al.,* 1999)

β-lactams in this study were reviewed as followed

1.1. Cephalosporins

Cephalosporins are derivatives of the fermentation products of *Cephalosporium acremonium* (also designated *Acremonium chrysogenum*). They contain a 7-aminocephalosporanic acid nucleus , which consists of a β-lactam ring fused to a dihydrothiazine ring (Figure1b). Various substitutions at positions 3 and 7 alter their antibacterial activities and pharmacokinetic properties. Addition of a methoxy group at position 7 of the β-lactam ring results resistant to a variety of β-lactamases (Murray *et al*., 1999).

Spectrum of Activity (Murray *et al*.,1999).

Cephalosporins are classified by a well accepted but somewhat arbitrary scheme of grouping by generations that is based on general features of their antibacterial activity (Table 1).

Table 1: Cephalosporins (Murray *et al*.,1999).

Narrow spectrum (first generation)	**Expand spectrum (second generation)**	**Broad spectrum (third generation)**	**Extended spectrum (fourth generation)**
Cefadroxil	Cefaclor	Cefdinir	Cefepime
Cefazolin	Cefamandole	Cefixime	Cefpirome
Cephaloridine	Cefonicid	Cefoperazone	
Cephalothin	Ceforanide	Cefotaxime	
Cephapirin	Cefuroxime	Cefpodoxime	
Cephradine	Cefprozil	Ceftazidime	
	Loracarbef	Ceftibuten	
	Cefmetazole	Ceftizoxime	
	Cefotetan	Ceftriaxone	
	Cefoxitin		

The first-generation (narrow-spectrum) drugs have good activity against Gram-positive bacteria and relatively modest activity against Gram-negative bacteria.

The second-generation (expanded-spectrum) cephalosporins are stable to certain β-lactamases found in Gram-negative bacteria and as a result have increased activity against Gram-negative organisms. The agents are more active than narrow-spectrum drugs against *Escherichia coli, Klebsiella* spp., and *Proteus* spp. Their activity also extends to correct some *Enterobacter* and *Serratia* strains, and they have good activity against *Haemophilus* spp., *Neisseria* spp., and many anaerobes.

The third-generation (broad-spectrum) cephalosporins are generally less active than the narrow-spectrum agents against Gram-positive cocci, but they are much more active against the *Enterobacteriaceae* and *Pseudomonas aeruginosa.* Their potent broad spectra of activity against Gram-negative bacteria are due to their stability to β-lactamases and their ability to pass through the outer cell envelopes of Gram-negative bacilli. Because of its long half-life in serum, ceftriaxone is used frequently in outpatient antibiotic therapy of serious infections.

The fourth-generation (extended-spectrum) cephalosporins are active against stably derepressed class I β-lactamases mutants of the *Enterobacteriaceae* and *P.aeruginosa* and penetrate well through Gram-negative bacterial outer membrane, due to a quaternary nitrogen substitution that makes them zwitterions (net neutral charge).

1.2. Clavulanic acid

Clavulanic acid, β-lactamase inhibitors, inhibits β-lactamases from staphylococci and many Gram-negative bacteria. This agent acts primarily as a "suicide inhibitor" by forming an irreversible acyl enzyme complex with the β-lactamase, leading to loss of activity of the enzyme. Clavulanic acid acts synergistically with various penicillins and cephalosporins against β-lactamase-producing bacteria. Recently discovered plasmid mediated TEM β-lactamase in ceftazidime-resistant strains of *Klebsiella pneumoniae* and *E.coli* are inactivated by this agent. However, the inducibleβ-lactamases (chromosomal class I) of *Enterobacter, Citrobacter, Proteus, Acinetobacter, Serratia* and *Pseudomonas* spp. are not inhibited by clavulanic acid (Wiedemann *et al.,*1989)

2. Mechanisms of Resistance to β-lactam

Bacterial resistance to antibiotic therapy may be caused by several mechanisms. Clinically important resistance is caused primarily by three mechanisms:

2.1 Enzymatic Destruction: (β-lactamases)

The best-known example of enzymatic inactivation of antibiotics is that of the β-lactamases. These enzymes render β-lactam antibiotics inactive by cleaving the lactam ring of susceptible antibiotics via an irreversible hydroxylation of the amide bond (Figure 2). β-lactamases are a common resistance mechanism among Gram-positive and Gram-negative organisms, both aerobic and anaerobic. Gram-positive bacteria produce large quantities of β-lactamase, which they must excrete into their external environment to inactivate the targeted antibiotic before it reaches the organism. For Gram-negative bacteria, the β-lactamases are contained in periplasmic space, and may be produced in much smaller quantities (Bush, 1995).

2.2 Alteration of the Target Site:

The target site of β-lactam antibiotics is a group of enzymes (peptidoglycan transpeptidases) known as penicillin-binding proteins (PBPs). These enzymes or PBPs are vital for synthesis and maintenance of the bacterial cell wall. Binding of a β-lactam antibiotic to a PBP results in rapid cell death. Some bacteria have adapted by changing the structure of their PBPs so that they bind less avidly to these antibiotics (Danziger and Pendland, 1995).

2.3 Decreased Access to Target Site: (Decreased Uptake or Increased Efflux)

Gram-negative bacteria have an outer phospholipid/lipopolysaccharide membrane with pores, called porins, which allow some antibiotics access to the bacterial cell wall. By modifying these porin channels in the outer membrane, bacteria can prevent various antibiotics from reaching the target site (Gold and Moellering, 1996).

3 Resistance to β-lactam Due to β-lactamases

Of the various mechanisms of acquired resistance to β-lactam antibiotics, resistance due to production of β-lactamases by the cell is the most prevalent. Alterations in the preexisting PBP, acquisition of a novel PBP insensitive to β-lactam, changes in the outer membrane proteins of Gram-negative organisms and active efflux, which prevent these compounds from reaching their targets, can also confer resistance (Quintiliani *et al.,* 1999).

β-lactamases comprise a family of tremendous diversity. A number of classification schemes have been suggested according to their hydrolytic spectrum, susceptibility to inhibitors, genetic localization (plasmidic or chromosomal), gene or amino-acid sequence. An updated version proposed by Bush, Jacoby, and Medeiros (1995) includes both plasmid- and chromosome-specified enzymes and β-lactamases are placed into functional group based on substrate and inhibitor profile and molecular structure as shown in Table 2 (Bush *et al.,* 1995).

Group 1 comprises cephalosporinases that are not well inhibited by clavulanic acid.

Group 2 includes penicillinases, cephalosporinases, and broad-spectrum ß-lactamases that are generally inhibited by active site-directed ß-lactamase inhibitors such as clavulanic acid.

Group 3 consists of metallo-ß-lactamases that hydrolyze penicillins, cephalosporins, and carbapenems and that are poorly inhibited by almost all ß-lactam-containing molecules.

Group 4 comprises penicillinases that are not well inhibited by clavulanic acid.

Subgroups were also defined according to rates of hydrolysis of carbenicillin or cloxacillin (oxacillin) by group 2 penicillinases. The classification initially introduced by Ambler (1980) and based on the amino-acid sequence recognizes four molecular classes designated A to D. Classes A, C, and D gather evolutionarily distinct groups of serine enzymes, and class B the zinc-dependent ("EDTA-inhibited") enzymes.

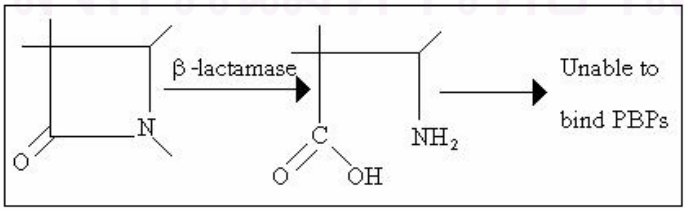


Figure 2: Mode of β-lactamase enzyme activity. By cleaving the β–lactam ring the molecule can no longer bind to penicillin binding proteins (PBPs) and is no longer able to inhibit cell wall synthesis (Forbe *et al.,* 1998).

Table 2: Classification scheme for β-lactamases (Bush *et al.*, 1995).

Functional group	Molecular class	Produced enzymes	Inhibited by		Representative enzymes	Estimated no. of enzymes	
			CA[A]	EDTA[B]		1995	2000
1	C	Ceph[C]	-	-	AmpC	32	51
2	A,D	Most enzymes				136	256
2a	A	Pen[D]	+	-	Pen from Gram positive bacteria	20	23
2b	A	Broad-spectrum enzymes	+	-	TEM-1, TEM-2, SHV-1	16	16
2be	A	ESBLs	+	-	TEM-3 to 28, SHV-2 to 6	36	119
2br	A	Broad-spectrum enzymes, Inhibitor-resistant TEM (IRT)	±[E]	-	TEM-30 to 36, TRC-1	9	24
2c	A	Carbenicillin-hydrolyzing enzymes	+	-	PSE-1,-3,-4, CARB-3	15	19
2d	D	Cloxacillin-(oxacillin)-hydrolyzing enzymes	±	-	OXA-1 to 11, PSE-2 (OXA-10)	18	31
2e	A	Ceph	+	-	Found in *P.vulgaris*	19	20
2f	A	Carbapenem-hydrolyzing nonmetallo-β-lactamases	+	-	IMI-1, NMC-A from *E.cloacae,* Sme-1 from *S.marcescens*	3	4
3 (3a, 3b, 3c)	B	Metallo-β-lactamases	-	+	L1 from *S.maltophilia,* CcrA from *B.fragilis*	13	24
4	ND[F]	Miscellaneous-Pen. Unsequenced enzymes that do not fit into other groups.	-	ND	Pen from *B.cepacia*	7	9

[A]CA = clavulanic acid, [B] EDTA = ethylenediaminetetraacetic acid, [C]Ceph = cephalosporinases, [D] Pen = penicillinases, [E] ± = low binding to CA, [F]ND = nondetermined.

By using this scheme, ESBLs (extended spectrum β-lactamases) are defined as β-lactamases capable of hydrolyzing oximino-cephalosporins that are inhibited by clavulanic acid and are placed into functional group 2be.

Knowledge of the amino acid sequence of many β-lactamases allows them to be classified in to one of the four evolutionary molecular classes (A, B, C, and D). β-lactamases of classes A, C, and D act by a serine-ester-linked acyl enzyme. Molecular class A comprises penicilinases, cephalosporinases, and broad-spectrum β-lactamases that are generally inhibited by β-lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam. This group includes the β-lactamases of *S. aureus* (Bush group 2a) and many of the plasmid specified β-lactamase of Gram-negative bacteria, such as the common TEM- and SHV-type enzymes (groups 2b and 2be). In Gram-negative bacteria, β-lactamases of molecular classes C (group 1) and D (group 2d) include the chromosomal cephalosporinases (AmpC enzymes) and oxacillin hydrolyzing enzymes (OXA), respectively. Class B (group 3) comprises the metallo-β-lactamases, which require Zn^{2+} as a cofactor. These enzymes, which have been identified in *Bacteroides fragilis, Stenotrophomonas maltophilia, Flavobacterium* spp., and *Legionella* spp., hydrolyze all classes of β-lactam including carbapenems (e.g., imipenem and meropenem), and are not inhibited by penicillinase inhibitors. Further dissemination of these potent enzymes should be expected since plasmid-mediated metallo β-lactamases have now been found in *Pseudomonas aeruginosa, B.fragilis, Serratia marcescens,* and *Klebsiella pneumoniae* (Ito *et al*., 1995; Minami *et al,* 1996; Osano *et al*., 1991).

4. History of Extended Spectrum-β-Lactamase (ESBLs)

The first plasmid-mediated β-lactamase in Gram-negative bacteria, TEM-1, was described in the early 1960s (Datta and Kontomichalou, 1965). The TEM-1 enzyme was originally found in a single strain of *E.coli* isolated from a blood culture from a patient named Temoniera in Greece, hence the designation TEM (Medeiros *et al*., 1984). Being plasmid and transposon mediated has facilitated the spread of TEM-1 to other species of bacteria. Within a few years after its first isolation, the TEM-1 β-lactamase spread worldwide and is now found in many different species of members of the family *Enterobacteriaceae, Pseudomonas aeruginosa, Haemophilus influenzae,* and *Neisseria gonorrhoeae*. Another common plasmid-mediated β-lactamase found in *Klebsiella pneumoniae* and *E.coli* is SHV-1 (for sulphydryl variable). The SHV-1 β-lactamase is chromosomally encoded in the majority of isolates of *K.pneumoniae* but is usually plasmid mediated in *E.coli* (Heritage *et al.,* 1999).

Over the last 20 years, many new β-lactam antibiotics have been developed and were specifically designed to be resistant to the hydrolytic action of β-lactamases. However, with each new class that has been used to treat patients, new β-lactamases emerged and caused resistance to that class of drug.

Presumably, the selective pressure of the use and overuse of new antibiotics in the treatment of patients has selected for new oxyimino-cephalosporins, which became widely used for the treatment of serious infections due to Gram-negative bacteria in the 1980s.

Not surprisingly, resistance to these expanded-spectrum β-lactam antibiotics due to β-lactamases emerged quickly. The first of these enzymes capable of hydrolyzing the newer β-lactams, SHV-2, was found in a single strain of *Klebsiella ozaenae* isolated in Germany (Kliebe *et al*., 1985). Because of their increased spectrum of activity, especially against the oxyimino-cephalosporins, these enzymes were called extended-spectrum β-lactamases (ESBLs). Today, over 150 different ESBLs have been described. These β-lactamases have been found worldwide in many different genera of *Enterobacteriaceae.*

5. Characterization of ESBLs (Susceptibility and Biochemical Characteristics)

ESBLs contain a number of mutations that allow them to hydrolyze expanded-spectrum β-lactam antibiotics. While TEM- and SHV-type ESBLs retain their ability to hydrolyze penicillins, they are not catalytically as efficient as the parent enzymes (Bush and Singer, 1989). In addition, the expansion of the active site that allows the increased activity against expanded-spectrum cephalosporins may also result in the increased susceptibility of ESBLs to β-lactamase inhibitors (Jacoby and Medeiros, 1991). ESBLs are not active against cephamycins, and most strains expressing ESBLs are susceptible to cefoxitin and cefotetan. However, it has been reported that ESBL-producing strains can become resistant to cephamycins due to the loss of an outer membrane porin protein (Martinez' *et al.,* 1996; Vatopoulos *et al*., 1990).

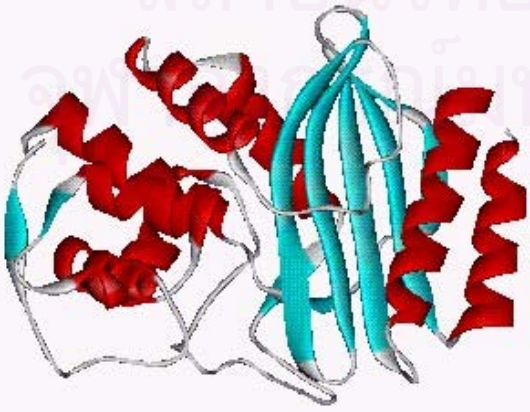


Figure 3: Tertiary structure of β-lactamase enzyme

6. Types of ESBLs

Most ESBLs are derivatives of TEM or SHV enzymes (Bush *et al*., 1995; Jacoby *et al*., 1991). There are now > 100 TEM-type β-lactamases and > 30 SHV-type enzymes With both of these groups of enzymes, a few point mutations at selected loci within the gene give rise to the extended-spectrum phenotype. TEM- and SHV-type ESBLs are most often found in *E.coli* and *K.pneumoniae*; however, they have also been found in *Proteus* spp., *Providencia* spp., *Pseudomonas aeruginosa* and other genera of *Enterobacteriaceae* (Nordmann *et al*., 1998).

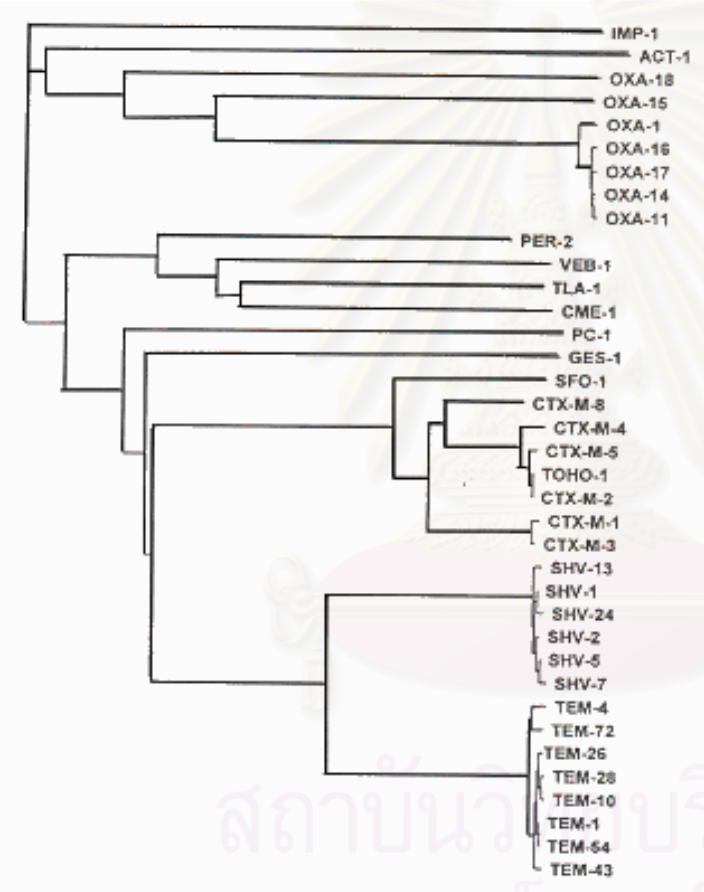


Figure 4: Phylogeny of ESBLs. Representative sequences of various ESBLs were obtained from GenBank. The PC1 (class A, *S.aureus* enzyme), IMP-1 (class B, metallo-enzyme), and ACT-1 (class C, AmpC-type enzyme) β-lactamases were included for comparison. (Bradford *et al*., 2001)

6.1 TEM

TEM-1, board spectrum β-lactamase, is the most commonly encountered β-lactamase in Gram-negative bacteria. Up to 90% of ampicillin resistance in *E.coli* is due to the production of TEM-1 (Livermore, 1995). This enzyme is also responsible for the ampicillin and penicillin resistance that is seen in *H.influenzae* and *N.gonorrhoeae* in increasing numbers. TEM-1 is able to hydrolyze penicillins and early cephalosporins such as cephalothin and cephaloridine. TEM-2, the first derivative of TEM-1, had a

single amino acid substitution from the original β-lactamase (Barthélémy *et al*., 1985). This caused a shift in the isoelectric point from a pI of 5.4 to 5.6, but it did not change the substrate profile. TEM-3, originally reported in 1989, was the first TEM-type β-lactamase that displayed the ESBL phenotype (Sougakoff *et al*, 1988). In the years since that first report, over 90 additional TEM derivatives have been described. Some of these β-lactamases are inhibitor-resistant enzymes, but the majority of the new derivatives are ESBLs. The characteristics of TEM-type-β-lactamases were shown in Table 3

The amino acid substitutions within the TEM enzyme occur at a limited number of positions as shown in Figure5. The combinations of these amino acid changes result in various subtle alterations in the ESBL phenotypes, such as the ability to hydrolyze specific oxyimino-cephalosporins such as ceftazidime and cefotaxime, or a change in their isoelectric points, ranging from a pI of 5.2 to 6.5. A number of amino acid residues are especially important for producing the ESBL phenotype when substitutions occur at the position (Bradford *et al*., 2001).

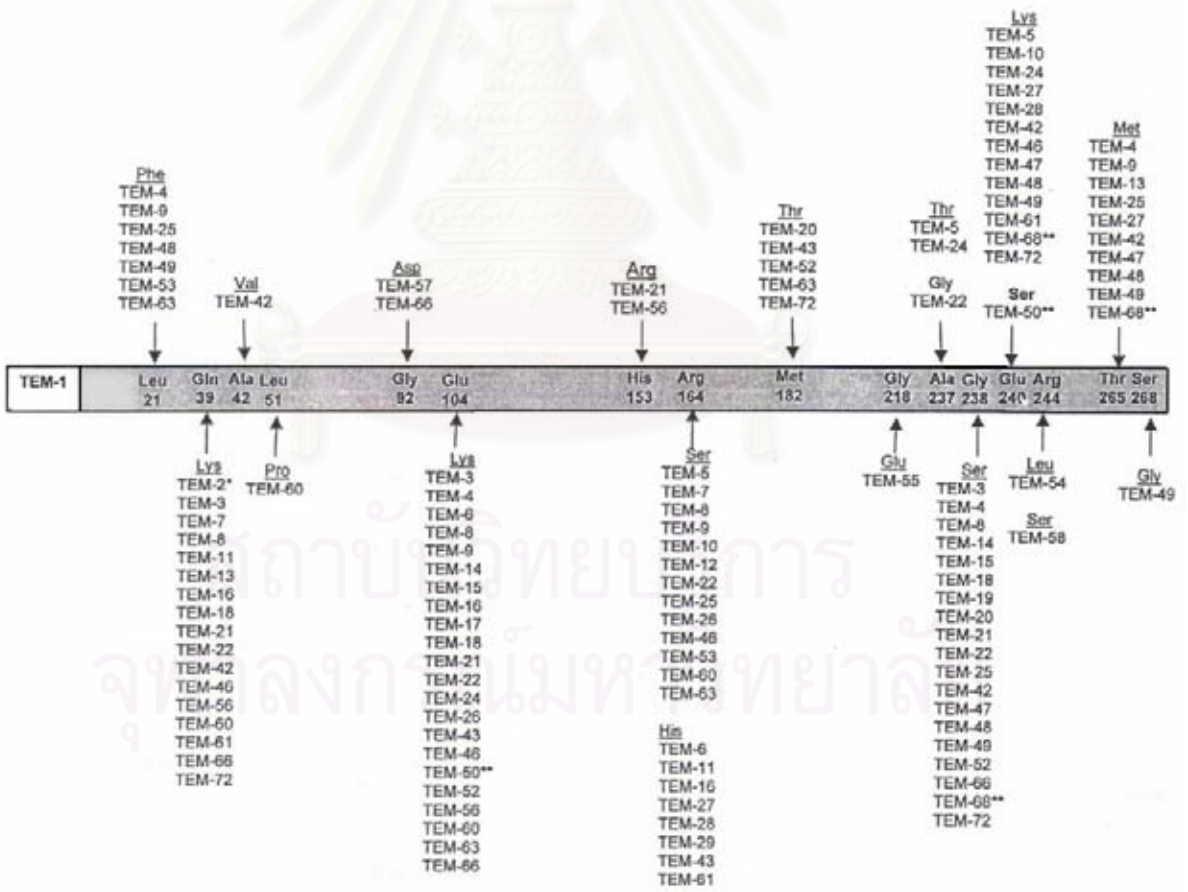


Figure 5: Amino acid substitutions in TEM ESBL derivatives (Bradford *et al*., 2001).

6.2 Inhibitor-Resistant β-Lactamases (IRT)

Although the inhibitor-resistant β-lactamases are not ESBLs, they are often discussed with ESBLs because they are also derivatives of the classical TEM- or SHV-type enzymes. In the early 1990s, β-lactamases resistant to inhibition by clavulanic acid were discovered. Nucleotide sequencing revealed that these enzymes are variants of the TEM-1 or TEM-2 β-lactamases. These enzymes were at first given the designation IRT for inhibitor-resistant TEM β-lactamases (Knox, 1995); however, all have subsequently been renamed with numerical TEM designations. There are at least 19 distinct inhibitor-resistant TEM β-lactamases. IRT have been found mainly in clinical isolates of *E.coli,* but also some strains of *K.pneumoniae, K.oxytoca, P.mirabilis,* and *Citrobacter freundii* (Bret *et al*., 1996; Lemozy *et a*l., 1995). The amino acid substitution in IRT is shown in Figure 6.

