ยีนที่ควบคุมการดื้อยาปฏิชีวนะกลุ่มเบต้าแลคแทมและยีโนทัยป์ของ
Escherichia coli จากสิ่งส่งตรวจของผู้ป่วย

นางสาวยุพิน ไตรภพสกุล

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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BETA – LACTAM ANTIBIOTIC RESISTANT GENES AND GENOTYPES OF *ESCHERICHIA COLI* FROM CLINICAL SPECIMENS

Miss Yupin Taipobsakul

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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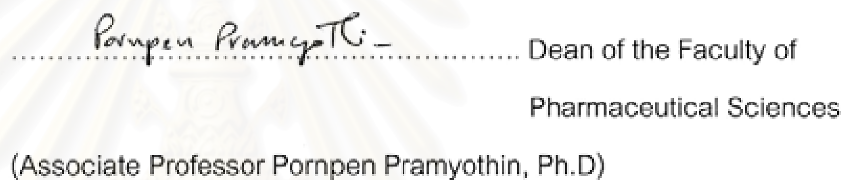

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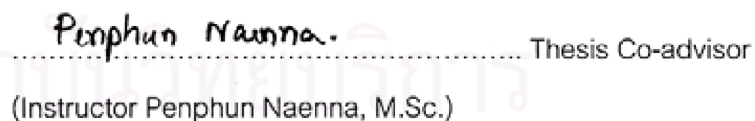

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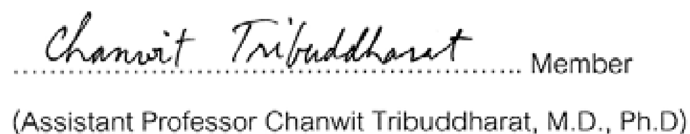

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เมื่อนำเชื้อ *Escherichia coli* จำนวน 120 สายพันธุ์ ซึ่งแยกมาจาก เลือด ,หนอง และ ปัสสาวะของผู้ป่วยที่โรงพยาบาลศิริราชระหว่างเดือนมิถุนายนถึงสิงหาคม พ.ศ. 2547 มาตรวจหาเชื้อที่สร้างเอนไซม์ extended spectrum beta-lactamase (ESBL) โดยใช้วิธี initial screen test และ phenotypic confirmatory test ตามข้อกำหนดของ The National Committee for Clinical Laboratory Standards (NCCLS) พบเป็นเชื้อที่สร้าง ESBL จำนวน 37 สายพันธุ์

สำหรับผลความไวรับของเชื้อที่สร้างเอนไซม์ ESBL พบว่าเมื่อทดสอบกับยากลุ่ม cephalosporin รุ่น 3 เชื้อทุกสายพันธุ์จะดื้อต่อ cefpodoxime ในขณะที่ไวต่อ ceftazidime (46.0%) มากกว่า cefotaxime (8.1%) และ ceftriaxone (5.4%) เมื่อทดสอบกับยาในกลุ่มbeta-lactam ชนิดอื่นๆ พบว่า 2.7% ไวต่อ ampicillin,10.8% ไวต่อ amoxicillin/clavulanic acid, 2.7% ไวต่อ cefazolin, 67.6% ไวต่อ cefoxitin, 19.0% ไวต่อ aztreonam และ 97.3% ไวต่อ imipenem สำหรับยาในกลุ่มอื่นๆ พบว่า 13.5% ไวต่อ gentamicin, 32.4% ไวต่อ norfloxacin และ 27.0% ไวต่อ trimethoprim/sulfamethoxazole