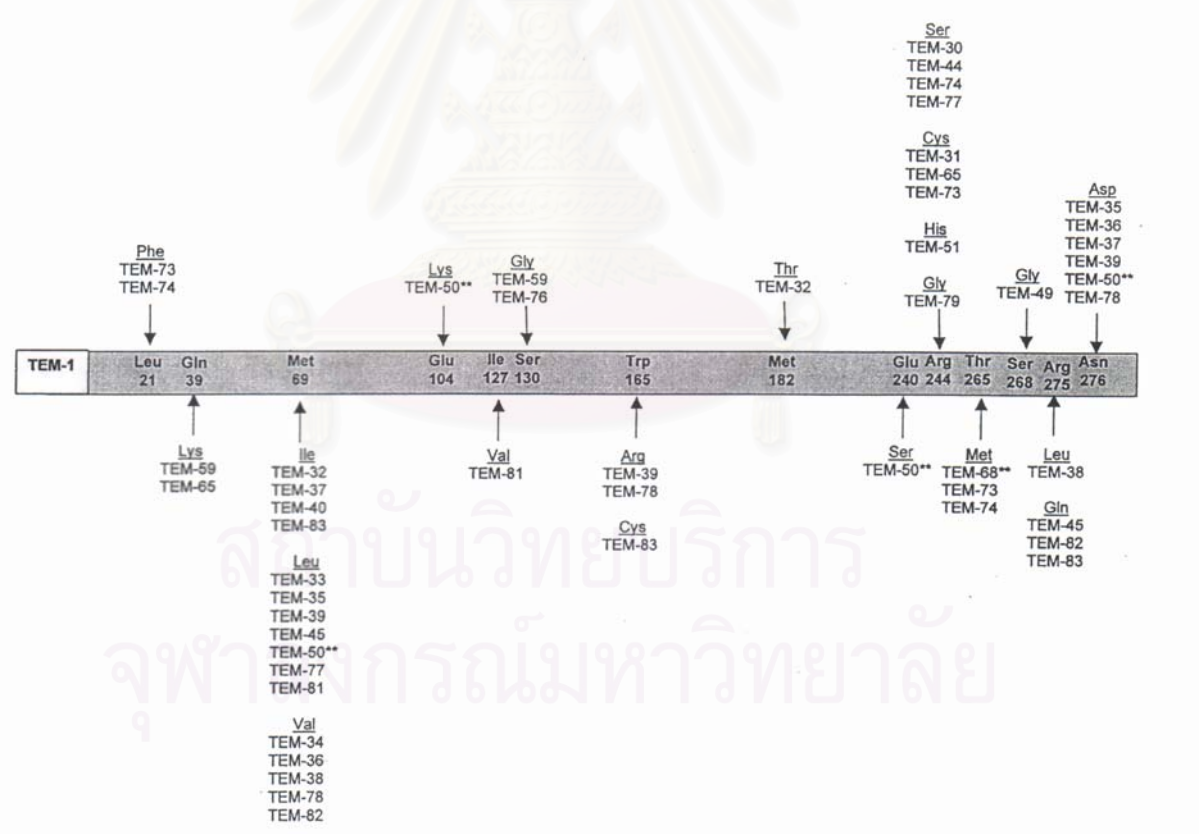


Figure 6: Amino acid substitutions in TEM IRT derivatives (Bradford *et al*., 2001).

Table 3: Characteristics of TEM-type-β-lactamases (Bradford *et al*., 2001).

pI*	Enzymes	Enzyme type		
		Broad spectrum	ESBL	IRT
5.2	TEM-12, TEM55, TEM-57, TEM-58		X	
	TEM-30, TEM-35, TEM-36,TEM-37,TEM-38,TEM-41,TEM-45			X
5.3	TEM-25		X	
5.4	TEM-1	X		
	TEM-7, TEM-19, TEM-20, TEM-65		X	
	TEM-32, TEM-33, TEM-34, TEM-39, TEM-40, TEM-44			X
5.42	TEM-29		X	
5.55	TEM-5, TEM-17		X	
5.59	TEM-9		X	
5.6	TEM-2	X		
	TEM-10, TEM-11, TEM-13, TEM-26, TEM-63		X	
	TEM-50		X	X
	TEM-59			X
5.7	TEM-68		X	X
5.8	TEM-42		X	
5.9	TEM-4, TEM-6, TEM-8, TEM-27, TEM-72		X	
6.0	TEM-15, TEM-47, TEM-48,TEM-49,TEM-52,TEM-66,TEM-92		X	
6.1	TEM-28, TEM-43		X	
6.2	TEM-3, TEM-16, TEM-21, TEM-22		X	
6.3	TEM-56, TEM-60		X	
6.4	TEM-56, TEM-60		X	
6.5	TEM-24, TEM-46, TEM-61		X	
Not determined	TEM-14, TEM-53, TEM-54		X	
	TEM-76, TEM-77, TEM-78,TEM-79,TEM-81,TEM-82,TEM-83,TEM-84			X

*pI ; Isoelectric point.

6.3 SHV

The majority of SHV-type ESBLs are found in strains of *K.pneumoniae.* However, these enzymes have also been found in *Citrobacter diversus, E.coli,* and *P.aeruginosa* (El Harrif-Heraud *et al*., 1997; Naas *et al*., 1999; Rasheed *et al*., 1997). Unlike the TEM-type β-lactamases, there are relatively few derivatives of SHV-1 β-lactamase (Table 4). Furthermore, the changes that have been observed in bla_{SHV} to give rise to the SHV variants occur in fewer positions within the structural gene (Figure.7). The majority of SHV variants possessing an ESBL phenotype are characterized by the substitution of a serine for glycine at position 238. A number of variants related to SHV-5 also have a substitution of lysine for glutamate at position 240. The serine residue at position 238 is critical for the efficient hydrolysis of ceftazidime, and the lysine residue is critical for the efficient hydrolysis of cefotaxime (Huletsky *et al*., 1993). To date, the majority of SHV-type derivatives possess the ESBL phenotype. However, one variant, SHV-10, is reported to have an inhibitor-resistant phenotype (Prinarakis *et al*., 1997).

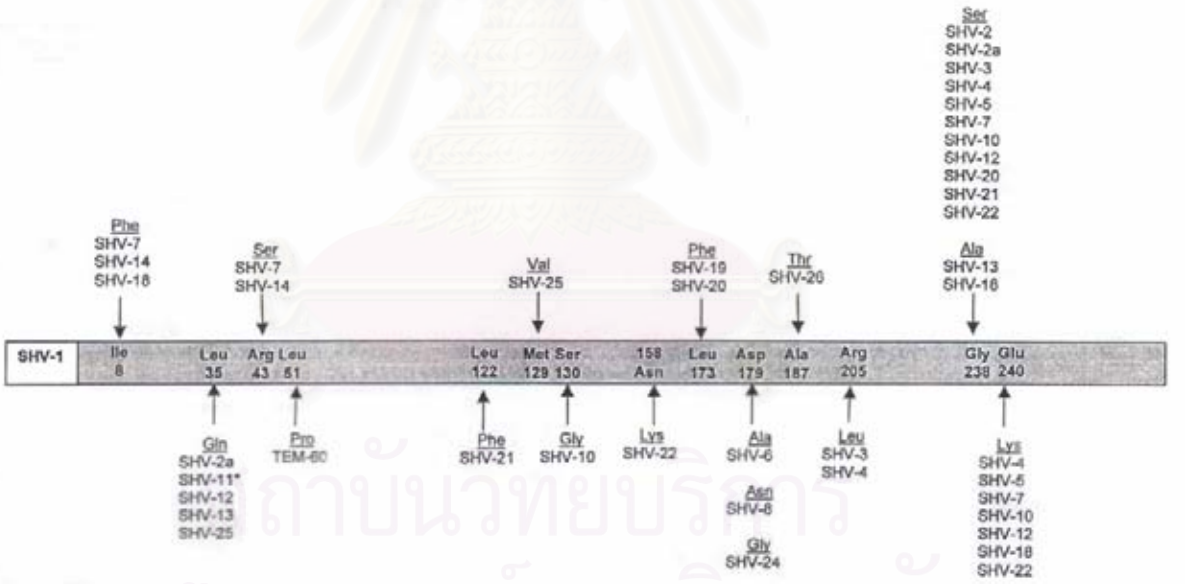


Figure 7: Amino acid substitutions in SHV ESBL derivatives (Bradford *et al*., 2001).

Table 4: Characteristics of SHV-type-β-lactamases (Bradford *et al*., 2001).

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

*pI ; Isoelectric point.

6.4 CTX-M

In recent years a new family of plasmid-mediated ESBLs, called cefotaximases (CTX-M), were characterized at the beginning of the 1990s. Until now, there are more than 20 types reported from many countries. In contrast to TEM and SHV type, CTX-M preferentially hydrolyze cefotaxime over ceftazidime (Bonnet *et al*.,2000). Although there is some hydrolysis of ceftazidime by these enzymes, it is usually not enough to provide clinical resistance to organisms in which they reside. CTX-M ESBLs have mainly been found in strains of *Salmonella enterica* serovar Typhimurium and *E.coli,* and have also been described in other species of *Enterobacteriaceae* (Bauernfeind *et al.,* 1990; Bonnet *et al*., 2000; Gazouli *et al*., 1998).

Strains expressing CTX-M-type β-lactamases have been isolated from many parts of the world, but have most often been associated with focal outbreaks in Eastern Europe (Bradford *et al*., 1998), South America, and Japan. Several institutions in the areas where outbreaks have occurred reported that the CTX-M type enzyme is the most frequently isolated ESBL among clinical isolates in their laboratories (Sabate *et al*.,2000).

6.5 OXA

The OXA-type enzymes are another growing family of ESBLs. These β-lactamases differ from the TEM and SHV enzymes in that they belong to molecular class D and functional group 2d. The OXA-type β-lactamases confer resistance to ampicillin and cephalothin and are characterized by their high hydrolytic activity against oxacillin and cloxacillin and the fact that they are poorly inhibited by clavulanic acid (Bush *et al*., 1995). While most ESBLs have been found in *E.coli* and *K.pneumoniae,* and other *Enterobacteriaceae,* the OXA-type ESBLs have been found mainly in *P.aeruginosa* (Philippon *et a*l., 1997).

6.6 Other ESBLs

While the majority of ESBLs are derived from TEM or SHV β-lactamases and others can be categorized with one of the newer families of ESBLs, a few ESBLs have been reported not to be closely related to any of the established families of β-lactamases (Figure 4). These enzymes, PER-1, PER-2, VEB-1, CME-1 and TLA-1 β-lactamases, confer resistance to oxyimino-cephalosporins, especially ceftazidime, and aztreonam (Rossolini *et al*., 1999; Silva *et al*., 2000). VEB-1 (Vietnamese extended-spectrum β-lactamase) was first found in a single isolate of *E.coli* in a Vietnamese patient in France, but was subsequently also found in a *P.aeruginosa* isolate from a patient from Thailand (Girlich *et al*.,2001).

7. Laboratory Identification : (Clinical Microbiology Techniques)

ESBL-mediated resistance may appear falsely susceptible to certain extended-spectrum cephalosporins in *in vitro* susceptibility tests, as the MICs of cephalosporins for producers are often 0.5-2 mg/l, and inhibition zones of disks are correspondingly large. Some of these strains will show zones of inhibition below the normal susceptible population but above the standard breakpoints for certain extended-spectrum cephalosporins or aztreonam; such strains may be screened for potential ESBL production by using the screening breakpoints listed in the Table 5. Nevertheless, such ESBLs have been associated with clinical failure in patients and in experimental animals (Brun-Buisson *et al*., 1987; Rice *et al*., 1991), susgesting that reliable detection is required. Various tests have, therefore, been developed to

detect ESBLs in *Klebsiella* (Table 6), the main host genus and are equally applicable to other *Enterobacteriaceae* with little or no chromosomal β-lactamase activity, e.g. *E.coli* and *P. mirabilis* (Livermore and Brown, 2001).

TEM- and SHV-derived ESBLs are the most common cause of resistance to oxyimino-aminothiazolyl cephalosporins in *Klebsiellae* and *E.coli* (Livermore and Williams, 1996). In order to detect ESBLs producers, ceftazidime or cefpodoxime should be included in all first-line susceptibility testing against isolates of these species and ESBL production should be suspected in those that show resistance. Ceftazidime and cefpodoxime are chosen because they are the best third-generation cephalosporins substrates for most TEM- and SHV-derived ESBLs (Emery *et al*., 1997). ESBL production can then be confirmed with the double disk or commercial tests. Other oxyimino-aminothiazolyl cephalosporins are less reliable indicators (Livermore and Brown, 2001). However, more recent data suggest that susceptibility testing with cefpodoxime can lead to a high number of false-positives if the current National Committee for Clinical Laboratory Standards (NCCLS) interpretive criteria, as shown in Table 5, are applied (Tenover *et al*., 2000).

7.1 NCCLS Initial and Confirmatory Test

Currently, the NCCLS recommends an initial screening by disk diffusion test. Diminished zones of inhibition around third-generation β-lactam disks were interpreted as positive results. The β-lactams and zone diameter breakpoints used were; cefpodoxime, ≤ 22 mm; ceftazidime, ≤ 22 mm; cefotaxime, ≤ 27 mm; ceftriaxone, ≤ 25 mm; and aztreonam, ≤ 27 mm as listed in Table 5. (NCCLS, 2001). A positive result is reported as suspicious for the presence of an ESBL. This screen is then followed by a phenotypic confirmatory test that consists of determining MICs of either ceftazidime or cefotaxime with and without the presence of clavulanic acid (4 μg/ml). A decrease in the MIC of ≥ 3 twofold dilutions in the presence of clavulanate is indicative of the presence of an ESBL. If an ESBL is detected, the strain should be reported as not susceptible to all expanded-spectrum cephalosporins and aztreonam regardless of the susceptibility testing result (NCCLS, 2001:39).

Table 5: Zone diameter interpretive standards for disk diffusion susceptibility testing (NCCLS, 2001:39).

Antibiotic	Zone diameter for susceptible strains	Zone diameter breakpoint for possible ESBL-producing strains
Cefpodoxime10 μg	≥ 21 mm	≤17 mm
Ceftazidime 30 μg	≥ 18 mm	≤22 mm
Cefotaxime 30 μg	≥ 23 mm	≤ 27 mm
Aztreonam 30 μg	≥ 22 mm	≤ 27 mm
Ceftriaxone 30 μg	≥ 21 mm	≤ 25 mm

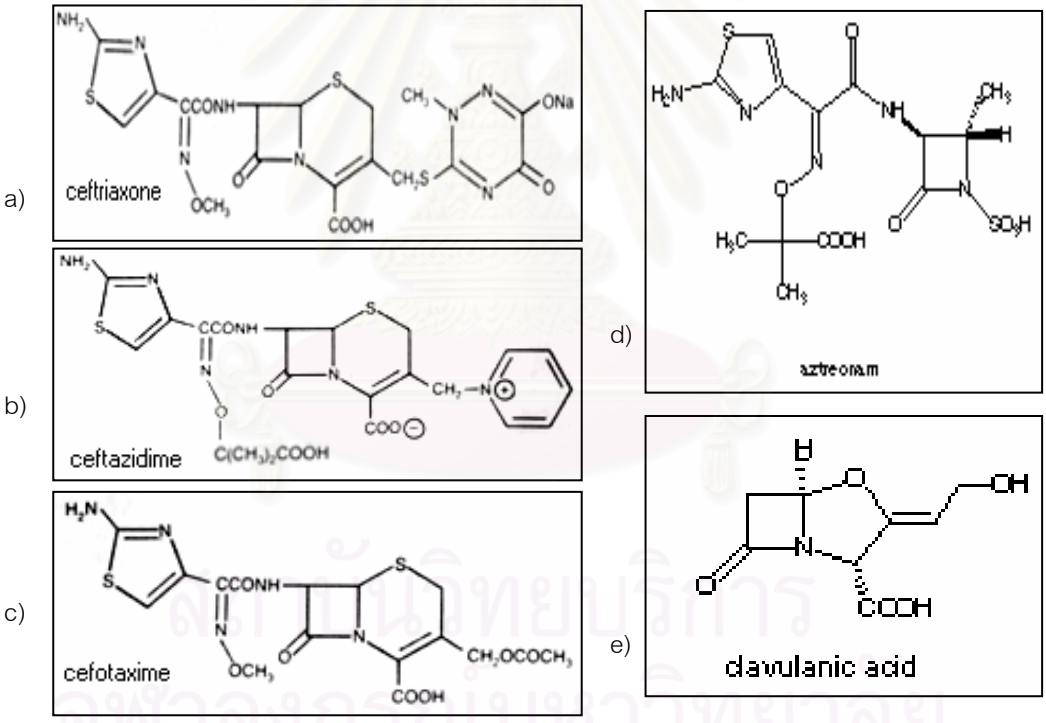


Figure 8: Chemical structures of β-lactam antibiotics used in screening tests (Woster, 1999).

7.2 Double-Disk Synergy Test (DDST).

This test is most widely used due to its simplicity and ease of interpretation. Expanded-spectrum β-lactam disks (ceftazidime, cefotaxime, ceftriaxone and aztreonam) were placed 25-30 mm (center to center) away from a disk containing a β-lactamase inhibitor (10/20 μg clavulanic acid/amoxicillin). A clear extension of the edge of the inhibition zone of any of the antibiotics towards the disk containing clavulanic acid was interpreted as positive for ESBL production (Jarlier *et al*., 1988).

7.3 Combined Disk Methods

Combined disk methods depend on comparing the zones given by disks containing extended-spectrum cephalosporins with and without clavulanic acid. ESBL production is inferred if the zones given by the disks with clavulanic acid are ≥ 5 mm larger than those without the inhibitor. (Livermore and Brown, 2001).

7.4 Inhibitor-Potentiated Disk Diffusion (IPD)

IPD test is another useful test for detection of ESBL activity by measuring the zone augmentation. The bacterial strains were tested in pairs of Mueller Hinton (MH) agar with and without clavulanic acid (4 μg/ml) using the disc diffusion method. Synergy with clavulanic acid was measured as augmentation zone widths. An augmentation zone width of ≥ 10 mm (to any of the agents: ceftazidime, cefotaxime, ceftriaxone, cefpodoxime, ceftibutem, aztreonam) in the clavulanate-containing MH agar was considered positive for ESBL production (Ho *et al*., 1998).

7.5 Three-Dimensional Test (3-D).

Three-dimensional test is based on the Kirby-Bauer disk diffusion test methodology, After inoculation of the test organism onto the surface of a Mueller-Hinton agar plate, a slit is cut into the agar, into which a broth suspension of the test organism is introduced. Subsequently, antibiotic disks are placed on the surface of the plate 3 mm from the slit. Distortion or discontinuity in the expected circular zone of inhibition is considered a positive test (Thomson and Sanders., 1992).

7.6 Etest ESBL Strip (AB Biodisk) .

The Etest ESBL, automated susceptibility testing systems, consist of CT/CTL and TZ/TZL strips. CT codes for the cefotaxime gradient and CTL for the cefotaxime gradient plus 4 μg/ml clavulanic acid. TZ codes for the ceftazidime gradient and TZL for the ceftazidime gradient plus 4 μg/ml clavulanic acid. Etest with a CT or TZ gradient at one end and a CT or TZ plus clavulanic acid at the other can be used to detect ESBLs. They should be used in accordance with the manufacturer's package insert. If the ratio of the MIC of CT or TZ to the MIC of CT or TZ plus clavulanic acid (CT/CTL or TZ/TZL) is $\geq$ 8, ESBL production is inferred.

7.7 VITEK ESBL Cards (bio Me'rieux).

VITEK ESBL cards, another commercial manufacturers, have developed ESBL detection tests that can be used along with MIC test methods already in place in the clinical laboratory. Organisms and the antimicrobial agents are brought together in a liquid medium that is distributed to 30 or 45 small wells contained in a small plastic card. Each well contains a specified concentration of antimicrobial agent. The inoculation of the card, which is automated through the use of a filling-sealer module, results in the distribution of inoculum to each of the antibiotic containing wells in the card. Once inoculated, the cards are placed in an incubator-reader module. A predetermined reduction in growth in wells containing clavulanic acid (4μg/ml) compared to those containing drug alone indicates the presence of an ESBL (Sander *et al*., 1996).

Table 6: Clinical Microbiology for ESBL detection techniques (Bradford, 2001).

Test	Advantages	Disadvantages
Standard NCCLS interpretive criteria	Easy to use, performed in every lab.	ESBLs not always "resistant"
NCCLS ESBL confirmatory test	Easy to use and interpret	Sensitivity depends on choice of oxyimino-cephalosporin
Double disk test	Easy to use, easy to interpret	Distance of disk placement for optimal sensitivity not standardized
Combined disk methods	Easy to use, easy to interpret	Sensitivity depends on choice of oxyimino-cephalosporin
Inhibitor-potentiated disk diffusion (IPD)	Easy to use, easy to interpret	Sensitivity depends on choice of oxyimino-cephalosporin
Three-dimensional test	Sensitive, easy to interpret	Not specific for ESBLs, labor intensive
Etest ESBL strips	Easy to use	Not always easy to interpret, not as sensitive as double-disk test
Vitek ESBL test	Easy to use, easy to interpret	Reduced sensitivity

8. Molecular Detection Methods

The tests described above presumptively identify the presence of an ESBL. The identification of specific ESBL present in a clinical isolate is more complicated and not used for the routine antimicrobial susceptibility test. The following methods have been identified the specific ESBLs (Table 7).

8.1 Isoelectric Focusing

In the early days of studying ESBLs, determination of the isoelectric point was usually sufficient to identify the presence of ESBL. However, with >90 TEM-type β-lactamases, many of which possess identical isoelectric points, determination of the ESBL by isoelectric point is no longer possible. A similar situation is found in the SHV, CTX-M, and OXA families of ESBLs (Matthew *et al*., 1975).

8.2 DNA Probe

Early detection of β-lactamase genes was performed using DNA probes that were specific for TEM and SHV enzymes (Arlet and Philippon, 1991). However using DNA probes can sometimes be rather labor intensive.

8.3 Polymerase Chain Reaction (PCR)

PCR is easiest and most common molecular method used to detect the presence of a β-lactamase belonging to a family of enzymes with oligonucleotide primer that are specific for a β-lactamase gene. Oligonucleotide primers can be chosen from sequences available in public databases such as Genbank (Genbank, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/Genbank/index.html). These primers are usually chosen to anneal to regions where various point mutations are not known to occur. However PCR will not discriminate among different variants of TEM or SHV.

8.4 Oligonucleotide Probe

Oligonucleotide probe is the first molecular method for the identification of β-lactamase was the oligotyping method developed by Ouellette *et al*., which was used to discriminate between TEM-1 and TEM-2. This method used oligonucleotide probes that are designed to detect point mutations under stringent hybridization conditions. Subsequently, Mabilat and Courvalin developed additional oligonucleotide probes to detect mutations at six positions within the bla_{TEM} gene. Using this method, several new TEM variants were identified within a set of clinical isolates. The probes used in oligotyping tests for TEM β-lactamases have been labeled either with a radioisotope or with biotin (Mabilat and Courvalin, 1990; Tham *et al*., 1990).

8.5 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP).

In this test, amplified PCR products were digested with several restriction endonucleases, and the subsequent fragments were separated by electrophoresis. The sizes of fragments generated by each restriction enzyme indicate point mutations within the structural bla_{TEM} gene. For detection and identification of SHV derivatives, the PCR product is digested with restriction enzyme *Nhe*I, which detects the G- to-A nucleotide change that gives rise to the glycine to serine substitution at position 238,

which is common to many of early SHV –type ESBL. Although this method cannot determine which SHV-type ESBL is present, it can detect the specific mutation at position 238 (Nüesch-Inderbinen and Hächler, 1996).

8.6 PCR-Single Strand Conformational Polymorphism (PCR-SSCP).

PCR-SSCP has been used to detect a single base mutation at specific location within the bla_{SHV} gene. In this test, a 475-bp amplimer is generated by using oligonucleotide primers that are internal to the coding sequence of the bla_{SHV} gene and is digested with restriction enzyme *Pst*I. The fragments are then denatured and separated on a 20% polyacrylamide gel. Genes for SHV-1, -2, -3, -4, -5, and –7 β-lactamases can be identified by the electrophoretic pattern of the digested amplimer (M'Zali *et al*.,1996).

PCR-RFLP was developed to identify some of the newer SHV variants. Following PCR, a variety of restriction endonucleases were used to detect 12 mutations at 11 positions within the bla_{SHV} structural gene. The combination of PCR-SSCP and PCR-RFLP allows the identification of 17 different SHV genes (Chanawong *et al*., 2000).

8.7 Ligase Chain Reaction (LCR)

LCR allows the discrimination of DNA sequences that differ by a single base pair by the use of a thermostable ligase with four oligonucleotide primers that are complimentary to the target sequence and hybridize adjacent to each other. A single base mismatch in the oligonucleotide junction will not be ligated and subsequently amplified. In this LCR test, the target DNA containing the bla_{SHV} gene is denatured in a thermocycler and annealed with biotinylated oligonucleotide primers that detect mutations at four positions. The LCR product is detected by an enzymatic reaction using NADPH-alkaline phosphatase. This method was able to detect seven of the SHV variants (Kim and Lee, 2000).

8.8 Nucleotide Sequencing

Nucleotide sequencing remains the standard for determination of the specific β-lactamase gene present in a strain. However, the variability can be seen in the sequences for some of the SHV β-lactamases due to compressions and difficulty in reading traditional sequencing autoradiographs, rather

than actual differences in the sequence. Amino acid sequences of the β-lactamases were deduced and compared with other on website www.lahey.org/studies/webt.htm (Jacoby and Bush.,2001).

Table 7: Molecular detection techniques for ESBL (Bradford, 2001).

Test	Advantages	Disadvantages
DNA probes	Specific for gene family (e.g., TEM or SHV)	Labor intensive, cannot distinguish between ESBLs and non-ESBLs, cannot distinguish between variants of SHV or TEM
PCR	Easy to perform, specific for gene family (e.g., TEM or SHV)	Cannot distinguish between ESBLs and non-ESBLs , cannot distinguish between variants of SHV or TEM
Oligotyping	Detects specific TEM variants	Requires specific oligonucleotide probes, labor intensive, cannot detect new variants
PCR-RFLP	Easy to perform, can detect specific nucleotide changes	Nucleotide changes must result in altered restriction site for detection
PCR-SSCP	Can distinguish between a number of SHV variants	Requires special electrophoresis conditions
LCR	Can distinguish between a number of SHV variants	Requires a large number of oligonucleotide primers
Nucleotide sequencing	The gold standard, can detect all variants	Labor intensive, can be technically challenging, can be difficult to interpret manual methods

9. Epidemiology

ESBLs are now a problem in hospitalized patients worldwide. The ESBL phenomenon began in western Europe, most likely because expanded-spectrum β-lactam antibiotics were first used there clinically. However, it did not take long before ESBLs had been detected in the United States and Asia. The prevalence of ESBLs among clinical isolates varies from country to country and from institution to institution. In the United States, occurrence of ESBL production in *Enterobacteriaceae* ranges from 0 to 25%, depending on the institution, with the national average being around 3% (EDC National Nosocomial infections Surveillance, http://www.cdc.gov/ncidod/hip/SUR-VEILL/NNIS.HTM) Among isolates of *K.pneumoniae*, the percentage of ceftazidime resistance ranges from 5 to 10% for non-intensive care unit (non-ICU) and ICU isolates respectively.

In Europe the prevalence of ESBL production among isolates of *Enterobacteriaceae* varies greatly from country to country. In the Netherlands, a survey of 11 hospital laboratories showed that < 1% of *E.coli* and *K.pneumoniae* strains possessed an ESBL (Stobberingh *et al*., 1999). However, in France, as many as 40% of *K.pneumoniae* isolates were found to be ceftazidime resistant (Branger *et al*., 1998). Across Europe, the incidence of ceftazidime resistance among *K.pneumoniae* strains was 20% for non-ICU isolates and 42% for isolates from patients in the ICU. In Japan, the percentage of β-lactam resistance due to ESBL production in *E.coli* and *K.pneumoniae* remains very low. In a recent survey of 196 institutions across Japan, < 0.1% of *E.coli* and 0.3% of *K.pneumoniae* strains possessed an ESBL (Yagi *et al*., 2000). Elsewhere in Asia, the percentage of ESBL production in *E.coli* and *K.pneumoniae* varies, from 4.8% in Korea to 8.5% in Taiwan and up to 12% in Hong Kong (Ho *et al*, 2000; Pai *et al*, 1999; Yan *et al*., 2000).

It is interesting that specific ESBLs appear to be unique to a certain country or region. For example, TEM-10 has been responsible for several unrelated outbreaks of ESBL-producing organisms in the United States for a number of years (Bradford *et al*., 1994; Naumovski *et al*., 1992; Rice *et al*., 1990; Urban *et al*., 1994). However, TEM-10 has only recently been reported in Europe with the same frequency (Barroso *et al*., 2000; Liu *et al*., 1992). Similarly, TEM-3 is common in France but has not been detected in the United States (Nordmann *et al.,* 1998; Soilleux *et al*., 1996). In recent years, there have been reports of outbreaks of TEM-47 producing organisms in Poland (Gniadkowski *et al.,* 1998), and the prevalence of TEM-52 in Korea is unique to that country (Pai *et al*., 1999). Another recent survey of Korea revealed that the SHV-12 and SHV-2a β-lactamases are the most common ESBLs found in Korea (Kim *et al*., 1998). In contrast, the SHV-5 β-lactamase is commonly encountered worldwide and has been reported in Croatia, France, Greece, Hungary, Poland, South Africa, the United Kingdom, and the United States (Gniadkowski *et al*., 1998; Pitout *et al*., 1998; Shannon *et al*., 1998; Szabo *et al*., 1999; Vatopoulos *et al.,* 1995).

In Thailand, the data of ESBL-producing strains remain limited. In this study, disk diffusion, double disk synergy test and Etest were used to screen ESBL producer. The positive results were subjected to amplification by polymerase chain reaction (PCR) and sequencing in order to identify specific ESBLs. The data will be useful in apply of techniques to detect ESBL-producing strains in our laboratory.

CHAPTER IV

MATERIALS AND METHODS

Methodology Scheme

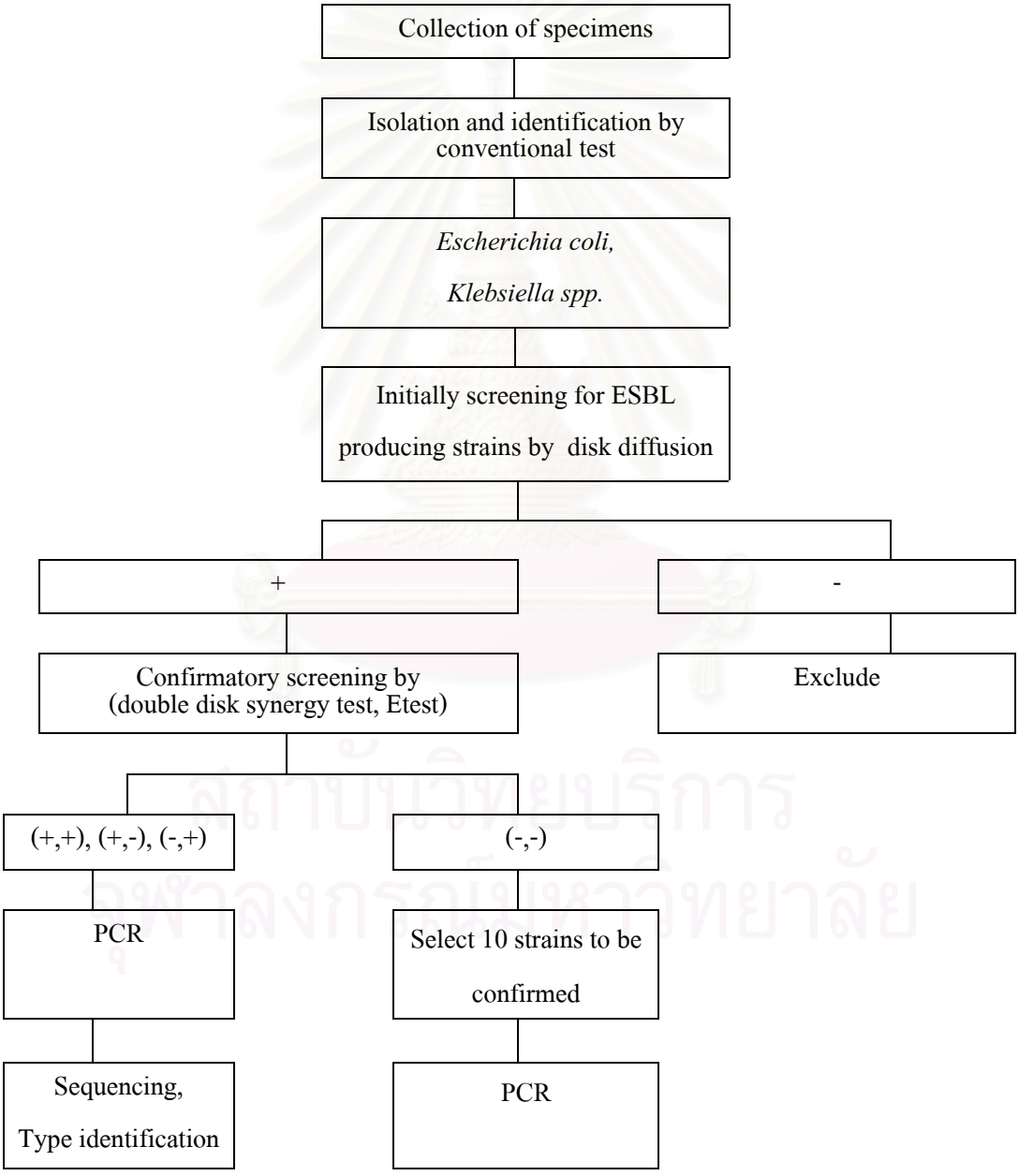


Figure 9: Methodology scheme

PART I : CLINICAL ISOLATES

The strains of *Escherichia coli* and *Klebsiella* spp. were isolated from clinical specimens such as urine, sputum, body fluid, pus from various sites of different patients and were collected from Department of Microbiology in King Chulalongkorn Memorial Hospital during February to May 2002. They were identified as *E.coli* and *Klebsiella* spp. respectively according to the Manual of Clinical Microbiology (Murrey *et al*., 1999). The strains were detected ESBLs by screening method then amplified by PCR and sequencing.

Criteria for Nosocomial Infections (Fauci *et al*., 1998).

The clinical data of patients were reviewed and analyzed for the prevalence of nosocomial infection using the criteria according to Fauci *et al.,* 1998. Illustrated that, a patient who had been hospitalized and developed an infection, which had been manifested after 48 hours are considered to be nosocomial. A patient may develop a nosocomial infection after being discharged from the hospital if the organism apparently was acquired in the hospital.

PART II : IDENTIFICATION

The pure culture of organisms was identified as followed.

1. Conventional Identification

1.1 Colony Morphology

The organisms were sub cultured on MacConkey agar plates and incubated at 37°C in ambient air for 18-24 hours. *E.coli* and *Klebsiella* spp. are lactose fermenter. *E.coli* are, flat, dry, pink colonies with a surrounding darker pink area of precipitated bile salts, 2-3 mm and *Klebsiella* spp. are mucoid, 3-4 mm.

1.2 Biochemical Characteristic test

Biochemical characteristic tests were selected according to Forbes *et al*.(1998), and Koneman EW *et al*.(1992). A single colony was picked and inoculated to testing media. The biochemical test lists and interpretation criteria for *E.coli* and *Klebsiella* spp. are shown as described below.

1.2.1 Oxidase Test

An 18-24 h growth colony from nonselective media was smeared with a sterile wooden applicator stick on a filter paper impregnated with *p*-phenylenediamine dihydrochloride. Enterobacteriaceae shows unchanging color on the paper with in 10 seconds, indicating non cytochrome oxidase activity.

1.2.2 Motility

Organisms were inoculated by stabbing in to the motility medium with a depth of 5 mm and incubated at 35°C for 18 to 24 hours. The migration throughout the medium (tubid) were indicated positive result.

1.2.3 TSI Medium Test

Organisms were inoculated to Triple Sugar Iron agar by stabbing the butt and streaking the slant and the tube was incubated at 35°C for 18 to 24 hours.

- The organisms, with ferment glucose but not lactose, were recorded as K/A (alkaline (purple) slant / acid (yellow) butt).
- The organisms, with ferment glucose and lactose, were recorded as A/A (acid (yellow) slant / acid (yellow) butt).
- A black precipitation in the butt indicates the production of H_2S.

1.2.4 Indole Production

Organisms were inoculated into peptone water and incubate at 35°C for 18 to 24 hours. Add few drops of Kovac's reagent and shake well. The development of red color at the interface of the reagent and the broth within seconds after adding the reagent was indicated positive test.

1.2.5 Methyl red Reaction (MR test)

Organisms were inoculated into MR-VP medium and incubate at 35°C for 18 to 24 hours. Add 2 drops of methyl red reagent. The development of a stable red color in the medium indicates positive test. An orange or yellow color indicates negative test.

1.2.6 Voges-Proskauer (VP test)

Organisms were inoculated into MR-VP medium and incubate at 35°C for 18 to 24 hours. Add 0.6 ml of VP-1 reagent then follow with 0.2 ml of VP-2 reagent, shake well. Developing a red color 15 min or more after addition of the reagents was represented a positive test. The negative test was shown by unchanging color of reagent after standing for over 1 hour

1.2.7 Citrate

Organisms were inoculated onto the slope of Simmons' citrate medium surface and incubate at 35°C for 18 to 24 hours. The positive test was represented by the development of deep blue color.

1.2.8 Urease Test

Organisms were inoculated onto the slope of the agar slant and incubate at 35°C for 18 to 24 hours. The positive test was represented by the development of red or pink color. The medium of a negative test remains original yellow color.

1.2.9 Decarboxylation Reaction

Organisms were inoculated into four tubes of Falkow's decarboxylation broth containing lysine, arginine, ornithine and control tube devoid of amino acid. Overlay all tubes with sterile mineral oil to cover about 1 cm of the surface before tighten screw caps and incubated at 35°C for 18 to 24 hours. Converstion of the control tube to a yellow color was indicated that the organism was viable and that the pH of the medium has been lowered sufficiently to activate the decarboxylase enzymes. Reversion of the color to purple color was indicated a positive test.

1.2.10 Orthonitrophenyl-β -D-Galactopyranoside (ONPG)

Organisms were inoculated into ONPG broth and incubate at 35°C for 18 to 24 hours. The yellow color indicates that the organism has produced orthonitrophenol from the ONPG substrate through the action of β-Galactosidase.

1.2.11 Phenylalanine Deaminase (PD)

Organisms were inoculated onto the slope of the agar slant and incubate at 35°C for 18 to 24 hours. Add 4 or 5 drops of the ferric chloride reagent directly to the surface of the agar. The positive test was represented by the development of green color.

1.2.12 Carbohydrate Utilization

Inoculate the organism into bromthymol blue broth containing glucose, lactose and incubate at 35°C for 18 to 24 hours. The development of a yellow color (acid production) in broth containing glucose or lactose indicates a positive test

1.3 Interpretation Criteria

The results from the biochemical reactions were interpreted according to Forbes *et al*., 1998, and Koneman EW *et al*., 1992 as showed in Table 8-10.

Table 8: Tests of identification of organisms with + + - - IMViC profiles (Forbes *et al*., 1998:519).

Organisms with I M V C + + - -	H_2S Production (IN TSI)	Urease	Phenylalanine Deaminase	Sucrose Fermentation	Lysine Decarboxylase
Citrobacter freundii	V	V	-	V	-
Edwardsiella tarda	+	-	-	-	+
Enterobacter agglomerans group*	-	V	V	V	-
Escherichia coli	-	-	-	V	+
Morganella morganii subsp. *morganii*	V	+	+	-	-
Proteus vulgaris	+	+	+	+	-
Shigella groups A, B, and C	-	-	-	-	-
Yersinia enterocolitica, frederiksenii, and *intermedia*	-	V	-	+	-

+ >90% of strains positive; v, variable; I, indole; M, methyl red; V, Voges-Proskauer; C, citrate; TSI, triple sugar iron agar.

*Because of the biochemical variability of this organism, all possible combinations of IMViC reactions can occur.

Table 9: Differentiation of species within the Genus Escherichia (Koneman *et al*., 1992:614).

Biochemical Test *	*E.coli*	*E.hermannii*	*E.fergusonii*	*E.blattae*	*E.vulneris*
Indole	+	+	+	-	-
Methly red	+	+	+	+	+
Voges-Proskauer	-	-	-	-	-
Citrate	-	-	V(17)	V(50)	-
Lysine decariboxylase	+	-	+	+	+
Arginine dihydrolase	V(17)	-	-	-	V(30)
Ornithine decarboxylase	V(65)	+	+	+	-
ONPG	+	+	+	-	+
Fermentation of Lactose	+	V(45)	-	-	V(15)

+ >90% or more strains are positive; -, 90% or more strains are negative; V, 11%-89% of strains are positive.

Table 10: Differentiation among the Species and Subspecies of *Klebsiella* (Macfaddin, 2000:757).

Test	*K.ornithinolytica*	*K.oxy toca*	*K.pneumoniae* Subsp.*ozaenae*	*K.pneumoniae* Subsn.*pneumoniae*	*K.pneumoniae* Subsp.*rhinoscieronmatis*	*K.planticola*	*K.tenigena*
Lactose	A	A	V	A	-	A	A
Indole	+	+	-	-	-	V[-]	-
Methyl red	+	V[-]	+	V[-]	+	+	V
Voges-Proskauer	V	+	-	+	+	+	+
Simmons citrate	+	+	V	+	-	+	V
Malonate	+	+	-	+	+	+	+
Christensen's Urease	+	+[a]	-	+[a]	-	+[a]	-
Lysine	+	+	V	+	-	+	+
ONPG	+	+	V[+]	+	-	+	+

[a]May be slow or delayed.

2. API20E (bioMerieux, Marcy-l' Etoile, France)

All ESBLs producing *Klebsiella* spp. were confirmed for their species by API20E. API20E is a standardized method combining 20 biochemical tests. The enzymatic tests were inoculated with a dense suspension of organisms, made from a pure culture, which was used to dehydrate the enzymatic substrates. The metabolic end products produced during the incubation period were either revealed through spontaneous colored reactions or by the addition of reagents. The fermentation tests were inoculated with an enriched medium, which reconstitutes the sugar substrates. Fermentation of carbohydrates was detected by a shift in the pH indicator.

The API20E strip consists of 20 microtubes containing dehydrated substrates for the demonstration of enzymatic activity or the fermentation of sugars.

1. Beta-galactosidase (ONPG test)
2. Arginine dihydrolase (ADH test)
3. Lysine decarboxylase (LDC test)
4. Ornithine decarboxylase (ODC test)
5. Citrate utilization (CIT test)
6. H_2S production (H_2S test)
7. Urease (URE test)
8. Tryptophane deaminase (TDA test)
9. Indole production (IND test)
10. Acetoin production (VP test)
11. Gelatinase (GEL) test
12. Fermentation/ oxidation (Glucose test)
13. Fermentation/ oxidation (Mannitol test)
14. Fermentation/ oxidation (Inositol test)
15. Fermentation/ oxidation (Sorbitol test)
16. Fermentation/ oxidation (Rhamnose test)
17. Fermentation/ oxidation (Sucrose test)
18. Fermentation/ oxidation (Melibiose test)
19. Fermentation/ oxidation (Amygdalin test)
20. Fermentation/ oxidation (Arabinose test)

Interpretation: After 18-24 hours of inoculation, the reactions were read by referring to the Analytical Profile Index.

PART III : CULTURE PRESERVATION

1. Media for Culture Preservation

Trypticase soy broth (BBL, Becton Dickinson and Company, Cockeysville, MD) containing 50% horse serum was used to preserve all culture isolates in this study.

2. Preservation Method

All culture isolates were grown entirely on tryptic soy agar (BBL, Becton Dickinson and Company,Cockeysville, MD) at 37°C. The overnight cultures were transferred to micro-centrifuged tubes of 1 ml trypticase soy broth containing 50% horse serum and mix-well suspensions were kept at -70°C until required as described by Ohashi *et al* (1978).

PART IV : REFERENCE BACTERIAL STRAINS

1. Reference strains of *E. coli* ATCC 25922, *E. coli* ATCC 35218 and *Klebsiella pneumoniae* ATCC 700603 were used for quality control in the disk diffusion test, double disk synergy test and E-test.

2. Reference strains of *E.coli* C600 (pCFF04) carrying $bla_{\text{TEM-3}}$, *E.coli* C600 (pUD) carrying $bla_{\text{TEM-4}}$ and *K. pneumoniae* ATCC 700603 carrying $bla_{\text{SHV-18}}$ were kindly provided by Assistant Professor Dr. Aroonwadee Chanawong, Khon Kaen University, and used as positive control strains in PCR amplification step.

PART V: SCREENING METHODS FOR THE ESBL PRODUCING STRAINS

Criteria for ESBL Production

ESBL production was detected by the initial screen test by mean of disk diffusion test as recommended by NCCLS (NCCLS.,2001). Any of positive isolates were confirmed by the confirmatory test; double disk synergy test and Etest ESBL. The positive results indicate the presence of an ESBL. The screening tests except Etest ESBL were run triplicate.

1. DISK DIFFUSION TEST (NCCLS.,2001)

1.1 Media and Antimicrobial Agents

Muller-Hinton agar (BBL, Becton Dickinson and Company, Cockeysville, MD, USA) with a depth of 4 mm in plastic plate (100 mm) was used to perform disk diffusion test. Antimicrobial susceptibility disks containing ceftazidime (CAZ 30μg), ceftriaxone (CRO 30μg), cefotaxime (CTX 30μg) and aztreonam (ATM 30μg), were purchased from BBL, Becton Dickinson and Company, Cockeysville,MD,USA. All antimicrobial susceptibility disks were stored at -20°C until required. The media and antimicrobial susceptibility disks must be allowed to reach room temperature prior to use.

1.2 Inoculum Preparation

The inoculum was prepared by the direct colony suspension method. The 3-5 single colonies of isolate with the same morphological type were selected from over night culture plates. The selected colonies were transferred into 0.85% normal saline tube and adjusted turbidity to a 0.5 Mcfarland standard tube.

1.3 Inoculation of Agar Plate

Sterile cotton-tipped swabs were dipped and rotated into the inoculum suspension. The excess liquid was removed by rotating the swab against the side of the tube. Muller-Hinton agar plates were streaked three times within 15 min of inoculum preparation by rotating the dish 60°C each time to ensure a distribution of inoculum. The inoculated agar plates were allowed to dry for approximately 10 minutes prior to apply antimicrobial susceptibility disks.

1.4 Application of Antimicrobial Susceptibility Disks

Ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg) and aztreonam (30 μg) disks were placed on Mueller Hinton agar plate.

1.5 Incubation

The agar plates were inverted and incubated within 15 min after antimicrobial susceptibility disks were applied at 35°C, overnight in ambient-air incubator.

1.6 Interpretation of the Result

Inhibition zone criteria of disk diffusion test were measured and interpreted to the nearest millimeter according to the recommendations of the National Committee for Clinical Laboratory Standards as followed (NCCLS.,2001).

Table 11: Inhibition zone criteria for the detection of ESBLs in *K.pneumoniae* and *E.coli* (NCCLS, 2001:39)

Antibiotic	Zone diameter for susceptible strains	Zone diameter breakpoint for possible ESBL-producing strains
Ceftazidime 30μg	≥ 18 mm	≤ 22 mm
Cefotaxime 30μg	≥ 23 mm	≤27 mm
Aztreonam 30μg	≥ 22 mm	≤ 27 mm
Ceftriaxone 30μg	≥ 21 mm	≤ 25 mm

1.7 Quality Control

Quality control strains, used according to NCCLS recommendations, were also included to check the quality of the reagents and the ability of the test device to confirm ESBL production. The expected results and interpretation for control strains were provided in Table 12.

Table 12: Acceptable limits for quality control strains used to monitor accuracy of disk diffusion testing (NCCLS, 2001:62)

Antibiotic	QC Recommendation	
	E.coli ATCC 25922 (ESBL -)	*K. pneumoniae* ATCC 700603 (ESBL +)
Ceftazidime 30μg	25-32 mm	10-18 mm
Cefotaxime 30μg	29-35 mm	17-25 mm
Aztreonam 30μg	28-36 mm	9-17 mm
Ceftriaxone 30μg	29-35 mm	16-24 mm

2. DOUBLE DISK SYNERGY TEST (DDST) (Jarlier *et al*., 1988)

2.1 Media and Antimicrobial Agents

Muller-Hinton agar (BBL, Becton Dickinson and Company, Cockeysville, MD,USA) with a depth of 4 mm in plastic plates (100 mm) were used to perform double disk synergy test. Antimicrobial susceptibility disks containing ceftazidime (CAZ 30μg), ceftriaxone (CRO 30μg), cefotaxime (CTX 30μg) and aztreonam (ATM 30μg), amoxicillin/clavulanate (AMC 20μg /10μg) were purchased from BBL, Becton Dickinson and Company, Cockeysville, MD, USA. All antimicrobial disks were stored at -20°C until required. The media and antimicrobial susceptibility disks must be allowed to reach room temperature prior to use.

2.2 Inoculum Preparation

The inoculum was prepared by the direct colony suspension method. The 3-5 single colonies of isolate with the same morphological type were selected from over night culture plates. The selected colonies were transferred into 0.85% normal saline tube and adjusted turbidity to a 0.5 Mcfarland standard tube.

2.3 Inoculation of Agar Plate

Sterile cotton-tipped swabs were dipped and rotated into the inoculum suspension. The excess liquid was removed by rotating the swab against the side of the tube. Muller-Hinton agar plates were streaked three times within 15 min of inoculum preparation by rotating the dish 60°C each time to ensure a distribution of inoculum. The inoculated agar plates were allowed to dry for approximately 10 minutes prior to apply antimicrobial susceptibility disks.

2.4 Application of Antimicrobial Susceptibility Disks

Ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg) and aztreonam (30 μg) disks were placed 25 mm (center to center) away from a 20 μg amoxicillin/ 10 μg clavulanic acid disk.

2.5 Incubation

The agar plates were inverted and incubated within 15 min after antimicrobial susceptibility disks were applied at 35°C, overnight in ambient-air incubator.

2.6 Interpretation of Result

Enhancement of the inhibition zone of any of the antibiotics towards the disk containing clavulanic acid was interpreted as positive for ESBL production (right hand side in the Figure 10).

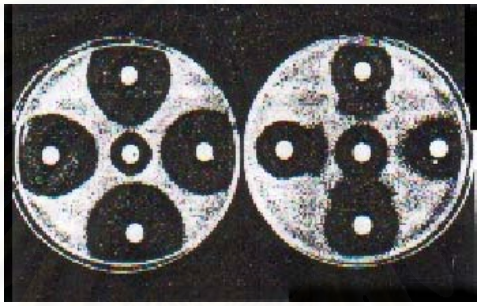


Figure 10: The double-disk diffusion ESBL detection test as suggested by Jarlier *et al*, 1998.

2.7 Quality Control

The quality control strains for the double disk synergy test were used according to NCCLS recommendations *E.coli* ATCC 35218 produces TEM-1 β—lactamase (non-ESBL) were used as a negative control there, and *K.pneumoniae* ATCC 700603, an ESBL positive genotype as a positive control strain.