เมื่อนำเชื้อสายพันธุ์ที่สร้าง ESBL มาตรวจหายีน bla_{TEM} , bla_{SHV} , bla_{CTX-M} และ bla_{VEB} โดยวิธี Polymerase Chain Reaction (PCR) พบว่าเชื้อมียีน bla_{TEM} 78.4% , bla_{SHV} 8.1% , bla_{CTX-M} 78.4% และ bla_{VEB} 8.1% ตรวจพบยีน *bla* อย่างน้อย 2 ชนิดในเชื้อสายพันธุ์เดียวกันสูงถึง 75.7% โดยตรวจพบยีน bla_{TEM} ร่วมกับbla_{CTX-M} มากที่สุด เมื่อนำ *E. coli* 120 สายพันธุ์มาตรวจหา Integrase (*IntI*1) gene ซึ่งเป็นยีนที่ทำหน้าที่ในการพา multiple antimicrobial resistant genes ไปยังเชื้อสายพันธุ์ต่างๆ โดยวิธี PCR และ Southern blot hybridization โดยพบ *IntI*1 สูงถึง 99.2% ซึ่งชี้ให้เห็นว่าอาจมีการแพร่กระจายของเชื้อดื้อยาในโรงพยาบาลศิริราชในอัตราที่สูง

จากการศึกษา pulsotype ของเชื้อ *E. coli* ที่สร้าง ESBL ทั้ง 37 สายพันธุ์ โดยวิธี Pulsed field gel electrophoresis (PFGE) สามารถจำแนกเชื้อออกได้ถึง 32 pulsotypes ความหลากหลายของสายพันธุ์ชี้ให้เห็นว่าการแพร่กระจายของเชื้อดื้อยาจะเป็นทั้งแบบ vertical และ horizontal gene transfer โดยส่วนใหญ่เป็นแบบ horizontal gene transfer

ภาควิชา จุลชีววิทยา.................... ลายมือชื่อนิสิต..........ยุพิน ไตรภพสกุล..........

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ปีการศึกษา 2548...................... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....เพ็ญพรรณ แน่นหนา.....

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KEY WORD: *Escherichia coli* / ESBLs / TEM / SHV / CTX-M / VEB/ Integrase (*IntI*1) gene

YUPIN TAIPOBSAKUL: BETA – LACTAM ANTIBIOTIC RESISTANT GENES AND GENOTYPES OF *ESCHERICHIA COLI* FROM CLINICAL SPECIMENS. THESIS ADVISOR : ASSOC. PROF DR. PINTIP PONGPECH, THESIS COADVISOR : INSTRUCTOR PENPHUN NAENNA, 167 pp. ISBN 974-53-2444-2.

Thirty-seven extended spectrum beta-lactamase (ESBL) producing strains were detected among the 120 *E. coli* isolated from blood, pus, and urine of the patients at Siriraj Hospital during June to August 2004 using the National Committee for Clinical Laboratory Standards (NCCLS) initial screen test and the phenotypic confirmatory test.

Antimicrobial susceptibility test of all ESBL producing *E. coli* strains were performed. Among the third generation cephalosporins, it was shown that all strains were resistant to cefpodoxime while these strains were more susceptible to ceftazidime (46.0%) than to cefotaxime (8.1%) and ceftriaxone (5.4%). For the other beta-lactam antibiotics, it was shown that 2.7% were susceptible to ampicillin, 10.8% to amoxicillin/clavulanic acid, 2.7% to cefazolin, 67.6% to cefoxitin, 19.0% to aztreonam, and 97.3% to imipenem. For non beta-lactam antibiotics, it was shown that 13.5% were susceptible to gentamicin, 32.4% to norfloxacin, and 27.0% to trimethoprim /sulfamethoxazole.

The purified DNA from ESBL producing strains were also subjected for Polymerase Chain Reaction (PCR) amplification of ESBL genes (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{VEB} genes). Among 37 ESBL producing *E. coli* strains, 78.4% were bla_{TEM} positive, 8.1% were bla_{SHV} positive, 78.4% were $bla_{CTX\text{-}M}$ positive, 8.1% were bla_{VEB} positive, and 75.7% were coharboured at least 2 different *bla* genes. As a result, the presence of bla_{TEM} and $bla_{CTX\text{-}M}$ in ESBL producing *E. coli* strains indicates the high prevalence of these genes. All 120 *E. coli* strains were investigated for the presence of an integrase (*intI*1) gene which acts as the antimicrobial resistant genes carries to the other bacterial strains by PCR. Southern blot analysis using *intI*1 as a probe was also performed for all isolated DNA. As a result, as high as 99.2% of the strains carried *intI*1. This indicated the wild spread of resistant bacteria in Siriraj Hospital.