3. Etest ESBL STRIPS

3.1 Media and Antimicrobial Agents

Muller-Hinton agar (BBL, Becton Dickinson and Company, Cockeysville, MD,USA) with a depth of 4 mm in plastic plates (100 mm) were used to perform Etest. Etest ESBL strips were purchased from AB BIODISK, Solna, Sweden. The Etest ESBL CT/CTL and TZ/TZL strips (Figure 11) consist of a thin, inert and non-porous plastic carrier (5 x 60 mm). One side of the strip is calibrated with MIC reading scales in μg/ml while the reverse surface carries two predefined exponential gradients. CT codes for the cefotaxime (0.25-16 μg/ml) gradient and CTL the cefotaxime (0.016-1 μg/ml) plus 4 μg/ml clavulanic acid. TZ codes for the ceftazidime (0.5-32 μg/ml) gradient and TZL the ceftazidime (0.064-4 μg/ml) plus 4 μg/ml clavulanic acid. The strips were stored in airtight container bedded with silica gel at –20°C until required. The media and Etest ESBL strips must be allowed to reach room temperature prior to use.

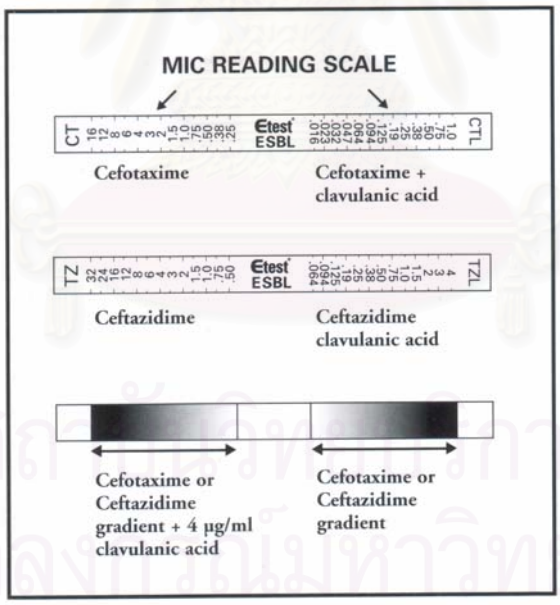


Figure 11: Configuration of Etest ESBL strips

3.2 Inoculum Preparation

The inoculum was prepared by the direct colony suspension method. The 3-5 single colonies of isolate with the same morphological type were selected from over night culture plates. The selected colonies were transferred into 0.85% normal saline tube and adjusted turbidity to a 0.5 Mcfarland standard tube.

3.3 Inoculation of Agar Plate

Sterile cotton-tipped swabs were dipped and rotated into the inoculum suspension. The excess liquid was removed by rotating the swab against the side of the tube. Muller-Hinton agar plates were streaked three times within 15 min of inoculum preparation by rotating the dish 60°C each time to ensure a distribution of inoculum. The inoculated agar plates were allowed to dry for approximately 10 minutes prior to apply strips

3.4 Application of Strips

Both CT/CTL and TZ/TZL E-test ESBLS strips were placed on the agar surface, do not move or remove it or replace on the agar.

3.5 Incubation

The agar plates were inverted and incubated within 15 min after strips were applied at 35°C, overnight in ambient-air incubator

3.6 Interpretation of Result

The ratio of MIC with and without clavulanic acid was $\geq$ 8, the strain produced an ESBL. The interpretative of Etest were shown in Table 13 and Figure 12

Table 13: Interpretation of Etest ESBL

ESBL	MIC (μg/ml) Ratio
Positive*	CT ≥ 0.5 and CT/CTL ≥ 8 **OR** TZ ≥ 1 and TZ/TZL ≥ 8 **OR** "phantom" zone or deformation of the CT or TZ ellipse
Negative	CT <0.5 and CT/CTL < 8 **OR** TZ < 1 and TZ/TZL < 8
Non determinable (ND)**	CT > 16 and CTL > 1 **AND** TZ > 32 and TZL > 4 **OR** When one strip is ESBL negative and the other ND.

TZ =ceftazidime, TZL=ceftazidime+clavulanic aicd, CT=cefotaxime, CTL =cefotaxime+clavulanic acid

*example of positive test were shown in Figure 12a, 12b and 12c;**example of ND test was shown in Figure12d

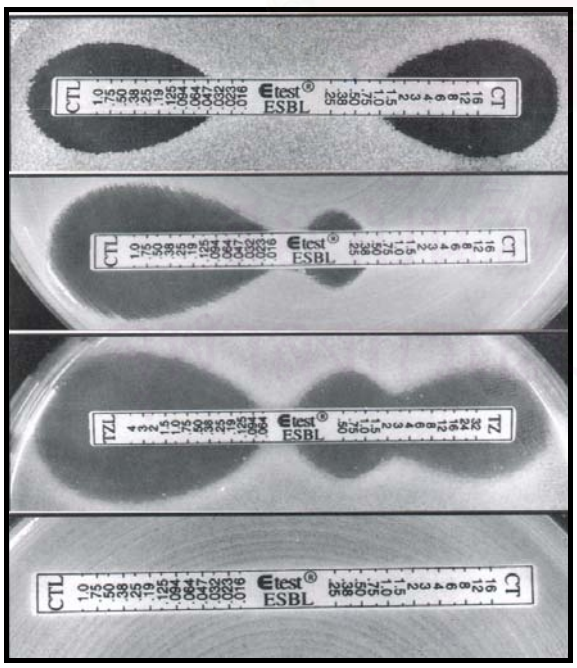


Figure 12: Different growth-inhibition patterns of Etest ESBL;

a.) : Clear-cut ESBL positive (MIC CT/CTL = 1.5/0.047 = 32).

b.) : A "rounded" phantom inhibition zone below CT indicative of ESBL.

c.) : Deformation of the TZ inhibition ellipse indicative of ESBL.

d.) : When MIC values are below or above the test ranges, interpretation is Non Deterinable (ND).

3.7 Quality Control

The quality control strains were used according to NCCLS recommendations and Etest technical guides. The results were shown in Table 14

Table 14: Quality control specifications for Etest ESBL CT/CTL and TZ/TZL strips.

Strain	MIC (μg/ml)			
	TZ	TZL	CT	CTL
E.coli ATCC 25922 / ESBL-	≤ 0.5*	0.064-0.25	≤ 0.25*	0.032-0.125
E.coli ATCC 35218 / ESBL- (TEM-1)	≤ 0.5*	< 0.064*	≤ 0.25*	0.016-0.064
K.pneumoniae ATCC 700603 / ESBL+	8- ≥32	0.125-0.5	1-4**	0.125-1

*; MIC Value below the strip range, **; Deformation of CT ellipse can be observed.

PART VI : AMPLIFICATION OF GENES ENCODING SHV- AND TEM-TYPE ESBLs BY PCR

1. DNA Extraction

Bacterial DNA was prepared by suspending one colony in 50 μl of sterile distilled water and heating at 95°C and centrifuged 13,000 rpm at room temperature. Supernatant was used as PCR template as described by Aroonwadee Chanawong *et al*., (2001). Extracted DNA were stored at -20°C

2. Polymerase Chain Reaction (PCR) Amplification

PCR amplification of bla_{TEM} and bla_{SHV} genes were performed according to the objective in this study, due to the most common ESBL types that were detected in the *Enterobacteriaceae.* However it was in preliminary study found that TEM ESBLs were not detect in most of the isolation of *E.coli* . Therefore identified CTX-M and VEB ESBLs which were common in Southeast Asia was included in this study.

2.1 Primer

The PCR primers TEM-A,TEM-B, SHV-A and SHV-B employed were those described by Yan, *et al*.,2000. Primer specific for the $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}3}$ genes, CTX-A and CTX-B were described by Bonnet *et al*.,2000. The synthesis of primer VEB-A and VEB-B were performed with the program Primer 3 input, which available at the website http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/egi. An internal fragment of 216 bp was amplified from position 460-675 ($bla_{VEB\text{-}1}$ numbering from accession number AF010416), which corresponded to the conserved regions of VEB ESBL gene as demonstrated in Table 15.

Table 15: Sequence of primer and size of PCR products

Specific for	Primer	Primer sequence	Location	Product size (bases)	Reference
bla_{TEM}	TEM-A	5'-CCCCTA TTTGTTTATTTTTC-3'	112-130	962	Yen *et al.,*
	TEM-B	5'-GACAGTTACCAATGCTTAATCA-3'	1074-1053		(2000)
bla_{SHV}	SHV-A	5'-GCCGGGTTATTCTTATTTGTCGC-3'	55-77	1012	Yen *et al.,*
	SHV-B	5'-TCTTTCCGATGCCGCCGCCAGTCA-3'	1067-1044		(2000)
bla_{VEB}	VEB-A	5'-CCTTTTGCCTAAAACGTGGA-3'	460-479	216	This study
	VEB-B	5'-TGCATTTGTTCTTCGTTTGC-3'	675-656		
bla_{CTX}	CTXM-A	5'-CGCTTTGCGATGTGCAG-3'	264-280	550	Bonnet *et al.,*
	CTXM-B	5'-ACCGCGATATCGTTGGT-3'	814-798		(2000)

2.2 Amplification of the bla_{TEM} by PCR

DNA (2 µl) was used as the template for PCR for the detection of bla_{TEM} gene. Primer TEM-A and TEM-B (Table 15) were used for amplification of entire bla_{TEM} gene in a 50 µl PCR reaction mixture. The PCR conditions were as followed: 1.5 mM $MgCl_2$, 1 X PCR buffer, 200 µM of each dNTP mixture (Promega, USA), 10 pmol of each primer, and 2.5 U *Taq* polymerase (Promega, USA). After an initial denaturation at 95°C for 3 min, the reactions were run for 35 cycles through a temperature profile of 95°C for 30 seconds (denaturation), 56°C for 30 seconds (annealing), and 72°C for 1.30 minutes (extension). A final extension was performed at 72°C for 1.30 minutes.

2.3 Amplification of the *bla*$_{SHV}$ by PCR

DNA (1 μl) was used as the template for PCR for the detection of *bla*$_{SHV}$ gene. Primer SHV-A and SHV-B (Table 15) were used for the amplification of entire *bla*$_{SHV}$ gene in a 50 μl PCR reaction mixture. The PCR conditions were as followed: 1.5 mM $MgCl_2$, 1 X PCR buffer, 200 μM of each dNTP mixture (Promega, USA), 10 pmol of each primer, and 2.5 U *Taq* polymerase (Promega, USA). After an initial denaturation at 95°C for 3 min, the reactions were run for 30 cycles through a temperature profile of 95°C for 30 seconds (denaturation), 65°C for 1 minutes (annealing and extension). A final extension was performed at 72°C for 1.30 minutes.

2.4 Amplification of the *bla*$_{VEB}$ and *bla*$_{CTX-M}$ by PCR

DNA (3 μl) was used as the template for PCR for the detection of *bla*$_{VEB}$ and *bla*$_{CTX-M}$ gene. Primer VEB-A, VEB-B, CTX-M-A and CTX-M-B (Table 15) were used in a 50 μl PCR reaction mixture. The PCR conditions were as followed: 1.5 mM $MgCl_2$, 1 X PCR buffer, 200 μM of each dNTP mixture (Promega, USA), 10 pmol of each primer, and 2.5 U *Taq* polymerase (Promega, USA). After an initial denaturation at 95°C for 3 min, the reactions were run for 35 cycles through a temperature profile of 95°C for 30 seconds (denaturation), 56°C for 30 seconds (annealing), and 72°C for 1.30 minutes (extension). A final extension was performed at 72°C for 1.30 minutes.

3. Analysis of PCR Product

1% agarose gel (GIBCO; Grand Island, N.Y. USA) in Tris-acetate (TAE) buffer containing 0.5 μg of ethidium bromide (Sigma, MO, USA) per ml was prepared. The pH of TAE buffer was pH 8.0. 100 volts electrophoretically separated the 5 μl of PCR products until the bromphenol blue indicator in the loading buffer had migrated to half the length of the gel. The amplified products were then visualized on a short-wavelength of UV light transilluminator. A 100 base pair DNA ladder (Promega, USA) was used as a DNA size marker.

4. Quality Control

- *E. coli* C600 (PCFF04) carrying bla_{TEM-3} was used as a positive control for TEM was kindly provided by Assistant Professor Dr. Aroonwadee Chanawong.
- *K. pneumoniae* ATCC 700603 carrying bla_{SHV-18} was used as a positive control for SHV as described by Rasheed *et al*., 2000.
- *K. pneumoniae* KU204-0345 carrying $bla_{VEB-1-like}$, obtained from this study, was used as a positive control for VEB.
- *E. coli* EU269-0445 carrying $bla_{CTX-M-15}$, obtained from this study, was used as a positive control for CTX-M.

PART VII : TYPING OF ESBL TEM AND SHV BY SEQUENCING

1. Purification of PCR Products

The PCR products of 962 bp bla_{TEM} and 1012 bp bla_{SHV} were purified by QIAquick PCR purification kit as described by the manufacturer (QIAGEN, Max-Volmer-StraBE4, Hilden, Germany). The QIAquick system is a combination of spin column technology with the selective binding properties of a uniquely designed silica-gel membrane. DNA was absorbed to the silica-membrane in the presence of high salt while contaminants pass through the column. Impurities were efficiently washed away and the pure DNA was then eluted with Tris buffer. The concentration of DNA was measured by spectrophotometer (BIO RAD, Smart Spec ^{tm} 3000, U.S.A) and approximately adjusted to 100 ng/μl for preparation of sequencing reaction.

2. Primers

Nucleotide sequences were determined by sequencing both strands of bla_{TEM} and bla_{SHV} genes. The PCR primers TEM-A, TEM-B, SHV-A and SHV-B employed were those described by Yan, *et al*.,2000. The synthesis of primer TEM-C, TEM-D, SHV-C and SHV-D were from position 414-432, 486-468, 447-464 and 510-492 respectively, which corresponded to the conserved regions of bla_{TEM} and bla_{SHV} gene as demonstrated in Table 16.

Table 16: Primer for sequencing of *bla*$_{TEM}$ and *bla*$_{SHV}$

Specific for	Primer	Primer sequence	Location
bla $_{TEM}$	TEM-A	5'-CCCCTA TTTGTTTATTTTTC-3'	112-130
	TEM-B	5'-GACAGTTACCAATGCTTAATCA-3'	1074-1053
	TEM-C	5'-CTGACAACGATCGGAGGA-3'	414-432
	TEM-D	5'-AAGGCGAGTTACATGATC-3'	486-468
bla $_{SHV}$	SHV-A	5'-GCCGGGTTATTCTTATTTGTCGC-3'	55-77
	SHV-B	5'-TCTTTCCGATGCCGCCGCCAGTCA-3'	1067-1044
	SHV-C	5'-GCCTTTTTGCGCCAGAT-3'	447-464
	SHV-D	5'-ATTCAGTTCCGTTTCCCA-3'	510-492

3. Sequencing Reaction Preparation

Approximately 100 ng of DNA sample was sequenced using two primer sets, *bla*$_{TEM}$ and *bla*$_{SHV}$ (Table 16) with ABI prism Big Dye terminator cycle sequencing ready reaction kit (Perkin Elmer Corporation, ABI prism, Applied Biosystem, Foster city, CA). The sequence reaction required 4 µl of BigDye terminator as described by manufacturer, with 3.2 *p*mole of primer and 90 ng of purified PCR product template in a total volume of 10 µl. The sequencing cycle was performed using 25 cycles of

Denaturing step : 10 sec at 96°C
Annealing step : 5 sec at 50°C
Extension step : 4 min at 60°C

4. Ethanol-Sodium Acetate Precipitation

The PCR products were precipitated using 2 µl of 3 M sodium acetate (NaOAc), pH 4.6 and 50 µl of 95% ethanol (EtOH) for each sequencing reaction as described by manufacturer. The precipitated DNA was stored at -20°C until use. Purify sequencing

reaction, which added with 15 μl template suppressor reagent (TSR) and then were heated 95 °C at 2 min and placed on ice immediately, was subjected to automated sequence analysis on ABI prism 310 automated sequencer (Perkin Elmer Corporation, ABI prism, Applied Biosystem, Foster city, USA).

5. Analysis

The nucleotide sequence and the deduced protein sequence were analyzed with the software available over the Internet at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Multiple sequence alignment and pairwise comparisons of sequences were analyzed by Chromas and ClustalW, version 1.74. All amino acid sequences were compared with the published data at website http://www.lahey.org/studies/webt.htm (Jacoby and Bush, 2001).

CHAPTER V

RESULTS

PART I : CLINICAL ISOLATES

A total of 270 *E.coli* and *K.pneumoniae* strains were used in the present study. These included 212 strains of *E.coli* and 58 strains of *K.pneumoniae* (Table 17). Most of them (246 strains, 91.1%) were isolated from patients with urinary tract infections; Among the 212 strains of *E.coli* , 197 (92.9%) strains were isolated form urine, 11 (5.2%) strains from body fluid, and 4 (1.9%) strains from sputum. While the 58 *K.pneumoniae* strains were isolated from various specimens; 49 (84.8%) strains from urine, 5 (8.6%) strains from sputum, 3 (5.2%) strains from pus and 1 (1.7%) strain from body fluid (Figure 13).

Table 17 : The number of isolates in this study.

Organisms	No. Of isolates
E.coli	212
K.pneumoniae	58
Total	270

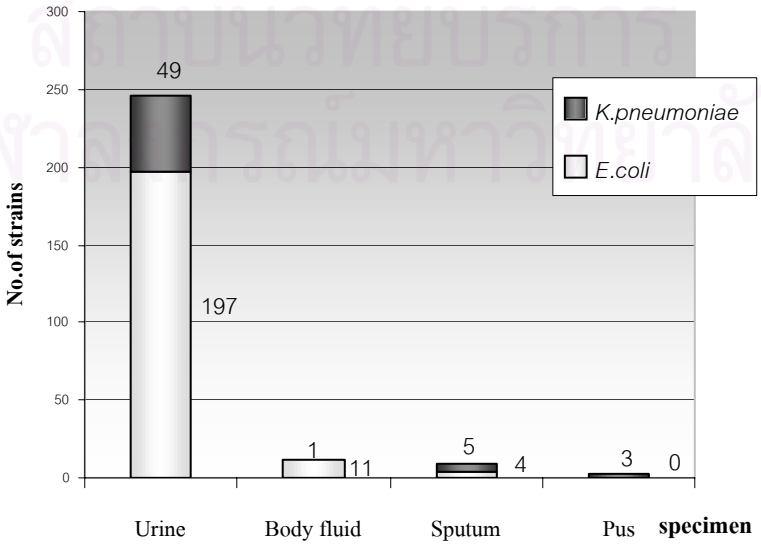


Figure 13 : Number of isolation of *E. coli* and *K.pneumoniae* from various clinical specimens

PART II : IDENTIFICATION OF *Escherichia coli* and *Klebsiella* spp.

Identification the strains of *E.coli* and *Klebsiella* spp. have been previously performed by microbiology department. However all strains were subcultured and identified by conventional test as a conformation.

1. Identification of *E.coli*

Gram stain and culture on blood agar and MacConkey agar plate were examined for the characteristic of lactose fermentation Gram-negative organisms. The biochemical characteristics of *E.coli*, conferred positive by disk diffusion test, were demonstrated in Table 18.

Table 18: Biochemical characteristics of the 43 *E.coli* isolates positive for disk diffusion test.

Strain no.	Oxidase	Colony on MacConkey agar (Lactose fermenter)	TSI, gas production	Indole	MR	VP	Citrate	Motility	Urease	Lactose	LD	AD	OD	Identified
EU7-0245	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU21-0145	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU23-0145	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU24-0145	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU27-0245	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU31-0245	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU38-0245	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU39-0245	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU41-0245	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU44-0245	-	+	A/A+	+	+	-	-	+	-	+	+	-	-	*E.coli*
EB48-0245	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EB49-0245	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU61-0245	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU78-0245	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*

TSI, Triple sugar iron test; MR, Methyl red reaction test; VP,Voges-Proskauer test; LD, lysine decarboxylase test; AD, Arginine dihydrolase test; OD, Ornithine decarboxylase test; K/A, alkaline slant and acidic butt; A/A, acid slant and acid butt; +, positive ; -, negative;

Table 18: Biochemical characteristics of *E.coli* in 43 isolates. (cont.)

Strain no.	Oxidase	Colony on MacConkey agar (Lactose fermenter)	TSI, gas production	Indole	MR	VP	Citrate	Motility	Urease	Lactose	LD	AD	OD	Identified
EU84-0245	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU91-0245	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU95-0245	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EB110-0245	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU115-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
ES128-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU134-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU141-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU147-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU148-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU161-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU165-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU173-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU176-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU185-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU195-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU220-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU226-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU228-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU231-0445	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU253-0445	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU254-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU256-0345	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU265-0445	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU269-0445	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU278-0545	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
EU286-0545	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
ES295-0545	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*
ES296-0545	-	+	A/A +	+	+	-	-	+	-	+	+	-	-	*E.coli*

TSI, Triple sugar iron test; MR, Methyl red reaction test; VP,Voges-Proskauer test; LD, lysine decarboxylase test; AD, Arginine dihydrolase test; OD, Ornithine decarboxylase test; K/A, alkaline slant and acidic butt; A/A, acid slant and acid butt; +, positive ; -, negative;

2. Identification of *Klebsiella* spp.

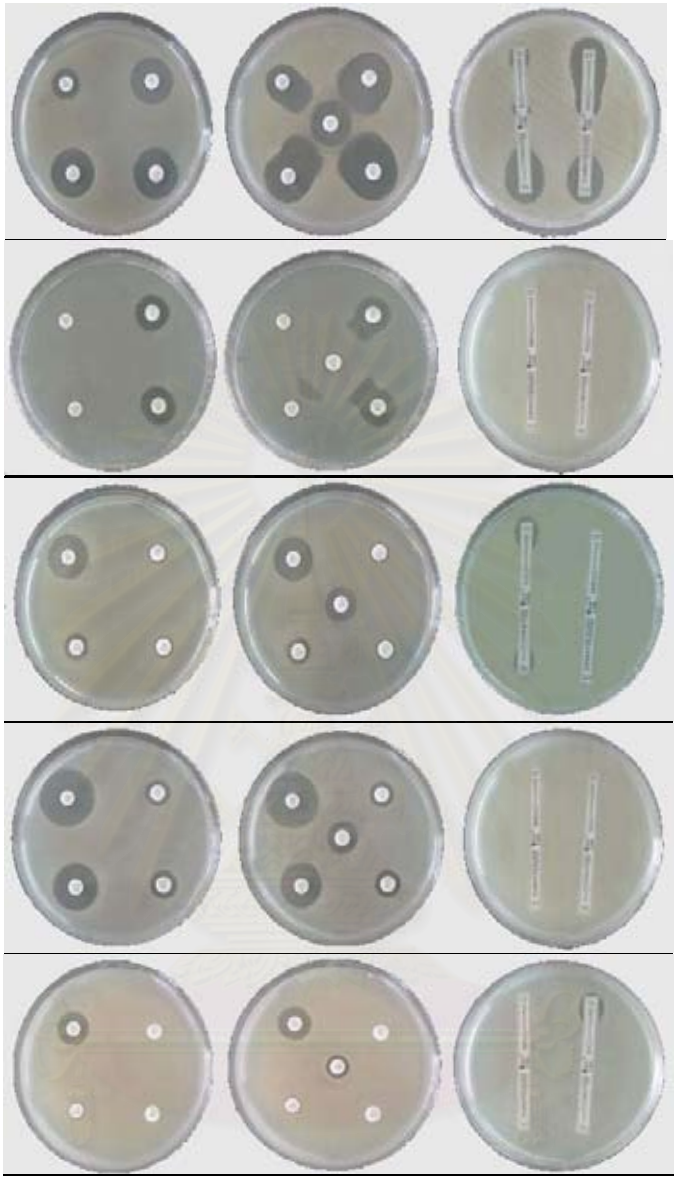
Gram stain and culture on blood agar and MacConkey agar plate were examined for the characteristic of lactose fermentation Gram-negative organisms. All 58 *Klebsiella* spp. were reconfirmed by conventional test. 24 *Klebsiella* spp., which conferred positive results in disk diffusion test, were confirmed their species by API 20E test strips. The biochemical characteristics of *Klebsiella* spp., conferred positive by disk diffusion test, were demonstrated in Table 19.

Table 19: Biochemical characteristics of *Klebsiella* spp. in 24 isolates.

Strain no.	Oxidase	Colony on MacConkey agar (Lactose fermenter)	TSI, gas production	Indole	MR	VP	Citrate	Motility	Urease	Lactose	LD	OD	Malonate	ONPG	Identified	% identity in API20E
KU14-0245	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU26-0145	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU46-0245	-	+	A/A +	-	-	+	+	-	+[a]	+	+	-	+	+	*K.pneumoniae*	97.6
KU47-0245	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KS53-0245	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K pneumoniae*	97.6
KU55-0245	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KS113-0245	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU127-0345	-	+	A/A+	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU152-0345	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU177-0345	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU178-0345	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU201-0345	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU203-0345	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU204-0345	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU206-0345	-	+	A/A+	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU213-0345	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU233-0445	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU237-0445	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU241-0445	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KP297-0545	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU299-0545	-	+	A/A +	-	-	+	+	-	+[a]	+	+	-	+	+	*K.pneumoniae*	97.6
KS312-0545	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU318-0545	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6
KU325-0545	-	+	A/A +	-	-	+	+	-	+	+	+	-	+	+	*K.pneumoniae*	97.6

TSI, Triple sugar iron test; MR, Methyl red reaction test; VP,Voges-Proskauer test; LD, lysine decarboxylase test; AD, Arginine dihydrolase test; OD, Ornithine decarboxylase test; K/A, alkaline slant and acidic butt; A/A, acid slant and acid butt; +, positive ; -, negative; a, deleyed, weak.

PART III : SCREENING FOR ESBL-PRODUCING STRAINS.



1).

2).

3).

4).

5).

Figure 14: Results of 3 ESBLs test.

1). Category 1 The positive of disk diffusion test (left), positive double disk synergy test (middle) and positive E test ESBL screen test (right).

2). Category 2 The positive of disk diffusion test (left), positive double disk synergy test (middle) and Non determination Etest ESBL screen test (right).

3). Category 3 The positive of disk diffusion test (left), negative double disk synergy test (middle) and positive Etest ESBL screen test(right).

4). Category 4 The positive of disk diffusion test (left), negative double disk synergy test (middle) and Non-determination Etest ESBL screen test (right).

5). Category 5 The positive of disk diffusion test (left), negative double disk synergy test (middle) and Negative Etest ESBL screen test (right).

Comparison the results of positive ESBLs strains of *E.coli* and *K.pneumoniae* performed by disk diffusion test, double disk synergy test and Etest ESBL. Strains giving positive results with at least two of the three methods were designated as ESBL producers. The results described into 5 categories (Figure 14, Table 20).

Table 20: ESBL producing *E.coli* and *K.pneumoniae* according to screening results.

Categories	Test			Total	No.of *E.coli* strains	No.of *K.pneumoniae* strains
	DT	DDST	Etest			
1.*	+	+	+	**46**	**31**	**15**
2.*	+	+	**ND**	**3**	**0**	**3**
3.*	+	-	+	**7**	**5**	**2**
4.	+	-	ND	5	2	3
5.	+	-	-	6	5	1
Total				67	43	24

DT, disk diffusion test; DDST, double disk diffusion test; ND, non –determinable, * categories 1,2,3 indicated the strains producing ESBLs.

The results of the positive ESBLs producing strains screened by 3 methods were shown in Figure 15 a. Of the 270 strains, 67 (24.8%) strains were positive for disk diffusion test. Among the 67 positive strains of initial screening 49 (18.2%) strains were positive by double disk synergy test and 53 (19.6%) strains were confirmed to be ESBL producer by Etest ESBL. Of the 212 clinical *E.coli* isolates, 43(20.3%) strains were positive for initial screening by disk diffusion. Subsequent confirmation of ESBL production among the *E.coli* isolates revealed that 31 (14.6%) strains demonstrated a clavulanic acid effect by double disk synergy whereas 36 (17.0%) strains were identified as ESBL producer by Etest ESBL. Of the 58 clinical *K.pneumoniae* isolates, 24 (41.2%) strains were positive for initial screening by disk diffusion. Subsequent confirmation of ESBL production among the *K.pneumoniae* isolates revealed that 18 (31.0%) strains demonstrated a clavulanic acid effect by double disk synergy whereas 17 (29.3%) strains were identified as ESBL producer by Etest ESBL.

Of 270 clinical isolates of *E.coli* and *K.pneumoniae,* 67 strains were positive for initial screening disk diffusion test. Among 67 strains, which confirmed by both double disk synergy test and Etest ESBL, indicated that 56 of 270 (20.7%) strains produced ESBL. Thirty-six of 212 (17 %) strains were *E.coli* and 20 of 58 (34.5%) strains were *K.pneumoniae.* (Figure 15 b).

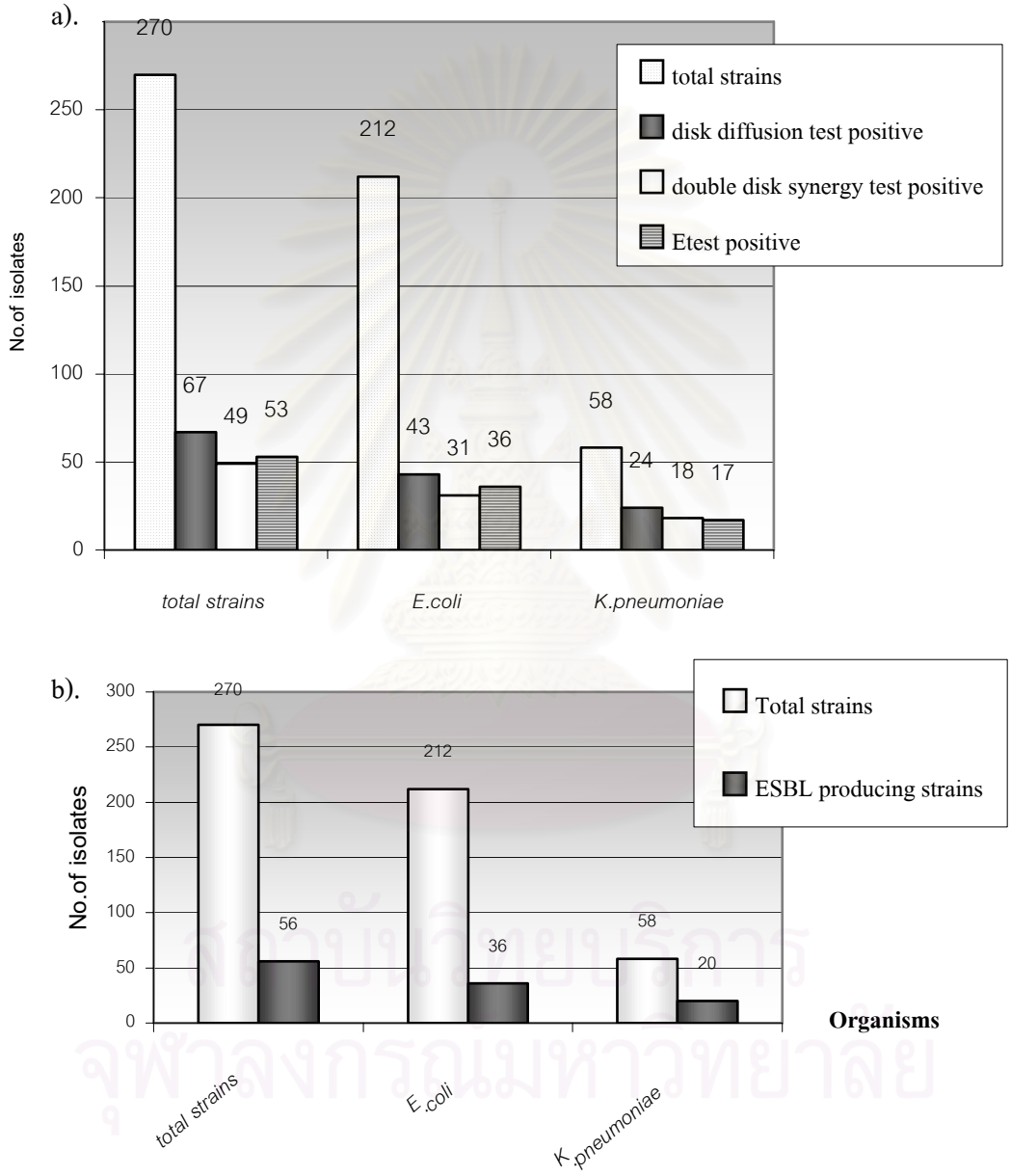


Figure 15 : Total number of *E.coli* and *K.pneumoniae* and ESBLs producing strains.

: a) Number of positive strains performed by disk diffusion test, double disk synergy test and Etest ESBL.

: b) Prevalence of ESBL-producing *E.coli* and *K.pneumoniae* .

PART IV: PCR AMPLIFICATION FOR DETECTION OF ESBLs

The presence of putative ESBLs was further confirmed using the Polymerase Chain Reaction (PCR) of the bla_{TEM}, bla_{SHV}, bla_{VEB} and bla_{CTX-M} genes amplification.

1. Control Strains for bla_{TEM} and bla_{SHV}

In order to determine the specificity of PCR assays using TEM-A, TEM-B, SHV-A, and SHV-B described by Yan et al., 2000, *E.coli* C600 (pCFF04) harbouring bla_{TEM-3}, *E.coli* C600 (pUD) harbouring bla_{TEM-4} and *K. pneumoniae* ATCC 700603 harbouring bla_{SHV-18} were used as the reference strains. (Figure 16)

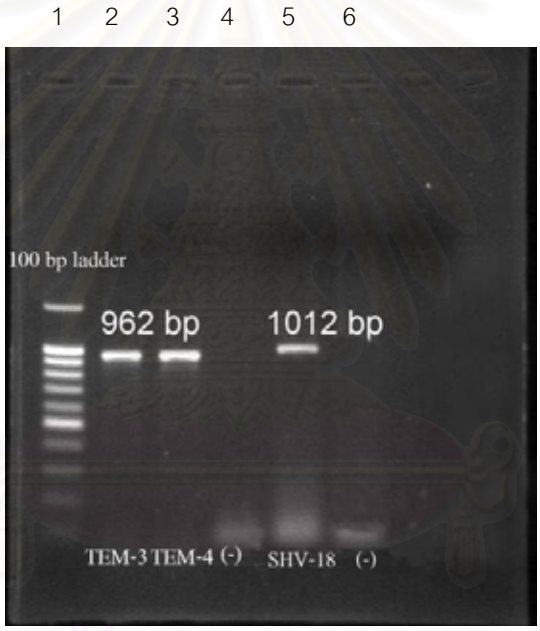


Figure 16: Agarose gel electrophoresis of specific 962 and 1012-bp amplicon of bla_{TEM} and bla_{SHV} respectively.

Lane 1 : 100bp DNA ladder

Lane 2 : bla_{TEM-3} [*E.coli* C600 (pCFF04)]

Lane 3 : bla_{TEM-4} [*E.coli* C600(pUD)]

Lane 4 : Negative control for bla_{TEM}

Lane 5 : bla_{SHV-18} [*K. pneumoniae* ATCC 700603]

Lane 6 : Negative control for bla_{SHV}

PCR amplification of TEM and SHV genes were performed for all positive isolates by screening in this study. However many isolates showed a phenotype of ESBL production but neither SHV nor TEM ESBLs was detected. Since CTX-M and VEB ESBLs have been reported from Asian countries, screening for the presence of these ESBLs was included in this study.

2. Control Strains for *bla*$_{VEB}$ and *bla* $_{CTX-M}$

In order to determine the specificity of PCR assays using VEB-A, VEB-B, CTX-M-A, and CTX-M-B. KU204-0345 harbouring *bla*$_{VEB-like}$ and EU269-0445 harbouring *bla* $_{CTX-M-15}$ were used as the control strains. (Figure 17)

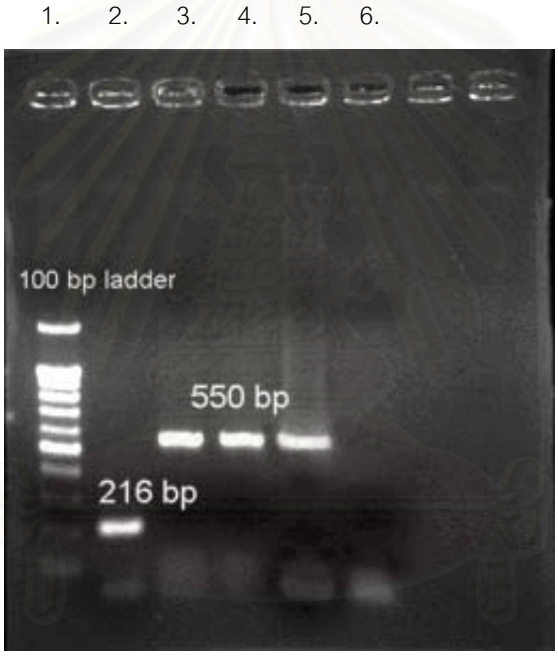


Figure 17: Agarose gel electrophoresis of specific 216 and 550-bp amplicon of *bla*$_{VEB}$ and *bla*$_{CTX-M}$ respectively.

Lane 1 : 100bp DNA ladder

Lane 2 : *bla*$_{VEB-like}$ (KU204-0345)

Lane 3 : *bla*$_{CTX-M-11}$ (EU95-0245)

Lane 4 : *bla*$_{CTX-M-15}$ (KU152-0345)

Lane 5 : *bla* $_{CTX-M-15}$ (EU269-0445)

Lane 6 : Negative control for *bla*$_{VEB}$ and *bla* $_{CTX-M}$

3. Clinical Isolates

Of 56 positive ESBLs producing strains of *E.coli* and *K.pneumoniae*, 36 (64.3%) strains were TEM positive, 22 (39.3%) were CTX-M positive,18 (32.1%) strains were SHV positive and 12 (21.4%) strains were VEB positive. Among 36 of *E.coli* ESBLs producing strains, 26(72.2%) strains produced TEM, 6 (16.7%) produced VEB, 19 (52.8%) produced CTX-M ESBLs, 4 strains were unidentified and none of SHV was found. While the 20 ESBL-producing *K.pneumoniae* were produced TEM, SHV, VEB and CTX-M ESBLs 10 (50%) strains, 18 (90%) strains,6 (30%) strains and 3 (15%) strains, respectively (Figure 18).

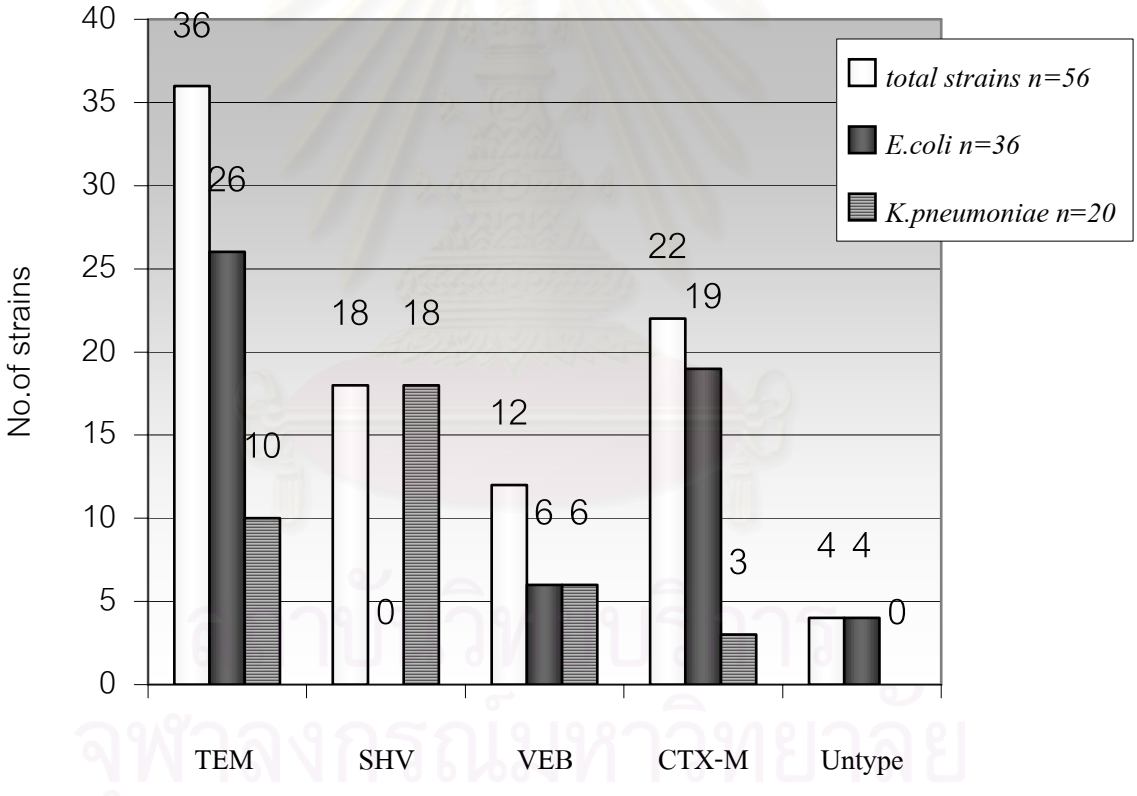


Figure 18: Frequencies of *bla* genes in the 56 ESBL producing *E.coli* and *K.pneumoniae* strains

Among 36 of *E.coli* ESBLs producing strains, 14 (38.9%) strains coharbouring between TEM and CTX-M ESBL were the most common. Of the 20 of *K.pneumoniae* ESBLs producing strains, 7 (35.0%) strains harbouring SHV were the most common. It was noteworthy that 30 (53.6%) out of 56 were coharbouring at least 2 enzymes. (Table 21)

Table 21: ESBL types identified in *E.coli* and *K.pneumoniae* by PCR.

ESBL		No.of strains (%)		
		E.coli	***K.pneumoniae***	**Total**
Harbouring	TEM	8 (22.2%)	1 (5.0%)	9 (16.1%)
	SHV	-	7 (35.0%)	7 (12.5%)
	VEB	2 (5.6%)	-	2 (3.6%)
	CTX-M	4 (11.0%)	-	4 (7.1%)
Co harbouring between	SHV, VEB	-	3 (15.0%)	3 (5.6%)
	TEM, SHV	-	3 (15.0%)	3 (5.6%)
	TEM, VEB	3 (8.3%)	1 (5.0%)	4 (7.1%)
	TEM, CTX-M	14 (38.9%)	-	14 (25.0%)
	TEM, SHV, VEB	-	2 (10.0%)	2 (3.6%)
	TEM, SHV, CTX-M	-	3 (15.0%)	3 (3.6%)
	TEM, VEB, CTX-M	1 (2.8%)	-	1 (1.8%)
Non-TEM,-SHV, -VEB, -CTX-M		4 (11.0%)	-	4 (7.1%)
Total		36	20	56

PART V : TYPE IDENTIFICATION BY PCR AND DNA SEQUENCING

Sequencing of bla_{TEM} and bla_{SHV} genes, using primer TEM-A, -B, -C, and –D and SHV-A, -B, -C and -D, were focused in this study. The nucleotide sequences were determined by sequencing both strands of positive strains including reference strain of *E.coli* C600 (pCFF04) and *K. pneumoniae* ATCC 700603 by automates sequencing method. The results were described into 5 categories according to the screening results.

The sequencing results revealed that all 45 positive TEM-PCR strains were similar to the wild-type TEM-1B, a narrow-spectrum β-lactamase. While sequencing of the SHV-type ESBL revealed 5 mutations located at positions 7, 35, 238, 240 and novel 254. Among 18 of 20 SHV ESBL-positive strains, SHV- 1, -2, -11, -12, -28 and the novel N254H were identified. Three (15%) out of 20 strains produced SHV-1, 5(25%) strains produced SHV-11, 7(35%) strains produced SHV-12, 1(5%) strain each produced SHV-2, SHV-28 and the novel N254H (Table 22).

We found 16 positive unusual phenotype, which confer positive screening but none of ESBLs were found, 9 of these were TEM-1, 1 was SHV-1, 2 were coharbouring between TEM-1B and SHV-11 and 4 untype (Table 22). Moreover we also found 2 ESBLs producing strains that have no synergistic effects, one produce SHV-12, CTX-M and TEM-1B and the another produce TEM-1B and VEB (Table 23).

Table 22: Type identification by PCR and sequencing of ESBL producing strains.

Type identification by		No.of strains (%)		
PCR	**Sequencing**	**Total**	***E.coli***	***K.pneumoniae***
TEM	TEM-1B (9)	9 (16.1%)	8 (22.2%)	1 (5.0%)
SHV	SHV-1, 2, 12 (4) Novel N254H	7 (12.5%)	-	7 (35.0%)
VEB*		2 (3.6%)	2 (5.6%)	-
CTX-M*		4 (7.1%)	4 (11.0%)	-
SHV, VEB*	SHV-1, 12, 28	3 (5.6%)	-	3 (15.0%)
TEM, SHV	SHV-11 (2), 12	3 (5.6%)	-	3 (15.0%)
TEM, VEB*	TEM-1B (4)	4 (7.1%)	3 (8.3%)	1 (5.0%)
TEM, CTX-M*	TEM-1B (14)	14 (25.0%)	14 (38.9%)	-
TEM, SHV, VEB*	TEM-1B (2), SHV-1,12	2 (3.6%)	-	2 (10.0%)
TEM, SHV, CTX-M*	TEM-1B (3), SHV-11 (3)	3 (3.6%)	-	3 (15.0%)
TEM, VEB*, CTX-M*	TEM-1B (1)	1 (1.8%)	1 (2.8%)	-
Non-TEM,-SHV, -VEB, -CTX-M		4 (7.1%)	4 (11.0%)	-
Total		56	36	20

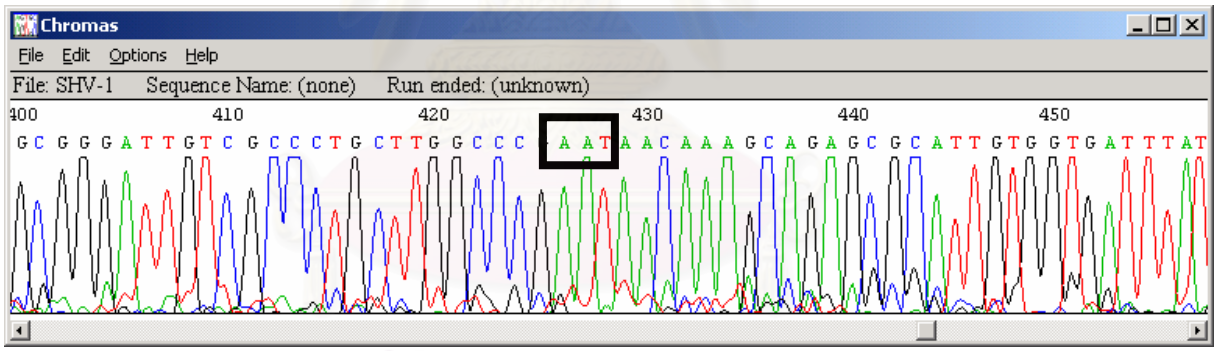
* = not performed by sequencing ** ( ) = number of strains

Table 23: Type identification by PCR and sequencing of non ESBL producing strains

Type identification by		No.of strains (%)		
PCR	**Sequencing**	**Total**	***E.coli***	***K.pneumoniae***
TEM-B	TEM-1B (5)	5	5	-
TEM-1B, SHV	TEM-1B (2), SHV-11 (2)	2	-	2
TEM-1B, VEB*	TEM-1B	1	-	1
TEM-1B, SHV, CTX-M*	TEM-1B ,SHV-12	1	-	1
Non-TEM,-SHV, -VEB, -CTX-M	-	2	2	-
Total	-	11	7	4

* = not performed by sequencing ** ( ) = number of strains

a)



b)

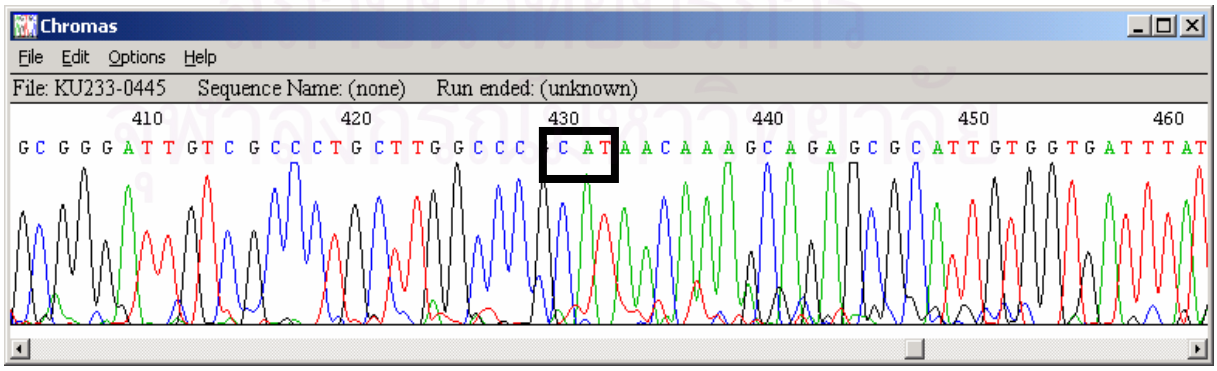


Figure 19. The chromatogram obtained from automate sequencing showed differentiation between nucleotide sequences within *bla*$_{SHV}$ of *K.pneumoniae* harbouring *bla*$_{SHV-1}$ gene GenBank accession numberX98100 (a) and KU233-0445 resistant mutant (b) at the codon 254 (AAT → CAT).

Figure 20: Alignments of the amino acid sequence of *K.pneumoniae* harbouring bla_{SHV-1} gene GenBank accession numberX98100 (a) and *K.pneumoniae* strains KU233-0445 (b) reveals non-published amino acid substitution from Asparagine (AAT) to Histidine (CAT) at the codon 254 (Ambler numbering system).