There were 32 different pulsotypes among all 37 ESBL producing *E. coli* strains. The various different types indicated that horizontal gene transfer was more predominate than vertical gene transfer for the mode of antibiotic resistance transmission.

Department	Microbiology.............	Student's signature. Yupin Taipobsakul
Field of study	Microbiology.............	Advisor's signature. Pintip Pongpech
Academic year	2005.......................	Co-advisor's signature. Penphun Naenna

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
bp	base pair
bla	beta-lactamase gene
$bla_{CTX\text{-}M}$	CTX-M beta-lactamase gene
bla_{SHV}	SHV beta-lactamase gene
bla_{TEM}	TEM beta-lactamase gene
bla_{VEB}	VEB beta-lactamase gene
°C	degree celsius
CTX-M	Cefotaximes
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
E. coli	*Escherichia coli*
e.g.,	exempli gratia (for example)
EMB	Eosin Methylene Blue Agar
ESBL	extended-spectrum beta-lactamase
et al.	et alii (and other people)
g	gram
*intI*1	integrase gene of class 1 integron
MHA	Mueller Hinton Agar

min	minute
ml	milliliter
NCCLS	National Committee for Clinical Laboratory Standards
NSS	Normal Saline Solution
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
SHV	Sulfydryl variable
TEM	Temoniera
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
v	volt
VEB	Vietnamese extended-spectrum beta-lactamase
μg	microgram
μl	microliter
%	percent

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CHAPTER I

INTRODUCTION

Escherichia coli is the most significant species in the family *Enterobacteriaceae*, genus *Escherichia*. It is normally considered as a non harmful member of the colon flora. However, sometimes, *E. coli* has been associated with a wide range of diseases and infections, including neonatal meningitis (particularly in the newborn), gastrointestinal infections, urinary tract infections, wound, and bacteremic infections in all age groups.

Beta-lactam antimicrobial agents represent the most common treatment for bacterial infections and continue to be leading cause of resistance to beta-lactam antibiotics among gram-negative bacteria worldwide.

E. coli can produce beta–lactamase enzyme that destroys beta-lactam antibiotics such as penicillins and cephalosporins, thus the treatment does not work. Extended spectrum beta-lactamases (ESBL) are enzymes that confer resistance to oxyimino cephalosporins, such as cefotaxime, ceftazidime and ceftriaxone and to monobactams, such as aztreonam. ESBLs predominantly are derivatives of plasmid-mediated TEM or SHV beta-lactamases, arise through a mutation or mutations that result in one or more amino acid substitutions.

Emerging multiple antibiotics resistant bacteria is a problem of current concern. *E. coli* strains from clinical specimens may be resistant to multiple antibiotics and a substantial proportion of multidrug resistant *E. coli* strains carry a mobile genetic element called integron element.

Since ESBL-producing organisms were first recognized in 1983, their emergence and rapid dissemination have been responsible for numerous outbreaks of infection throughout the world. The ESBL frequency has again increased sharply worldwide and they now are a major treatment problem in clinic. Clinically, they need for accurate detection of ESBLs of undividual therapy and efficient infection control to prevent outbreaks.

Nevertheless, the microbiology ESBL detection method only confirm whether an ESBL is produced but cannot detect which ESBL subtype is present. Definitive identification is very demanding and only possible by molecular detection methods. The techniques, which are necessary for the task of identifying the exact ESBL subtype (e.g., polymerase chain reaction, DNA-DNA hybridization, restriction fragment length polymorphism, DNA sequencing) are important because of the transmission of antibiotic resistant genes among clinical strains of bacteria.