PART VI : CORRELATION BETWEEN ESBLs PRODUCING STRAINS AND THE CLINICAL DATA

1. ESBLs in Clinical Specimens

Among 270 of clinical isolates, 212 strains were *E coli* and 58 strains were *K.pneumoniae.* Of the 212 isolates of *E.coli,* 197 strains were isolated from urine, 11 strains from body fluid, and 4 strains from sputum. Of the 58 strains of *K.pneumoniae*., 49 strains were isolated from urine, 5 strains from sputum, 3 strains from pus, and 1 strain from body fluid.

Among 36 of 212(17%) strains of ESBL-producing *E.coli* from clinical specimens, 31 (14.6%) strains were isolated form urine, 3 (1.4%) strains from body fluid, and 2 (0.9%) strains from sputum. Among 20 of 58 (34.5%) strains of ESBL-producing *K.pneumoniae* from clinical specimens, 16 (27.6%) strains were isolated from urine, 3 (5.2%) strains from sputum, and 1 (1.7%) strain from pus. (Figure 21).

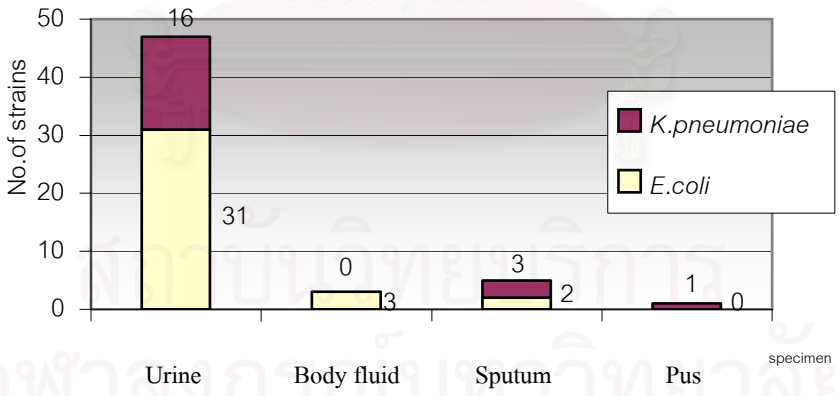


Figure 21: No. of ESBL-producing *E. coli* and *K.pneumoniae* isolated from various clinical specimens (total 56 strains)

2. ESBLs in Nosocomial Infection

The results of the *E.coli* and *K.pneumoniae* isolated with nosocomial infection have been determined. Among 270 strains of both organisms from clinical specimens, 65 (30.7%) strains of *E.coli* and 18 (31.0%) strains of *K.pneumoniae*, were isolated from patients indicated nosocomial infection.

Among 83 strains of clinical isolate with nosocomial infection, 18 (27.7%) strains of *E.coli* and 9 (50.0%) strains of *K.pneumoniae* were ESBLs producer.

Table 24: Number of ESBL-producing *E.coli* and *K.pneumoniae*, and total number isolated from patient with nosocomial infection.

Organism	No.of strains	
	Total	ESBL producing strains from nosocomial infection (%)
***E.coli* (n=212)**	65	18 (27.7%)
***K.pneumoniae* (n=58)**	18	9 (50.0%)
Total (n=270)	83	27 (32.5%)

CHAPTER VI

DISCUSSION

The prevalence of ESBL production among isolates of *Enterobacteriaceae* varies greatly from country to country. In Asia the percentage of ESBL production in *E.coli* and *K.pneumoniae* varies, from 4.8% in Korea to 8.5% in Taiwan and up to 12% in Hong Kong (Ho *et al*, 2000; Pai *et al*, 1999; Yan *et al*., 2000). In the present study, we found that 20.7% of clinical isolates were ESBLs-producing strains with higher rate than other study in Asia. However 16.98% of *E.coli* produced ESBLs with similar rate to those found in the multicenter study in Thailand (15.7%),whereas the prevalence of ESBL producing *Klebsiella* spp. were 34.48%, which was lower than those found in the previous report (45.6%) (Biedenbach *et al*., 1999).

Leelarutsame *et al.*, 2001 described the ESBL producing strains isolation in nosocomial infection in Thailand, forty percent of *E.coli* and 30.2% of *K.pneumoniae* were obtained, comparing with 27.7% and 50% in our study concerning the higher rate of ESBL producing *K.pnuemoniae* in nosocomial infection. Most of the isolated were obtained from urine culture, therefore ESBLs producing strains distribution in various clinical specimens cannot be evaluated.

This study focused on the distribution of SHV and TEM ESBLs, since they are distributed worldwide and limited data concerning these enzymes has been published in Thailand. However many isolates were detected a phenotype of ESBL production, neither SHV nor TEM ESBLs. Therefore CTX-M and VEB ESBLs have been further included since there were report the detection of these ESBL enzymes in Asian.

CTX-M ESBL, which preferentially hydrolyze cefotaxime, have mainly been found in strains of *Salmonella enterica* serovar Typhimurium and *E.coli*. It has been identified in numerous countries of Africa, South America, Asia and Europe during the last decade (Baraniak *et al*., 2002). Several institutions in the areas where the outbreaks of nosocomial reported that the

CTX-M ESBLs were the most frequently isolated ESBLs among clinical isolated (Baraniak *et al*., 2002 ; Sabate *et al*., 2000). We found that CTX-M ESBLs were the most common ESBL (52.8%) which were detected in ESBLs producing *E.coli*, but no significant information concerning these enzymes has been reported in Thailand.

Many reports reviewed that SHV enzymes are often found in clinical isolates of *K.pneumoniae* (Nüesch-Inderbinen *et al.,* 1996; Bush *et al.,* 1995). Similar in this study, SHV ESBLs were the most common ESBL in *K.pneumoniae* (90%). We found that in 18 SHV producing *K.pneumoniae* SHV-12 was the most common (35%). SHV-12 have been reported in Thailand by Chanawong *et al*. (2001) and also reported in China, Korea, Japan and Taiwan (Chanawong *et al*., 1999; Yagi *et al*., 2000; Kim *et al*, 1998; Yan *et al*., 2000). SHV-12 β-lactamase is the most common ESBL, emerging apparently as the best-adapted ESBL for the prevailing antibiotic selection pressure in the Far East Asia. Our findings augment others concerning the occurrence of SHV ESBLs in the former report in the Far East Asia.

We found VEB ESBLs at 16.7% and 30% in *E.coli* and *K.pneumoniae* respectively. VEB-1, that was first found in a single isolate of *E.coli* in a patient from Vietnam, but was subsequently also found in a *P.aeruginosa* isolate from a patient from Thailand (Naas *et al*.,1999). Girlich *et al*., 2001 reported that VEB ESBLs seemed to be highly prevalent in Thai isolates since it accounted for 60% of the ESBL-possessing *Enterobacteriaceae* isolates in their study.

In order to the study of limited ESBL primer, the positive ESBL strains by screening test, is possible due to the production of other ESBLs such as SFO-1, TOHO-1,-2, SME-1, NMC-A, IMI-1, PER-1,-2, GES-1 (Brown *et al*.,2000), or other CTX-M, which can't be detected by our CTX-M primer. The false positive of screening tests can occur with any possess an ESBL. Several groups have been reported that the high-level expression of TEM-1 or SHV-1 plasmid-encoded β-lactamases in *E.coli* and *K.pneumoniae* are responsible for resistance to inhibitor and can cause the MIC of ceftazidime to rise to levels at which an ESBL would be suspected (Miró *et al*.., 1998; Rice *et al*., 2000). In addition, Rasheed *et al*. reported that the production of SHV-1 in a strain of *K.pneumoniae* that was also lacking an outer membrane porin protein caused a false-

positive in ESBL detection tests that looked at the differential between MICs of oxyimino-β-lactam antibiotics with and without clavulanate (Wu *et al*.,2001).

Moreover, confirmation of ESBL production by clavulanic acid inhibition can be difficult in some strains, not only because the activity of the β-lactamases varies with different substrates, but also because organisms may contain additional resistance mechanisms that can mask the presence of ESBL activity. Our negative ESBL strains by screening test, one produce SHV-12, CTX-M and TEM-1B and another produce TEM-1B and VEB with no synergistic effects. These could include AmpC-type enzymes, porin changes, TEM and SHV β-lactamases that are no longer inhibited by clavulanic acid due to mutations in the coding sequences (Martinez *et al*.,2000 ; Rice *et al*., 2000).

In conclusion, it is important for clinical laboratories to detect these enzymes as effectively or rapidly as is needed. The clinicals will be able to selecting empirec regimens for serious infections in patients hospitalized in Thailand and also prevented the nosocomial infection.

The double disk diffusion test should be added routinely in the antibiotic susceptibility test for the *Enterobacteriaceae.* It is simple to performed, easy to interprete and economic. On the other hand, the Etest ESBL is the method of choice. However some positive ESBLs strains were unable to interprete probably the high concentration MIC and rather expensive.

It is demonstrated that VEB and CTX-M -ESBL are playing role in resistant associate ESBL in *E.coli* and *K.pneumoniae* in Thailand. The novel SHV enzymes which is mutate at position 254 have been detected. The further study in the characterization of this new enzyme and sequencing of bla_{VEB} and $bla_{\text{CTX-M}}$ genes of these isolated should be performed.

CHAPTER VII

CONCLUSION

The study indicated that epidemiology of extended-spectrum β-lactamases (ESBL) producing organisms in Thailand were increasing concern. Two hundred and seventy strains, 212 were *E.coli* and 58 were *K.pneumoniae,* were isolated from clinical specimens of patients in Chulalongkorn Memorial hospital during February to May 2002. They were initially screened by mean of disk diffusion test then confirmed by double disk synergy test and Etest ESBL. Overall, 20.7% (56/270) of the strains produced ESBLs. The frequency of ESBL production in *E.coli* was less than *K.pneumoniae* (17% versus 34.5%, respectively), In addition ESBLs producing strains, which were isolated from Nosocomial infection, found in *E.coli* (27.7%) less than in *K.pneumoniae* (50%).

Fifty-six ESBL-producing isolates were investigated ESBL-type by PCR method using bla_{TEM}, bla_{SHV}, bla_{VEB}, and bla_{CTX-M} primer. 64.3% were TEM positive, 32.1% SHV, 21.4% VEB and 39.3% were CTX-M. Among 20 of *K.pneumoniae* producing strains, 90% bla_{SHV}, 50% bla_{TEM}, 30% $bla_{VEB-like}$ and 15% $bla_{CTX-M-like}$ were found. Of the 36 of *E.coli* ESBLs producing strains, 72.2% bla_{TEM}, 52.8% $bla_{CTX-M-like}$ and 16.7% $bla_{VEB-like}$ were found. Bla_{SHV} was not detected in ESBL-producing *E.coli*, whereas it predominated in *K.pneumoniae*. Of the 56 ESBL producing strains, 30 (53.6%) coharboured at least 2 different *bla* genes. All TEM identified, were TEM-1B, which is not ESBL. CTX-M ESBLs were the most common in *E.coli*.

The sequence analysis showed the 45 positive TEM-PCR were TEM-1B, a narrow-spectrum β-lactamase, whereas among 20 SHV ESBL-producing *K.pneumoniae* were SHV- 1, -2, -11, -12, -2, which revealed 5 mutations located at positions 7, 35, 238, 240. SHV-12 were mostly found (35%). We found 1 strain that showed the mutations at codon 254 resulting in amino acid substitution from asparagine (AAT) to histidine (CAT). This novel mutation might be a new ESBL. ESBLs-producing *E.coli* and *K.pneumoniae* in Thailand may predominantly

derivatives of VEB and CTX-M enzymes, the further study suggested to perform the characterization of novel SHV and sequencing of $bla_{\text{VEB-like}}$ and $bla_{\text{CTX-M-like}}$ genes in Thailand.

REFERENCES

Ambler R P, Coulson A F W, Frere J M, Ghuysen J M, Joris B, Forsman M, Levesque R C, Tiraby G, Waley S G. A standard numbering scheme for the class A β-lactamases. Biochem J 1991; 276: 269-272.

Amino Acid Sequences for TEM, SHV and OXA Extended-Spectrum and Inhibitor Resistant β-Lactamases [online]. Available from: http://www.lahey.org/studies/webt.htm [2001, June last date accessed].

Arlet G, Philippon A. Construction by polymerase chain reaction and intragenic DNA probes for three main types of transferable b-lactamases (TEM, SHV, CARB). FEMS Microbiol Lett 1991; 82: 19-26.

Baraniak A, Fiett J, Hryniewicz W, Nordmann P, Gniadkowski. Ceftazidime-hydrolysing CTX-M-15 extended-spectrum β-lactamase (ESBL) in Poland. Antimicrob Agents Chemother 2002; 50:1936-1942.

Barroso H, Freitas-Vieira A, Lito LM, Cristino JM, Salgado MJ, Neto HF, Sousa JC, Soveral G, Moura T, Duarte A. Surver of *Klebsiella pneumoniae* producing extended-spectrum β-lactamases at a Portuguese hospital: TEM-10 as the endemic enzyme. J Antimicrob Chemother 2000; 45:611-616.

Barthelemy MJ, Peduzzi, Labia R. Distinction entre les structures primaries des β-lactamases TEM-1 et TEM-2. Ann Inst Pasteur Microbiol 1985; 136(A): 311-321.

Bauernfiend., A., H. Grimm, and S. Schweighart. A new plasmidic cefotaximase in a clinical isolate of Escherichia coli. Infection 1990; 18:294-298.

Biedenbach DJ, Johnson DM, Jones RN. In vitro evaluation of cefepime and other broad-spectrum β-lactams in eight medical centers in Thailand. Diagn Microbiol Infect Dis 1999; 35 : 325-331.

BLAST 2 program. Blast 2 program for submitted from NCBI [Online]. (n.d.). Available form :http;//www.ncbi.nlm.nih.gov/BLAST [2001,January 20].

Bolmstrom A, Qwarnstrom A, Karlsson A, Ho P, Lewis M T, Johnson D M, Jones R N. Validation of new Etest. ESBL strips for the detection and confirmation of strains producing extended spectrum β-Lactamases (ESBL): a trial using FDA criteria for susceptibility testing devices. Etest reference No. 1258.

Bonnet R, Sampail JLM, Labia R, Champs C D, Sirot D, Chanal C, Sirot J. A Novel CTX-M β-Lactamase (CTX-M-8) in Cefotaxime-Resistant *Enterobacteriaceae* Isolated in Brazil. Antimicrob Agents Chemother 2000: 1936-1942.

Bonomo R A, Rice L B. Inhibitor resistant class A beta-lactamases. Frontiers in bioscience 4 1999; 15: 34-41.

Bradford P A. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev 2001; 14: 933-951.

Bradford PA, Cherubin CE, Idemyor V, Rasmussen BA, Bush K. Multiply resistant *Klebsiella pneumoniae* strains from two Chicago Hospitals: identification of the extended-spectrum TEM-12 and TEM-10 ceftazidime-hydrolyzing β-lactamases in a single isolate. Antimicrob Agents Chemother 1994; 38:761-766.

Bradford PA, Urban C, Jaiswal A, Mariano N, Rasmussen BA, Projan SJ, Rahal JJ, Bush K. SHV-7, a novel cefotaxime-hydrolyzing β-lactamase, identified in Escherichia coli isolates from hospitalized nursing home patients. Antimicrob Agents Chemother 1995; 39:899-905.

Bradford PA, Yang Y, Sahm D, Grope I, Gardovska D, Storch G. CTX-M-5, a novel cefotaxime-hydrolyzing β-lactamases from an outbread of *Salmonella typhimurium* in Latvia. Antimicrob Agents Chemother. 1998: 1894-1980.

Branger C, Lesimple A L, Bruneu B, Berry P, Lambert-Zecho-vsky N. Long-term investigation of the clonal dissemination of *Klebsiella pneumoniae* isolates producing extended-spectrum β-lactamases in a university hospital. J Med Microbiol 1998; 47:210-209.

Bret L, Chanel C, Sirot D, Labia R, Sirot J. Characterization of an inhibitor-resistant enzyme IRT-2 derived from TEM-2 β-lactamase produced by Proteus mirabilis strains. J Antimicrob Chemother 1996; 38:183-191.

Bush K Table 1. The major groups of β-Lactamases. Functional and molecular characteristics of the major groups of beta-lactamases. [Online]. Available from http://gold.aecom.yu.edu/id/almanac/betalactamases.htm[2000, June 10].

Bush K, Jacoby G. Nomenclature of TEM β-Lactamases. J Antimicrob Chemother. 1997; 39:1-3.

Bush K, Jacoby G A, Medeiros A A. A Functional Classification Scheme for β-Lactamases and Its Correlation with Molecular Structure. Antimicrob Agents Chemother. 1995: 1211-1233.

Bush K, Singer S B.. Biochemical characteristics of extended broad-spectrum β-Lactamases. Infection 1989; 17: 429-433.

Chanawong A, M'Zali F H, Heritage J, Lulitanond A, Hawkey PM. Characterization of extended-spectrum β-Lactamases of the SHV family using a combination of PCR-single strand conformational polymorphism (PCR-SSCP) and PCR-restriction fragment length polymorphism (PCR-RFLP). FEMS Microbiol Lett 2000; 184:85-89.

Chanawong A, M'Zali F H, Heritage J, Lulitanond A, Hawkey P M. SHV-12, SHV-5, SHV-2a and VEB-1 extended-spectrum β-Lactamases in Gram-negative bacteria isolated in a university hospital in Thailand. Antimicrob Agents Chemother 2001; 48:839-852.

Chanawong A, M'Zali F H, Heritage J, Lulitanond A, Xiong J, Hawkey PM. Widespread distribution of an SHV-5-like extended spectrum β-lactamase among Enterobacteriaceae isolated from Thailand and the people's Republic of China J Antimicrob Chemother. 1999; 44, Suppl. A, Abstract P235, p.92.

Chang FY, Siu LK, Fung CP, Huang MH, Ho M. Diversity of SHV and TEM β-Lactamases in *Klebsiella pneumoniae*: Gene evolution in northern Taiwan and β-Lactamases, SHV-25 and SHV-26. Antimicrob Agents Chemother. 2001; 45(9) : 2407-2413.

Champs CD, Sirot D, Chanal C, Bonnet R, Sirot J. A 1998 survey of extended-spectrum β-Lactamases in *Enterobacteriaceae* in France. Antimicrob Agents Chemother 2000; 4(11):3177-3179.

Courvalin P. Genotypic approach to the study of bacterial resistance to antibiotics. Antimicrob Agents Chemother 1991; 35:1019-1023.

Datta N, Kontomichalou P. Penicillinase synthesis controlled by infectious R Factors in Enterobacteriaceae. Nature 1965; 208: 239-244.

Danziger LH, Pendland SL. Bacterial resistance to β-lactam antibiotics. Am J Health-Syst Pharm 1995;52(suppl 2):S3-S8.

El Harrif-Heraud Z, Arpin C, Benliman S, Quentin C. Molecular epidemiology of a nosocomial outbreak due to SHV-4 producing strains of Citrobacter diversus. J Clin Microbiol1997; 35: 2561-2567.

Essack SY, Lucinda M, Hall C, Pillay DG, Mcfadyen ML, Livermore DM. Complexity and diversity of *Klebsiella pneumoniae* Strains with extended-spectrum β-lactamases isolated in 1994 and 1996 at a teaching hospital in Durban, South Africa. Antimicrob Agents Chemother 2001;45(1):88-95.

Fauci A S, Braunwald E, Isselbacher K J, Wilson J D, Martin J B, Kasper D L, Hauser S L, Longo D L.1998. Harrison's principles of internal medicine. 14th edition, 846-848.

Ferraro MJ, Jacoby GA, Katsanis G. Improving the reliability of extended-spectrum beta-lactamase detection by the VITEK system. Abstract, 32nd Interscience conference on Antimicrobial agents and chemotherapy, Anaheim, October 1992.

Fiett J, Palucha A, Miaczynska B, Stankiewicz M, Mordarska HP, Hryniewicz W, Gniadkowski M. A novel complex mutant β-lactamase, TEM-68, identified in a *Klebsiella pneumoniae* isolate from an outbreak of extended-spectrum β-lactamase-producing Klebsiellae. Antimicrob Agents Chemother 2000; 44(6): 1499-1505.

Forbes BA, Sahm DF, Weissfeld AS.1998.Bailey & scott's Diagnostic Microbiology. 10th edition, 234-580.

Gazouli M. Tzelepi E, Markogiannakis A, Legakis N J, Tzouvelekis L S. Two novel plasmid-medicated cefotaxime-hydrolyzing β-lactamases (CTX-M-5 and CTX-M-6) from *Salmonella typhimurium*. FEMS Microbiol Lett 1998; 165:289-293.

Girlich D, Poirel L, Leelaporn A, Karim A, Tribuddharat C, Fennewald M, Nordmann P. Molecular Epidemiology of the integron-located VEB-1 extended-spectrum β-lactamase in nosocomial enterobacterial isolateds in Bangkok, Thailand. . J Clin Microbiol 2001; 39(1) : 175-182.

Gniadkowski M, Palucha A, Grzesioski P, Hryniewicz W. Outbreak of ceftazidime-resistant *Klebsiella pneumoniae* in a pediatric hospital in Warsaw, Poland: Clonal spread of the TEM-47 extended-spectrum β-lactamases (ESBL)-producing strain and transfer of a plasmid carrying the SHV-5-like ESBL-encoding gene. Antimicrob Agents Chemother1998; 42:3079-3085.

Gniadkowski M, Schneider I, Jungwirth R, Hryniewicz W, Bauernfeind A. Ceftazidime-resistant Enterobacteriaceae isolates from three polish hospitals: identification of three novel TEM- and SHV-5 type extended-spectrum β-lactamases. Antimicrob Agents Chemother1998; 42:514-520.

Gold HS, Moellering, RC, Jr. Antimicrobial-drug resistance. N Eng J Med 1996; 335:1445-1453.

Heritage J, M'Zali F H, Gascoyne-Binzi D, Hawkey P M. Evolution and spread of SHV extended-spectrum β-Lactamases in gram-negative bacteria. J Antimicrob Chemother. 1999; 44: 309-318.

Ho PL, Chow KH, Yuen KY, Ng WS. Chau PY. Comparison of a novel, inhibitor-potentiated disk-diffusion test with other methods for the detection of extended-spectrum beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae.* J Antimicrob Chemother 1998; 42:49-50.

Ho P L, Tsang D N C, Que T L, Ho M, Yuen K Y. Comparison of screening methods for detection of extended-spectrum β-lactamases and their prevalence among *Escherichia coli* and *Klebsiella* species in Hong Kong. APMIS 2000; 108:237-3697.

Huletsky A, Knox JR, Levesque R C. Role of Ser-238 and Lys-240 in the hydrolysis of 3rd-generation cephalosporins by SHV-type beta-lactamases probed by site-directed mutagenesis and 3-dimensional modeling. J Biol Chem 1993; 268:3690-3697.

Ito H, Arakawa Y, Ohsuka S, Wacharotayankun R, Kato N, Ohta M. Plasmid-mediated dissemination of the metallo-β-lactamase gene bla_{IMP} among clinically isolated strains of *Serratia marcescens.* Antimicrob Agents Chemother1995; 39:824-829.

Jacoby G A, Medeiros A A. Minireview: More extended-spectrum β-Lactamases. Antimicrob Agents Chemother. 1991; 35: 1697-1704.

Jarlier V, Nicolas M H, Fournier G, Philippon A. Extended broad-spectrum β-Lactamases conferring transferable resistance to newer β-Lactam agents in Enterobacteriaceae: Hospital prevalence and susceptibility patterns. Reviews of infectious diseases 1988; 10(4): 867-878.

Joseph DCY, Robert C, Moellering JR.1999, Antibacterial agents, Manual of clinical microbiology. 1474-1504.

Katsanis G P, Spargo J, Ferraro M J, Sutton L, Jacoby G A. Detection *of Klebsiella pneumoniae* and *Escherichia coli* strains producing extended-spectrum β-Lactamases. J Clin Microbiol 1994; 32(3) : 691-696.

Kim J, Kwon Y, Pai H, Kim JW, Cho DT. Survey of *Klebsiella pneumoniae* strains producing extended-spectrum β-lactamases: prevalence of SHV-12 and SHV-2a in Korea. J. Clin. Microbiol 1998; 36:1446-1449.

Kim J, Lee H J. Rapid discriminatory detection of genes coding for SHV β-Lactamases by ligase chain reaction. Antimicrob Agents Chemother 2000; 44:1860-1864.

Kliebe C, Nies BA, Meyer JF, Tolxdorff-Neutzling RM, Wiedemann B. Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. Antimicrob Agents Chemother 1985; 28:302-307.

Knox J R. Minireview, extended sprctrum and inhbitor-resistant TEM-type β-Lactamases: Mutations, Specificity, and three-dimensional structure. Antimicrob Agents Chemother 1995; 39(12) : 2593-2601.

Koneman E W, Allen S D, Janda W M, Schreckenberger P C, Winn JrW C. 1992. Bacterial resistance to antimicrobial agents: Color Atlas and Textbook of Diagnostic Microbiology 4th edition. J.B.Lippincott Company, Philadelphia: 612-614.

Leelarutsame A. Update in ESBL-producing *E.coli* and *Klebsiella* spp.: nosocomial strain. Medical Time 2001;(feb): 7-10.