At present, there has been very few reports concerning the ESBL producing *E. coli* and the prevalence of integron element in Thailand. In the present study, the epidemiology of ESBL producing *E. coli* strains and the prevalence of integrase (*intI*1) gene, a marker of multiple antibiotic resistance class 1 integron elements in Siriraj Hospital were determined. The ESBL producing *E. coli* detection was performed using the initial screen test and the phenotypic confirmatory test according to the National Committee for Clinical Laboratory Standards (NCCLS). The detection of beta-lactam antibiotic resistant genes of ESBL producing *E. coli* associated with TEM, SHV, CTX-M and VEB by Polymerase Chain Reaction (PCR) amplification of ESBL genes (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{VEB}) was performed as well as an integrase (*intI*1) gene in all *E. coli* strains from clinical specimens by PCR amplification of *intI*1. The presence of *intI*1 was confirmed by the southern blot hybridization using *intI*1 as a probe. The pulsotyping of ESBL producing *E. coli* was done using Pulse-Field Gel Electrophoresis (PFGE) technique.

The results from this study would provide useful information on the prevalence of class 1 integron elements among *E. coli* strains from clinical specimens and the spread of antibiotic resistance among the ESBL producing *E. coli* strains. It should be benefit for the treatment of the patients who have been infected with ESBL producing *E. coli* strains and also for medical personnel in the control and prevention of the occurrence of this organism.

CHAPTER II

OBJECTIVES

1. To detect ESBL producing *E. coli* strains from clinical specimens using by initial screen test and phenotypic confirmatory test.

2. To perform antimicrobial susceptibility test of *E. coli*.

3. To detect ESBL genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX\text{-}M}$ and *bla*$_{VEB}$ genes) in the ESBL producing *E. coli* strains by Polymerase Chain Reaction (PCR).

4. To assess a prevalence of an integrase (*intI*1) gene, a marker of multiple antibiotic resistance class 1 integron element among *E. coli* strains from clinical specimens.

5. To preliminary assess the spread of antibiotic resistance by pulsotyping ESBL producing *E. coli* strains using Pulsed-Field Gel Electrophoresis (PFGE).

CHAPTER III

LITERATURE REVIEWS

1. *Escherichia coli*

1.1 Classification

E. coli is a member of the family *Enterobacteriaceae*. It is the most significant species in the genus *Escherichia*, and recognized as an important potential pathogen in humans. *E. coli* is a gram-negative bacillus, commonly isolated from the colon.

Most strains of *E. coli* are motile and generally possess both sex pili and adhesive fimbriae. The organism also possesses O, H, and K antigens. *E. coli* O groups have shown cross-reactivity with similar antigens in other members of *Enterobacteriaceae*, notably with shigellae. Typing for H antigens is useful in completing the serogrouping of a particular strain. The capsular K antigen during bacterial agglutination by specific antiserum. Some *E. coli* K antigens are identical to capsular antigens of other species. The K1 antigen has been found to be identical to the capsular antigen in group B *Neisseria meningitidis*, suggesting a virulaence property of K antigens.

1.2 Characterization

E. coli has a distinctive colony morphology on certain laboratory media, such as MacConkey agar. Although may appear as a non-lactose-fermenter or as a mucoid colony, *E. coli* usually produces a dry, pink (lactose positive) colony with a surrounding pink area of precipitated bile salts on MacConkey agar.

E. coli typically produces positive tests for indole, lysine decarboxylase, and mannitol fermentation and produces gas from glucose. It is grown initially on a blood agar plate and on a differential medium, such as EMB (Eosin Methylene Blue) agar or MacConkey agar. It ferments lactose, forms pink colonies, whereas lactose-negative

organisms are colorless. On EMB agar, the colonies have a characteristic green sheen (Mahon and Manuselis, 2000).

1.3 Pathogenesis importance

E. coli has been associated with a wide range of diseases and infections, including meningitis (particularly in the newborn), gastrointestinal tract, urinary tract, wound and even septicemia in all age groups (Levinson and Jawetz, 2000).