Lemozy J, Sirot D, Chanal C, Hue C, Labia R, Dabernat H, Sirot J. First characterization of inhibitor-resistant TEM (IRT) β-lactamases in Klebsiella pneumoniae strains. Antimicrob Agents Chemother 1995; 33:2580-2582.

Liu P Y F, Gur D, Hall L M C, Livermore D M. Survey of the prevalence of β-lactamases amongst 1000 gram-negative bacilli isolated consecutively at the Royal London Hospital. J Antimicrob Chemother 1992; 30:429-447.

Livermore DM. β-lactamases in laboratory and clinical resistance. Clin Microbiol Rev1995; 8:557-584.

Livermore D M, Brown D F J. Detection of β-Lactamase mediated resistance. J Antimicrobial Agents Chemother 2001 ; 48 : 59-64.

Livermore DM, Williams JD. β-lactam : mode of action and mechanisms of bacterial resistance. In : Lorian V, ed. Antibiotics in laboratory medicine, 4 th ed. U.S.A. : Williams & Wilkins, 1996 : 502-78.

Lorian V. 1996. β-Lactam : Mode of action and mechanisms of bacterial resistance: Antibiotics in laboratory medicine 4th edition. J.B.Lippincott Company, Philadelphia: 502-578.

Mabilat C, Courvalin P. Development of "oligotyping for characterization and molecular epidemiology of TEM β-Lactamases in members of the family *Enterobacteriaceae.* Antimicrob Agents Chemother 1990; 34:2210-2216.

Macfaddin J F. 2000. Biochemical tests for identification of medical bacteria 3th edition. J.B.Lippincott Company, Philadelphia: 734-757.

Martinez-Martinez L, Hernandez-Alles S, Alberti S, Tomas J, Benedi V, Jacoby G. In vivo selection of porin-deficient mutants of Klebsiella pneumoniae with increased resistance to cefoxitin and expanded-spectrum cephalosporins. Antimicrob Agents Chemother 1996; 40(2): 342-348.

Matthew M, Harris A M, Marshall M J, Ross G W. The use of analytical isoelectric focusing for detection and identification of β-Lactamases. J Gen Microbiol 1975; 88:169-178.

Medeiros, A.A. β-lactamases. Br Med Bull 1984; 40: 18-27.

Minami S, Akama M, Narita H, Iyobe S, Mitsuhashi S. Imipenem and cephem resistant *Pseudomonas aeruginosa* carrying plasmids coding for class B β-lactamase. J Antimicrob Chemother 1996; 37:433-444.

Miro E, del Cuerpo M, Navarro F, Sabate M, Mireleis B, Prats G. Emergence of clinical isolates with decreased susceptibility to ceftazidime and synergistic effect with co-amoxiclav due to SHV-1 hyperproduction. J Antimicrob Agents Chemother 1998; 42:535-538.

Murray PR, Baron E J, Pfaller M A, Temover F C, Yolken R H. 1999. Manual of clinical microbiology: 1474-1543.

M'Zali F H, Chanawong A, Kerr K G, Birkenhead D, Hawkey P M. Detection of extended-spectrum β-Lactamases in members of the family Enterobacteriaceae: comparison of the MAST DD test, the double disc and the Etest ESBL. J Antimicrob Chemother. 2000; 45 : 881-885.

M'Zali F H, Gascoyne-Binzi DM, Heritage J, Hawkey P M. Detection of mutations conferring extended-spectrum activity on SHV β-Lactamases using polymerase chain reaction single strand conformational polymorphism (PCR-SSCP). J Antimicrob Chemother 1996;34 : 797-802.

Naas T, Philippon L, poirel L, Ronco E, Nordman P. An SHV-derived extended-spectrum β-lactamase in Pseudomonas aeruginosa. Antimicrob Agents Chemother1999; 43:1281-1284.

Naas TL, Poirel B, Karim A, Nordmann P. Molecular characterization of In50, a class1 integron encoding the gene for the extended-spectrum β-lactamase VEB-1 in *Pseudomonas aeruginosa.* FEMS Microbiol. Lett. 1999; 176: 411-419.

National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing; Approved standard 7th edition. 2002; 22(1): 27-62.

Naumovski L, Quinn J P, Miyashiro D, Patel M, Bush K, Singer S B, Graves D, Palzkill T, Arvin A M. Outbreak of ceftazidime resistance due to a novel extended-spectrum β-lactamases in isolates from cancer patients. Antimicrob Agents Chemother 1992; 36:1991-1996.

Negri MC, Lipsitch M, Blazquez J, Levin BR, Baquero F. Concentration-dependent selection of small phenotypic differences in TEM β-lactamase-mediated antibiotic resistance. Antimicrob Agents Chemother 2000; 44(9): 2485-2491.

Nordmann P. Trends in β-lactam resistance among Enterobacteriaceae. Clin Infect Dis 1998; 27:S100-S106.

Nordmann P, Guibert M. Extended spectrum β-lactamases in *Pseudomonas aeruginosa.* J Antimicrob Chemother 1998; 42:128-131.

Nüesch-Inderbinen MT, Hächler FHKH. Detection of genes coding for extended-spectrum SHV beta-lactamases in clinical isolates by a molecular genetic method, and comparison with the Etest. Eur J Clin Microbiol Infect Dis 1996; 15: 398-402.

Ohashi M, Murakami H, Kudo Y, Sakai S. 1978. Manual for the Laboratory diagnostic of bacteria . Southeast Asian Medical Information Center. Institute of Basic medical Science, The University of Tsukuba.

Osano E, Arakawa Y, Wacharotayankun R, Ohta M, Horii T, Ito H, Yoshimura F, Kato N. Moleculasr characterization of an enterobacterial metallo β-lactamases found in a clinical isolate of *Serratia marcescens* that showa imipemen resistance. Antimicrob Agents Chemother 1994; 38: 71-78.

Ouellette M, Paul GC, Philippon AM, Roy PH. Oligonucleotide probes (TEM-1, OXA-1) versus isoelectric focusing in β-lactamases characterization of 114 resistant strains Antimicrob Agents Chemother 1988; 32: 397-399.

Pai H, Lyu S, Lee J H, Kim J, Kwon Y, Kim J-W, Choe K W. Survey of extended-spectrum β-lactamases in clinical isolates of *Escherichia coli* and *Klebsiella pnemoniae*: prevalence of TEM-52 in Korea. J Chin Microbiol 1999; 37:1758-1763.

Philippon A, Labia R, Jacoby G. Minireview extened spectrum β-Lactamases. Antimicrob Agents Chemother 1989; 33(8): 1131-1136.

Philippon L N, Naas T, Bouthors A-T, Barakett V, Nordmann P. OXA-18, a class D clavulanic acid-inhibited extended-spectrum β-latamase from Pseudomonas aeruginosa. Antimicrob Agents Chemother 1997; 41:2188-2195.

Pitout J D D, Thompson K S, Hanson N D, Ehrhardt A F, Moland E S, Sanders C C. β-lactamases responsible for resistance to expanded-spectrum cephalosporins in Klebsiella pneumoniae, Escherichia coli, and Proteus milabilis isolates recovered in South Africa. Antimicrob Agents Chemother 1998; 42:1350-1354.

Poirel L, Naas T, Thomas IL, Karim A, Bingen E, Nordmann P. CTX-M-type extended-spectrum β-Lactamase that hydrolyzes ceftazidime through a single amino acid substitution in the omega loop. Antimicrob Agents Chemother 2001; 45(12): 3355-3361.

Primer 3 pick primers from a DNA sequence tool [online]; Available from : http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/eg

Prinarakis E E, Miriagou V, Tzelepi E, Gazouli M, Tzouvelekis L S. Emergence of an inhibitor-resistant β-lactamese (SHV-10) derived from an SHV-5 variant. Antimicrob Agents Chemother 1997; 41:838-840.

Rasheed J K, Anderson G J ,Yigit H, Queenan A M, Sanchez A D, Swenson J M, Biddle J W, Ferraro M J, Jacoby G A, Tenover F C. Characterization of the extended-spectrum β-Lactamase reference strain, *Klebsiella pneumoniae* K6 (ATCC 700603), which produces the novel enzyme SHV-18. Antimicrob Agents Chemother 2000; 44(9): 2382-2388.

Rasheed J K, Jay C, Metchock B, Berkowitz F, Weigel L, Crellin J, Steward C, Hill B, Medeiros A A, Tenover F C. Evolution of extended-spectrum β-lactam resistance (SHV-8) in a strain of Escherichia coli during multiple episodes of bacteremia. Antimicrob Agents Chemother 1997; 41:647-653.

Rice LB, Carias LL, Hujer A M, Bonafede M, Hutton R, Hoyen C, Bonomo RA. High-level expression of chromosomally encoded SHV-1 β-lactamase and an outer membrane protein change confer resistance to ceftazidime and piperacillin-tazobactam in a clinical isolate of *Klebsiella pneumoniae*. Antimicrob Agents Chemother 2000; 44(2):362-367.

Rice LB, Willey SH, Pananicolaou GA, Medieros AA, Eliopoulos G M, Moellering JRC, Jacoby GA. Outbreak of ceftazidime resistance caused by extended-spectrum β-lactamases at a Massachusetts chronic-care facility. Antimicrob Agents Chemother 1990; 34:2193-2199.

Richard Quintiliani, JR., Daniel F. Sahm, and Patrice Courvalin, 1999. Mechanisms of resistance to antimicrobial agents. Manual of clinical microbiology. 1505-1543.

Rossolini GM, Franceschini N, Lauretti L, Caravelli B, Riccio ML, Galleni M, Frere JM, Amicosante G Cloning of a Chryseobacterium (Flavobacterium) menigiosepticum chromosomal gene ($blaA_{CME}$) encoding an extended-spectrum class A β-lactamese related to the Bacteroides cephalosporinases and the VEB-1 and PER B-lactameses. Antimicrob Agents Chemother 1999; 43:2193-2199.

Sabate M, Tarrago T, Navarro F, Miro E, Verges C, Verges C, Barbe J, Prats G. Cloning and sequence of the gene encoding a novel cefotaxime-hydrolyzing β-lactamase (CTX-M-9) from *Escherichia coli* in Spain. Antimicrob Agents Chemother 2000; 44:1970-1973.

Sambrook J, Fritsch EF, Maniatis T.1989. Molecular Cloning: a Laboratory Manual, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.;1.25-1.28.

Sanders CC, Barry AL, Washington JA, Shubert C, Moland ES, Traczewski MM. Detection of extended-spectrum β-lactamase-producing members of the family*Enterobacteriaceae* with Vitek ESBL test. J Chin Microbiol 1996; 34: 2997-3001.

Shannon K, Stapleton P, Xiang X, Johnson A, Beattie H, Bakri FE, Cookson B, French G. Extended-spectrum β-lactamase-producing Klebsiella pneumoniae strains causing nosocomial outbreaks of infection in the United Kingdom. J Clin Microbiol 1998; 36:3105-3110.

Silva J, Aguilar C, Ayala G, Estrada M A, Garza-Ramos U, Lara-Lemus R, Ledezma L. TLA-1: a new plasmid-mediated extended-spectrum β-latamese from Escherichia coli. Antimicrob Agents Chemother 2000; 44:997-1003.

Sirot D. Extended-spectrum plasmid-mediated β-Lactamases. J Antimicrob Chemother 1995; 36 : 19-34.

Steward C D, Rasheed J K, Hubert S K, Biddle J W, Raney P M, Anderson G J, Williams P P, Brittain K L, Oliver A, Mcgowan J E, Tenover F C. Characterization of clinical isolates of *Klebsiella pneumoniae* from 19 laboratories using the National Committee for Clinical Laboratory Standards Extended-spectrum β-Lactamases detection methods. J Clin Microbiol 2001; 39(8): 2864-2872.

Soilleux M J, Morand A M, Arlet G J, Scavizzi M R, Labia R. Survey of *Klebsiella pneumoniae* producing extended-spectrum β-lactamases: prevalence of TEM-3 and first identification of TEM-26 in France. Antimicrob Agents Chemother 1996; 40:1027-1029.

Sougakoff W, Goussard S, Courvalin P. The TEM-3 B-lactamase, which hydrolyzes broad-spectrum cephalosporins, is derived from the TEM-2 penicillinase by two amino acid substitutions. FEMS Microbiol Lett 1998; 56:343-348.

Stobberingh EE, Arends J, Hoogkamp-Korstanje JAA, Goessens WHF, Visser MR, Buiting AGM, Debets-Ossenkopp YJ, Ketel RJV, Ogtrop MLV, Sabbe LJM, Voorn GP, Winter HLJ, van Zeiji JH. Occurrence of extended-spectrum beta-lactamases in Dutch hospitals. Infection 1999; 27:348-354.

Szabo D, Filetoth Z, Szentandrassy J, Nemedi M, Toth E, Jeney C, Kispal G, Rozgonyi F. Molecular epidemiology of a cluster of cases due to Klebsiella pneumoniae producing SHV-5 extended-spectrum β-lactamase in the premature intensive care unit of a Hungarian hospital. J Clin Microbiol 1999; 37:4167-4169.

Tenover FC, Mohammed JM, Gorton T, Dembek ZF. Detection and reporting of organisms producing extended spectrum β-lactamases: survey of laboratories in Connecticut. J Clin Microbiol 1999; 37: 4065-4070.

Thomson KS, Sanders CC. Detection of Extended-Spectrum β-lactamase in Members of the Family *Enterobacteriaceae*: Comparison of the double disk and three-dimensional tests. Antimicrob Agents Chemother1992; 36 (9): 1877-1882.

Translation tool [online]. Available from: http://us.expasy.org/tools/dna.html [1999, feb].

Tschantz WR, Sung M, Delgado-Partin V M, Dalbey RE. A serine and a lysine residue implicated in the catalytic machanism of the *Escherichia coli* leader peptidase. J boil chem. 1993; 268(36):27349-27354.

Tzouvelekis LS, Bonomo RA. SHV-type β-lactamases. Curr Pharm1999; Des.5: 847-864.

Urban C, Meyer KS, Mariano N, Rahal JJ, Flamm R, Rasmussen BA, Bush K. Identification of TEM-26 β-lactamases responsible for a major outbreak of ceftazidime-resistant *Klebsiella pneumoniae*. Antimicrob Agents Chemother 1994; 38:392-395.

Vatopoulos AC, Philippon A, Tzouvelekis LS, Komninou Z, Legakis NJ. Prevalence of a transferable SHV-5 type β-lactamase in clinical isolates of Klebsiella pneumoniae and Escherichia coli in Greece. J Antimicrob Chemother1990; 26:635-648.

Wiedemann B, Kliebe C, Kresken M. The Epidemiology of beta-lactamases. J Antimicrob Chemother1989; 24(Suppl.B):1-22.

Woster PM. Chemistry of Beta Lactam Antibiotics PHA 421 – Infectious Disease Module [online]. Available from: http://wizard.pharm.wayne.edu/medchem/betalactam.html

Wu TL, Siu LK, Su LH, Lauderdale TL, Lin FM, Leu HS, Lin TY, Ho M. Outer membrane protein change combined with co-existing TEM-1 and SHV-1 β-lactamases lead to false identification of ESBL-producing *Klebsiella pneumoniae.* J Antimicrob Chemother 2001; 47:755-761.

Yagi T, Krukawa H, Shibata N, Shibayama K, Arakawan Y. A preliminary survey of extended-spectrum β-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Japan. FEMS Microbiol Lett 2000; 184:53-56.

Yan J, Wu S, Tsai S, Wu J, Su I. Prevalence of SHV-12 among clinical isolates of *Klebsiella pneumoniae* producing extended-spectrum β-Lactamases and identification of a novel AmpC enzyme (CMY-8) in southern Taiwan. Antimicrob Agents Chemother. 2000; 44(6): 1438-1442.

APPENDICES

APPENDIX I

THE RESULTS OF ALL TESTS IN THIS STUDY

Category 1: ESBLs data identified in *E.coli* and *K.pneumoniae,* which confer positive and positive, by double disk synergy test and Etest respectively(+, +) (total:46 strains)

STRAINS positive ESBLs	Isolation from NOSO	Results of test.																
		Disk diffusion*				Double disk**					E-test***			PCR				Sequencing
		CAZ	CRO	CTX	ATM	CAZ	CRO	CTX	ATM	AMC	TZ/TZL	CT/CTL	Results	SHV	TEM	VEB	CTX-M	
EU7-0245	-	23	**12**	**12**	**20**	-	+	+	+	19	1/.50	R/.25	+	-	-	-	+	-
EU21-0145	+	**20**	**8**	**8**	15	+	+	+	+	18	8/1.5	**R/.38**	+	-	-	-	+	-
EU23-0145	+	**25**	**13**	**14**	**25**	+	+	+	+	19	e/.38	R/.094	+	-	+	-	+	TEM-1B
EU24-0145	+	**22**	**9**	**9**	**19**	+	+	+	+	19	3/1.5	R/.50	+	-	+	-	+	TEM-1B
EU27-0245	-	**28**	**14**	**17**	**28**	+	+	+	+	20	e/.38	R/.19	+	-	+	-	-	TEM-1B
EU31-0245	-	**R**	**R**	**R**	**8**	+	+	+	+	14	R/.50	R/.125	+	-	+	-	+	TEM-1B
EU38-0245	-	**20**	**R**	**R**	**15**	+	-	-	+	17	24/2	R/R	+	-	-	-	-	-
EU39-0245	+	**16**	**11**	**12**	**16**	+	+	+	+	17	R/.025	R/.094	+	-	+	+	-	TEM-1B
EU44-0245	-	**25**	**13**	**13**	**23**	+	+	+	+	20	2/.75	R/.50	+	-	+	-	+	TEM-1B
EB48-0245	-	**R**	**11**	**10**	**R**	-	+	+	+	16	R/.3	R/.75	+	-	-	-	-	-
EB49-0245	+	**18**	**9**	**10**	**17**	+	+	+	+	17	R/1.5	R/.38	+	-	+	-	+	TEM-1B
EU61-0245	-	**29**	**22**	**25**	**30**	+	+	+	+	19	.094/<.50	e/.032	+	-	+	-	-	TEM-1B
EU78-0245	-	**R**	**13**	**13**	**14**	+	+	+	+	19	R/.38	R/.125	+	-	-	+	-	-
EU84-0245	-	**10**	**8**	**9**	**12**	+	+	+	+	15	R/.75	R/.38	+	-	+	-	-	TEM-1B
EU91-0245	-	**22**	**10**	**12**	**22**	+	+	+	+	18	e/.50	R/.125	+	-	-	-	+	-
EU95-0245	-	**16**	**9**	**10**	**14**	+	+	+	+	16	R/.75	R/.19	+	-	+	-	+	TEM-1B
EB110-0245	-	**28**	**12**	**13**	**28**	+	+	+	+	22	e/.38	R/.19	+	-	+	-	-	TEM-1B
EU134-0345	+	**11**	**19**	**20**	**20**	+	+	+	+	21	e/.25	e/.094	+	-	-	+	-	-
EU147-0345	-	**23**	**10**	**12**	**20**	+	+	+	+	18	e/.38	R/.25	+	-	+	-	-	TEM-1B

*;disk diffusion test; zone of inhibition showed as a bold printed = positive test; + = zone of inhibition of CAZ, CRO, CTX, ATM more than or equals 22,25,27,27 cm.,respectively. ** double disk synergy test, += synergistic zone ***E test; + = TZ/TZL or CT/CTL ≥ 8 :Noso= nosocomial infection; +=positive; - = negative; ND= Non determination; R = no inhibition zone; e = ellipse; CAZ = ceftazidime; CRO= ceftriaxone; CTX=cefotaxime; ATM=aztreonam;AMC=amoxicillin/clavulanic acid; TZ/TZL=ceftazidime/ceftazidime+clavulanic aicd; CT/CTL, cefotaxime/cefotaxime+clavulanic acid.

Category 1: ESBLs data identified in *E.coli* and *K.pneumoniae,* which confer positive and positive, by double disk synergy test and Etest respectively (+, +) (Cont.)

STRAINS positive ESBLs	Isolation from NOSO	Results of test.																
		Disk diffusion*				Double disk**					E-test***			PCR				Sequencing
		CAZ	CRO	CTX	ATM	CAZ	CRO	CTX	ATM	AMC	TZ/TZL	CT/CTL	Results	SHV	TEM	VEB	CTX-M	
EU148-0345	+	20	R	R	9	+	+	+	+	-	R/.50	e/.25	+	-	-	-	+	-
EU173-0345	+	16	9	9	15	+	-	-	+	16	1.5/.38	R/.38	+	-	+	-	-	TEM-1B
EU176-0345	-	25	13	13	23	+	+	+	+	17	2/1	R/.75	+	-	+	-	+	TEM-1B
EU195-0345	+	16	8	8	15	+	+	+	+	14	R/.50	R/.19	+	-	+	-	+	TEM-1B
EU226-0345	+	15	11	13	18	+	+	+	+	14	R/.75	R/.75	+	-	+	+	-	TEM-1B
EU231-0445	+	R	R	R	R	+	+	+	+	R	R/.50	R/.094	+	-	+	+	+	TEM-1B
EU253-0445	+	25	10	11	22	+	+	+	+	22	2/.25	R/.19	+	-	+	-	+	TEM-1B
EU265-0445	+	17	11	11	15	+	+	+	+	17	e/.25	R/.064	+	-	+	-	+	TEM-1B
EU269-0445	+	19	10	10	19	+	+	+	+	16	R/.50	R/.19	+	-	+	-	+	TEM-1B
EU278-0545	-	10	23	22	17	+	+	+	+	15	R/.50	6/.38	+	-	+	+	-	TEM-1B
EU-286-0545	-	25	13	13	24	+	+	+	+	19	≤.50/.25	R/.15	+	-	-	-	-	-
ES-295-0545	+	14	R	R	10	+	+	+	+	14	R/.75	R/.19	+	-	+	-	+	TEM-1B
KU14-0245	+	R	16	18	10	+	+	+	+	13	R/1.0	R/.094	+	+	-	+	-	SHV-12
KU26-0145	-	R	8	9	10	-	-	-	+	16	R/.50	R/.25	+	+	-	+	-	SHV-28
KU46-0245	-	22	11	9	20	+	+	+	+	18	6/1.5	R/.19	+	+	+	-	+	SHV-11,TEM-1B
KU47-0245	-	R	11	13	R	-	+	+	+	17	R/.75	R/.094	+	-	+	-	-	TEM-1B
KU55-0245	+	13	12	17	15	+	+	+	+	17	e/.25	R/.125	+	+	-	-	-	SHV-12
KS113-0245	+	25	12	15	23	+	+	+	+	20	e/.25	R/.125	+	+	+	-	-	SHV-11, TEM-1B
KU177-0345	-	10	20	21	18	+	+	+	+	15	R/.50	e/.25	+	-	+	+	-	TEM-1B
KU178-0345	-	10	15	13	12	+	+	+	+	21	R/.25	R/.19	+	+	-	-	-	SHV-12
KU203-0345	-	16	23	25	23	+	+	+	+	17	e/.25	e/.125	+	+	+	+	-	TEM-1B,SHV-1
KU233-0445	+	23	12	12	23	+	+	+	+	18	e/.25	R/.064	+	+	-	-	-	Novel N254H
KU237-0445	+	20	10	11	18	+	+	+	+	19	8/1	R/R	+	+	-	-	-	SHV-1
KP-297-0545	+	22	20	22	26	+	+	+	+	22	.75/.25	e/.064	+	+	-	-	-	SHV-2
KU-299-0545	_	R	16	18	R	-	+	+	-	13	R/2	R/.25	+	+	+	+	-	TEM-1B, SHV-12
KS-312-0545	+	14	24	25	24	+	+	+	+	18	24/.25	e/.094	+	+	-	+	-	SHV-1
KU-325-0545	+	10	R	R	8	+	+	+	+	16	R/1.5	R/R	+	+	+	-	+	TEM-1B,SHV-11

*;disk diffusion test; zone of inhibition showed as a bold printed = positive test; + = zone of inhibition of CAZ, CRO, CTX, ATM more than or equals 22,25,27,27 cm.,respectively. ** double disk synergy test, += synergistic zone ***E test; + = TZ/TZL or CT/CTL ≥ 8 :Noso= nosocomial infection; +=positive; - = negative; ND= Non determination; R = no inhibition zone; e = ellipse; CAZ = ceftazidime; CRO= ceftriaxone; CTX=cefotaxime; ATM=aztreonam;AMC=amoxicillin/clavulanic acid; TZ/TZL=ceftazidime/ceftazidime+clavulanic aicd; CT/CTL, cefotaxime/cefotaxime+clavulanic acid.

Category 2: ESBLs data identified in *E.coli* and *K.pneumoniae,* which confer positive and non determination, by double disk synergy test and Etest respectively (+, ND) (total ;3 strains).

STRAINS positive ESBLs	Isolation from NOSO	Results of test.															
		Disk diffusion*				Double disk**					E-test***			PCR				Sequencing
		CAZ	CRO	CTX	ATM	CAZ	CRO	CTX	ATM	AMC	TZ/TZL	CT/CTL	Results	SHV	TEM	VEB	CTX-M	
KU201-0345	+	**R**	**14**	**15**	**R**	+	+	+	+	**13**	R/R	R/R	ND	+	+	-	-	TEM-1B, SHV-12
KU213-0345	-	**R**	**11**	**11**	**R**	+	+	+	+	**8**	R/R	R/R	ND	+	-	-	-	SHV-12
KU241-0445	_	**R**	**10**	**10**	**R**	+	+	+	+	**10**	R/R	R/R	ND	+	-	-	-	SHV-12

*;disk diffusion test; zone of inhibition showed as a bold printed = positive test; + = zone of inhibition of CAZ, CRO, CTX, ATM more than or equals 22,25,27,27 cm.,respectively. ** double disk synergy test, += synergistic zone ***E test; + = TZ/TZL or CT/CTL ≥ 8 :Noso= nosocomial infection; +=positive; - = negative; ND= Non determination; R = no inhibition zone; e = ellipse; CAZ = ceftazidime; CRO= ceftriaxone; CTX=cefotaxime; ATM=aztreonam;AMC=amoxicillin/clavulanic acid; TZ/TZL=ceftazidime/ceftazidime+clavulanic aicd; CT/CTL, cefotaxime/cefotaxime+clavulanic acid.

Category 3: ESBLs data identified in *E.coli* and *K.pneumoniae,* which confer positive and non determination by double disk synergy test and Etest respectively (-, +) (total ;7 strains).

STRAINS positive ESBLs	Isolation from NOSO	Results of test.																
		Disk diffusion*				Double disk**					E-test***			PCR				Sequencing
		CAZ	CRO	CTX	ATM	CAZ	CRO	CTX	ATM	AMC	TZ/TZL	CT/CTL	Results	SHV	TEM	VEB	CTX-M	
EU115-0345	-	**11**	**R**	**R**	**10**	-	-	-	-	19	8/.75	R/R	+	-	-	-	-	-
ES128-0345	+	**18**	**R**	**R**	**15**	-	-	-	-	14	R/.38	R/.25	+	-	+	-	-	TEM-1B
EU141-0345	+	**15**	**R**	**R**	**13**	-	-	-	-	**12**	R/1.0	R/.25	+	-	+	-	+	TEM-1B
EU165-0345	+	**18**	**R**	**R**	**13**	-	-	-	-	15	12/1.5	R/R	+	-	+	-	+	TEM-1B
EU256-0345	-	**17**	**R**	**R**	**10**	-	-	-	-	**12**	12/1.5	R/R	+	-	+	-	-	**TEM-1B**
KS53-0245	-	**22**	**12**	**14**	**21**	-	-	-	-	**13**	2/.50	R/.19	+	+	+	-	+	TEM-1B,SHV-11
KU206-0345	-	**16**	**R**	**9**	**13**	-	-	-	-	**13**	6/1.0	R/.38	+	+	+	-	-	TEM-1B,SHV-11

*;disk diffusion test; zone of inhibition showed as a bold printed = positive test; + = zone of inhibition of CAZ, CRO, CTX, ATM more than or equals 22,25,27,27 cm.,respectively. ** double disk synergy test, += synergistic zone ***E test; + = TZ/TZL or CT/CTL ≥ 8 :Noso= nosocomial infection; +=positive; - = negative; ND= Non determination; R = no inhibition zone; e = ellipse; CAZ = ceftazidime; CRO= ceftriaxone; CTX=cefotaxime; ATM=aztreonam;AMC=amoxicillin/clavulanic acid; TZ/TZL=ceftazidime/ceftazidime+clavulanic aicd; CT/CTL, cefotaxime/cefotaxime+clavulanic acid.

Category 4: ESBLs data identified in *E.coli* and *K.pneumoniae* ,which confer negative and Non determination by double disk synergy test and Etest respectively (-,ND) (total ;5 strains).

STRAINS positive ESBLs	Isolation from NOSO	Results of test.															
		Disk diffusion*				Double disk**					E-test***			PCR				Sequencing
		CAZ	CRO	CTX	ATM	CAZ	CRO	CTX	ATM	AMC	TZ/TZL	CT/CTL	Results	SHV	TEM	VEB	CTX-M	
EU41-0245	+	**15**	**19**	**20**	**22**	-	-	-	-	**12**	R/R	R/R	ND	-	-	-	-	-
EU161-0345	-	**R**	R	**R**	**R**	-	-	-	-	**17**	R/R	R/R	ND	-	-	-	-	-
KU127-0345	-	**12**	**R**	**R**	**14**	-	-	-	-	**14**	R/R	R/R	ND	+	+	-	-	TEM-1B,SHV-11
KU152-0345	-	**R**	**R**	**R**	**R**	-	-	-	-	**9**	R/R	R/R	ND	+	+	-	+	TEM-1B,SHV-12
KU204-0345	+	**R**	**R**	**R**	**R**	-	-	-	-	**12**	R/R	R/R	ND	-	+	+	-	TEM-1B

*;disk diffusion test; zone of inhibition showed as a bold printed = positive test; + = zone of inhibition of CAZ, CRO, CTX, ATM more than or equals 22,25,27,27 cm.,respectively. ** double disk synergy test, += synergistic zone ***E test; + = TZ/TZL or CT/CTL ≥ 8 :Noso= nosocomial infection; +=positive; - = negative; ND= Non determination; R = no inhibition zone; e = ellipse; CAZ = ceftazidime; CRO= ceftriaxone; CTX=cefotaxime; ATM=aztreonam;AMC=amoxicillin/clavulanic acid; TZ/TZL=ceftazidime/ceftazidime+clavulanic aicd; CT/CTL, cefotaxime/cefotaxime+clavulanic acid.

Category 5: ESBLs data identified in *E.coli* and *K.pneumoniae,* which confer negative and Negative, by double disk synergy test and Etest respectively (-, -) (total ;6 strains).

STRAINS positive ESBLs	Isolation from NOSO	Results of test.															
		Disk diffusion*				Double disk**					E-test***			PCR				Sequencing
		CAZ	CRO	CTX	ATM	CAZ	CRO	CTX	ATM	AMC	TZ/TZL	CT/CTL	Results	SHV	TEM	VEB	CTX-M	
EU185-0345	+	**18**	**9**	**9**	**18**	-	-	-	-	**10**	6/R	R/R	-	-	+	-	-	TEM-1B
EU220-0345	+	**20**	**24**	**24**	**25**	-	-	-	-	**12**	6/R	8/R	-	-	+	-	-	TEM-1B
EU228-0345	+	**21**	**10**	**10**	**20**	-	-	-	-	**13**	3/1.0	R/R	-	-	+	-	-	TEM-1B
EU254-0345	+	**21**	**10**	**10**	**19**	-	-	-	-	**10**	12/R	R/R	-	-	+	-	-	TEM-1B
ES-296-0545	+	**16**	**20**	**19**	**17**	-	-	-	-	**11**	8/R	R/R	-	-	+	-	-	TEM-1B
KU-318-0545	-	**18**	**R**	**R**	**13**	-	-	-	-	**13**	8/R	R/R	-	+	+	-	-	SHV-1, TEM-1B

*;disk diffusion test; zone of inhibition showed as a bold printed = positive test; + = zone of inhibition of CAZ, CRO, CTX, ATM more than or equals 22,25,27,27 cm.,respectively. ** double disk synergy test, += synergistic zone ***E test; + = TZ/TZL or CT/CTL ≥ 8 :Noso= nosocomial infection; +=positive; - = negative; ND= Non determination; R = no inhibition zone; e = ellipse; CAZ = ceftazidime; CRO= ceftriaxone; CTX=cefotaxime; ATM=aztreonam;AMC=amoxicillin/clavulanic acid; TZ/TZL=ceftazidime/ceftazidime+clavulanic aicd; CT/CTL, cefotaxime/cefotaxime+clavulanic acid.

APPENDIX II

REAGENTS AND EQUIPMENT

1) Media and reagents

Absolute ethanol	Merck, Germany.
Agarose (ultrapure)	GibcoBRL, USA.
Bacteriological peptone	Oxoid, USA.
Boric acid	Sigma, USA.
Bromthymol blue	Fluka, Germany.
Decarboxylase medium base	Difco, USA.
dNTPs	Promega, USA
EDTA	Amresco, USA.
Ethidium bromide	Amresco, USA.
Glacial acetic acid	Merck, Germany.
Horse serum	GibcoBRL, USA.
Hydrochloric acid	Merck, Germany.
L-Arginine hydrochloride	Sigma, USA.
Lactose	Oxoid, USA.
L-Lysine monohydrochloride	Sigma, USA.
L-Ornithine monohydrochloride	Sigma, USA.
MacConkey's agar	BBL, USA.
Malonate	BBL, USA.
Methyl red	Riedel-deHaen, Germany.
Motility medium	BBL, USA.
Mueller Hinton agar	Biorad, France.
N,N,N'.N'-tetramethyl-*p*- Phenylenediamine Dihydrochloride	Sigma, USA.

Orthonitrophenyl-β- D-Galactopyranoside	Oxoid, USA.
Potassium hydroxide	Merck, Germany.
Simmon citrate	BIOTEC, UK
Sodium acetate	Merck, Germany.
Sodium Chloride	Merck, Germany.
Taq DNA polymerase	Promega, USA.
Triple sugar iron agar	Oxoid, USA.
Tris base	Sigma, USA.
Trypticase soy agar	Oxoid, USA.
Trypticase soy broth	Oxoid, USA.
Urea medium	BBL, USA.
100 bp DNA ladder	Promega, USA.

2) Equipments

ABI prism 310 automated sequencer	Perkin Elmer, USA.
Automatic pipette	Gilson, Lyon, France.
Chemi Doc	BIORAD, USA.
Gelmate 2000 electrophoresis	Toyobo, Japan.
Incubator	Forma Scientific, USA.
Microcentrifuge	Eppendorf, USA.
Mixer-Vertex-Genic	Scientific industries,USA.
PCR machine Gene Amp PCR System 2400	Perkin Elmer, USA.
Spectophotometer	BIORAD, USA.

APPENDIX III

REAGENTS PREPARATION

1. Reagents for bacterial identification

1.1 Oxidase test reagent

Prepare a fresh solution of tetramethyl –*p*-phenylenediamine dihydrochloride each time of use by adding a loopful of it to about 3 ml of sterile distilled water or saline. Do not use if it becomes blue. Autoxidation of the reagent occurs rapidly and although this can be related by the addition of 1% ascorbic acid. It is not sufficiently stable in aqueous solution for storage.

1.2 Kovac's reagent

p-Dimethylaminobenzaldehyde	5 g
Iso amyl alcohol	75 ml
Conc. HCl	25 ml

Dissolve the aldehyde in the alcohol by gently warming in a water bath (about 50-55 °C). Cool and add the acid with care. Protect from light and store at 4°C.

1.3 Methyl red solution

Methyl red	0.04 g
Absolute ethanol	40 ml

Dissolve the methyl red in the ethanol and dilute to volume 100 ml with distilled water.

1.4 VP reagent

Solution 1

α-Naphthol	5 g
Absolute ethanol	100 ml

Dissolve α-Naphthol in ethanol then mix well and store at 4°C.

Solution 2

Potassium hydroxide	40 g
Distilled water	100 ml

Dissolve Potassium hydroxide in distilled water then mix well and store at 4°C.

2. Reagents for screening of ESBL production

2.1 McFarland 0.5 turbidity standard

1.175% $BaCl_2.2H_2O$	0.5 ml
1% H_2SO_4	99.5 ml

Measured the absorbance at 625 nm with a spectrophotometer; the optimal O.D. value is 0.08 to 0.10.

2.2 Sterile saline solution (suspending of bacterial inocula)

Sodium chloride	8.5	g/L
Distilled water	1	L

Sterilze by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. Store at room temperature.

2.3 Mueller- Hinton agar (Susceptibility testing)

Mueller-Hinton agar	35	g/L
Distilled water	1	L

The medium was sterilized by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. The sterile medium was cooled to 45°C to 50°C. Add blood after cooling base medium. Dispense 20 ml per petri dish. Cool and store at 4°C until used.

3. Reagents for agarose gel electrophoresis

3.1 0.5 M EDTA, pH 8.0

Disodium ethylene diamine tetraacetate.$2H_2O$	186.1 g
Distilled water	800 ml

Adjust pH to 8.0 with sodium hydroxide pellet then adjust volume to 1,000 ml

3.2 50X Tris-acetate buffer (TAE)

Tris base	242.0 g
Glacial acetic acid	57.1 ml
0.5 M EDTA pH 8.0	100 ml

Adjust the volume to 1 liter with deionized distilled water and sterilize by autoclaving at 121°C for 15 min.

3.3 1OX Tris-borate buffer (TBE)

Tris base	108 g
Boric acid	55 g
0.5 M EDTA pH 8.0	40 ml

Adjust volume to 1,000 ml with H_2O then sterilize by autoclaving.

3.4 10 mg/ml Ethidium bromide (Et-Br) (Stock)

Ethidium bromide	1 g
DDW	100 ml

Stir on a magnetic stirrer for several hours to ensure that dye has dissolved. Wrap the container in aluminum foil or transfer to a dark bottle and stores at 4°C

3.5 1% Agarose gel

Agarose ultrapure	0.4 g
1X TAE or TBE buffer	40 ml

4. Reagents for PCR product purification

4.1 **Buffer PB** (Ready to used)

4.2 **Buffer PE**

Buffer PE is supplied as a concentrate. Before using for the first time, add the 55-ml of ethanol (96-100%) to buffer PE concentrate as indicated on the bottle.

5. Reagents for Sequencing

5.1 **3 M Sodium acetate, pH 8.0**

Sodium acetate, 3 H_2O	408.1 g
Distilled water	800 ml

Adjust pH to 8.0 with glacial acetic acid, Adjust volume to 1,000 ml.

APPENDIX IV

Amino Acid Sequences for TEM and SHV

1. TEM

ß-Lactamase	Alternate Names(s)	Amino Acid at position																																						pI
		4	5	6	21	39	42	51	69	80	84	92	104	115	124	127	130	145	153	163	164	165	182	184	196	204	218	237	238	240	244	262	265	268	275	276	280	289	
TEM-1	RTEM-1	S	I	Q	L	Q	A	L	M	V	V	G	E	D	S	I	S	P	H	D	R	W	M	A	G	R	G	A	G	E	R	V	T	S	R	N	A	H	5.4
TEM-2						K																																		5.6
TEM-3	CTX-1 TEM-14					K							K																S											6.3
TEM-4					F								K																S				M							5.9
TEM-5	CAZ-1																				S							T		K										5.6
TEM-6													K								H																			5.9
TEM-7						K															S																			5.4
TEM-8	CAZ-2					K							K								S								S											5.9
TEM-9	RHH-1				F								K								S												M							5.5
TEM-10	MGH-1 TEM-E3 TEM-23																				S									K										5.6
TEM-11	CAZ-lo					K															H																			5.6

[a] Abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine, M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine: T, threonine; V, valine; W, tryptophan; Y,Tyrosine

TEM (cont.)

ß-Lactamase	Alternate Names(s)	Amino Acid at position																																							pI
		4	5	6	21	39	42	51	69	80	84	92	104	115	124	127	130	145	153	163	164	165	182	184	196	204	218	237	238	240	244	262	265	268	275	276	280	289	
TEM-12	YOU-2 CAZ-3 TEM-E2																				S																			5.3
TEM-13													K																				M							5.6
TEM-15													K																S											6
TEM-16	CAZ-7					K							K								H																			6.3
TEM-17													K																											5.5
TEM-18						K							K																											6.3
TEM-19																													S										5.4
TEM-20																							T						S										5.4
TEM-21						K							K						R										S										6.4
TEM-22						K							K															G	S											6.3
TEM-24	CAZ-6					K							K								S							T		K										6.5
TEM-25	CTX-2				F																								S				M							5.3
TEM-26	YOU-1												K								S																			5.6
TEM-27																					H									K			M							5.9
TEM-28																					H									K										6.1
TEM-29																					H																			5.4

[a] Abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine, M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine: T, threonine; V, valine; W, tryptophan; Y,Tyrosine

TEM (cont.)

ß-Lactamase	Alternate Names(s)	Amino Acid at position																																					pI
		4	5	6	21	39	42	51	69	80	84	92	104	115	124	127	130	145	153	163	164	165	182	184	196	204	218	237	238	240	244	262	265	268	275	276	280	289	
TEM-30	IRT-2 TRI-2 E-GUER																														S								5.2
TEM-31	IRT-1 TRI-1 E-SAL																														C								5.2
TEM-32	IRT-3								I														T																5.4
TEM-33	IRT-5								L																														5.4
TEM-34	IRT-6								V																														5.4
TEM-35	IRT-4								L																											D			5.2
TEM-36	IRT-7								V																											D			5.2
TEM-37									I																											D			5.2
TEM-38	IRT-9								V																										L				5.2
TEM-39	IRT-10								L													R														D			5.4
TEM-40	IRT-167 IRT-11								I																														5.4
TEM-41 (withdrawn)	IRT-12																														T								5.2
TEM-42						K	V																						S	K			M						5.8

[a] Abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine, M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine: T, threonine; V, valine; W, tryptophan; Y,Tyrosine

TEM (cont.)

ß-Lactamase	Alternate Names(s)	Amino Acid at position																																						pI
		4	5	6	21	39	42	51	69	80	84	92	104	115	124	127	130	145	153	163	164	165	182	184	196	204	218	237	238	240	244	262	265	268	275	276	280	289	
TEM-43													K								H		T																	6.1
TEM-44	IRT_2-2 IT-13					K																									S								5.4
TEM-45	IRT-14								L																										Q				5.2
TEM-46	CAZ-9					K							K								S									K									6.5
TEM-47																													S	K			M						6
TEM-48					F																								S	K			M						6
TEM-49					F																								S	K			M	G					6
TEM-50	CMT-1								L				K																S							D			5.6
TEM-51	IRT-15																														H								5.2
TEM-52													K										T						S										6
TEM-53					F																S																		
TEM-54																															L								
TEM-55																											E												5.2
TEM-56						K							K						R																				6.4
TEM-57												D																												5.2
TEM-58																															S	I							5.2
TEM-59	IRT-17					K											G																							5.6

[a] Abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine, M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine: T, threonine; V, valine; W, tryptophan; Y,Tyrosine

TEM (cont.)

ß-Lactamase	Alternate Names(s)	Amino Acid at position																																						pI
		4	5	6	21	39	42	51	69	80	84	92	104	115	124	127	130	145	153	163	164	165	182	184	196	204	218	237	238	240	244	262	265	268	275	276	280	289	
TEM-60						K		P					K								S																			6.4
TEM-61	CAZ-hi					K															H									K									6.5
TEM-62	Reserved																																							
TEM-63	TEM-64				F								K								S		T																	5.6
TEM-65	IRT-16					K																									C								5.4
TEM-66						K						D	K																S											6
TEM-67					I	K																									C								5.2
TEM-68																													S	K			M		L				5.7
TEM-69	Withdrawn																																							
TEM-70																										Q														5.2
TEM-71																													S	K									6
TEM-72						K																	T						S	K									5.9
TEM-73	IRT-18				F																										C		M						5.2
TEM-74	IRT-19				F																										S		M						5.2
TEM-75					F																H												M						
TEM-76	IRT-20																G																							
TEM-77	IRT-21								L																						S									
TEM-78	IRT-22								V													R															D			

[a] Abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine, M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine: T, threonine; V, valine; W, tryptophan; Y,Tyrosine

TEM (cont.)

ß-Lactamase	Alternate Names(s)	Amino Acid at position																																					pI
		4	5	6	21	39	42	51	69	80	84	92	104	115	124	127	130	145	153	163	164	165	182	184	196	204	218	237	238	240	244	262	265	268	275	276	280	289	
TEM-79																															G								
TEM-80	IRT-24								L							V																			D			5.2
TEM-81									L							V																						
TEM-82									V																									Q				
TEM-83									I													C												Q				
TEM-84																																			D			
TEM-85					F																S								K			M					
TEM-86					F																S						T		K			M					
TEM-87				K									K								C		T															
TEM-88													K										T		D				S									5.6
TEM-89						K							K				G												S									6.3
TEM-90	TLE-1													G																								5.6
TEM-91																					C		T						K									
TEM-92				K									K										T						S									6
TEM-93																							T						S	K								
TEM-94					F								K										T						S				M					
TEM-95																		A																				
TEM-96																				G																		

[a] Abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine, M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine: T, threonine; V, valine; W, tryptophan; Y,Tyrosine

TEM (cont.)

ß-Lactamase	Alternate Names(s)	Amino Acid at position																																							pI
		4	5	6	21	39	42	51	69	80	84	92	104	115	124	127	130	145	153	163	164	165	182	184	196	204	218	237	238	240	244	262	265	268	275	276	280	289	
TEM-97		D	P						V																													L	
TEM-98		D	P						M																													L	
TEM-99		D	P																												S							L	
TEM-100	Not yet released																																						
TEM-101						K																							S	K						V		
TEM-102		D	P		F																S												M						
TEM-103	IRT-28																																		L				5.2
TEM-104																																					V		
TEM-105															N																								
TEM-106													K										T																6
TEM-107													K								H		T					S											6
TEM-108		D	P							E															S										S			
TEM-109	Not yet released																																						
TEM-110					F																												M					
TEM-111													K															D					M					
TEM-112	Not yet released																																						

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2. SHV

ß-Lactamase	Alternate Name	Amino acid at position																																							pI
		7	8	18	25	35	43	48	54	69	75	80	89	113	122	126	129	130	140	141	142	146	149	156	158	173	179	187	188	192	193	205	226	238	240	243	267	278	
SHV-1		Y	I	T	A	L	R	E	G	M	V	V	E	L	L	A	M	S	A	T	V	A	T	G	N	N	D	A	A	K	L	R	P	G	E	A	T	Q	7.6
SHV-2																																		S					7.6
SHV-2A						Q																												S					7.6
SHV-3																																L		S					7
SHV-4																																L		S	K				7.8
SHV-5																																		S	K				8.2
SHV-6																											A												7.6
SHV-7			F				S																											S	K				7.6
SHV-8																											N												7.6
SHV-9	SHV-5a								Del										R											N	V			S	K				8.2
SHV-10									Del									G	R											N	V			S	K				8.2
SHV-11	SHV-1-2a					Q																												S	K				7.6
SHV-12	SHV-5-2a					Q																												S	K				8.2
SHV-13						Q																												A					7.6
SHV-14			F				S																																7
SHV-15						Q		K			M	M	K																					S	K				
SHV-16	163DRWET 167 insertion																																						7.6

[a] Abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine, M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine: T, threonine; V, valine; W, tryptophan; Y,Tyrosine

SHV (cont.)

ß-Lactamase	Alternate Name	Amino acid at position																																						pI
		7	8	18	25	35	43	48	54	69	75	80	89	113	122	126	129	130	140	141	142	146	149	156	158	173	179	187	188	192	193	205	226	238	240	243	267	278	
SHV-17	Withdrawn																																							
SHV-18			F				S																											A	K				7.8
SHV-19																										F													7.6
SHV-20																										F								S					7.6
SHV-21															F											F								S					7.6
SHV-22																									K									D	K				7.6
SHV-23	Not yet released																																							
SHV-24																											G													
SHV-25				A		Q											V																							7.5
SHV-26																												T											7.6
SHV-27																								D																
SHV-28		F																																						
SHV-29						Q	S																											A					
SHV-30	Not yet released																																							
SHV-31	Not yet released																																							
SHV-32																V								D																

[a] Abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine, M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine: T, threonine; V, valine; W, tryptophan; Y,Tyrosine

SHV (cont.)

ß-Lactamase	Alternate Name	Amino acid at position																																										pI
		7	8	18	25	35	43	48	54	69	75	80	89	113	122	126	129	130	140	141	142	146	149	156	158	173	179	187	188	192	193	205	226	238	240	243	267	278	
SHV-33																																	S						
SHV-34			F				S		G																								S					
SHV-35						Q							K																							G			
SHV-36			V			Q																																	
SHV-37				A		Q											V																					H	
SHV-38																						V																	7.6
SHV-39																																	S			S		
SHV-40						Q																														G			
SHV-41																					F																		
SHV-42					S												V																						
SHV-43														F									S																8
SHV-44	Not yet released																																						
SHV-45																								D									S	K				8.2
SHV-46	Not yet released																																						
SHV-47				A						I										I																		

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3. 64 codon on DNA

First Position	Second Position				Third Position
	T	C	A	G	
T	F	S	Y	C	**T**
	F	S	Y	C	**C**
	L	S	STOP CODON	STOP CODON	**A**
	L	S	STOP CODON	W	**G**
C	L	P	H	R	**T**
	L	P	H	R	**C**
	L	P	Q	R	**A**
	L	P	Q	R	**G**
A	I	T	N	S	**T**
	I	T	N	S	**C**
	I	T	K	R	**A**
	M	T	K	R	**G**
G	V	A	D	G	**T**
	V	A	D	G	**C**
	V	A	E	G	**A**
	V	A	E	G	**G**

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BIOGRAPHY

Miss Mayteera Dansuputra was born on September 25, 1977 in Bangkok, Thailand. She graduated with the Bachelor degree of Science in Biotechnology from the Faculty of Science, King Mongkut Institute of Technology Ladkrabang in 1997.