1.3.1 Septicemia

Typically, septicemia caused by gram negative bacilli such as *E. coli* originates from infections in the urinary or gastrointestinal tract (e.g., an intra-abdominal infection with sepsis following intestinal perforation). The mortality associated with *E. coli* septicemia is high for patients in whom immunity is compromised or the primary infection is in the abdomen or central nervous system (Murray et al., 2002).

1.3.2 Urinary tract infection

E. coli is the most common cause of urinary tract infection and accounts for approximately 90% of first urinary tract infections in young women. The symptoms and signs include urinary frequency, dysuria, hematuria and pyuria. Flank pain is associated with upper tract infection. None of these symptoms or signs is specific for *E. coli* infection. Urinary tract infection can result in bacteremia with clinical signs of sepsis.

Nephropathogenic *E. coli* typically produce a hemolysin. Most of the infections are caused by *E. coli* of small number of O antigen types. K antigen appears to be important in the pathogenesis of upper tract infection. Pyelonephritis is associated with a specific type of pilus, P pilus, which binds to the P blood group substance (Brooks et al., 2001).

1.3.3 Neonatal Meningitis

E. coli and group B streptococci cause the majority of central nervous system infections in infants younger than 1 month. Approximately 75% of the *E. coli* strains

possess the K1 capsular antigen. This serogroup is also commonly present in the gastrointestinal tracts of pregnant women and newborn infants. However, the reason this serogroup has a predilection for causing disease in newborns is not understood (Brooks et al., 2001).

Table1: Gastroenteritis caused by *E. coli* (Murray et al., 2002)

Organism	Site of action	Disease	Pathogenesis
Enterotoxigenic *E.coli* (ETEC)	Small intestine	Traveler's diarrhea; infant diarrhea in underdeveloped countries; watery diarrhea, vomiting, cramps, nausea, low-grade fever	Plasmid-mediated heat-stable and/or heat-labile enterotoxins that stimulate hypersecretion of fluids and electrolytes
Enteropathogenic *E.coli* (EPEC)	Small intestine	Infant diarrhea in underdeveloped countries; fever, nausea, vomiting, nonblood stool	Plasmid-mediated A/E histopathology with disruption of normal micro-villus structure resulting in malabsortion and diarrhea
Enteroinvasive *E.coli* (EIEC)	Large intestine	Disease in underdeveloped countries; fever, cramping, watery duarrhea; may progress to dysentery with scant, bloody stools	Plasmid–mediated invasion and destruction of epithelial cells lining colon
Enterohemorrhagic *E.coli* (EHEC)	Large intestine	Haemorrhagic colitis (HC) with severe abdominal cramps, initial watery diarrhea, followed by grossly blood diarrhea; little or no fever; may progress to hemolytic uremic syndrome	Mediated by cytotoxic Shiga toxins (Stx-1 , Stx-2), which disrupt protein synthesis; A/E lesions with destruction of intestinal microvillus resulting in decreased absorption
Enteroaggregative *E.coli* (EAEC)	Small intestine	Infant diarrhea in underdeveloped countries; persistant watery diarrhea with vomiting, dehydration, and low-grade fever	Plasmid-mediated aggregative adherence of bacilli (stacked bricks) with shortening of microvilli, mononuclear infiltration, and hemorrhage; decreased fluid absortion
Diffuse aggregative *E.coli*	Small intestine	Watery diarrhea in infants 1 to 5 years of age	Stimulates elongation of microvilli

1.3.4 Gastroenteritis

The strains of *E. coli* that cause gastroenteritis are subdivided into the following six groups, and each group cause disease by a different mechanism, as shown in Table 1 (Murray et al., 2002).

2. Beta–lactam antibiotics

Beta-lactams comprise a very large family of different groups of compounds all containing the beta-lactam ring (Rang et al., 2001).

Penicillins, cephalosporins, monobactams and carbapenems are members of this family. The major antibacterial action, beta-lactams act by binding to inhibit a number of bacterial enzymes, namely, penicillin-binding protein (PBPs) of susceptible organisms, which are essential for synthesis of peptidoglycan of bacterial cell wall. In addition, these beta-lactam agents may produce bactericidal effects by triggering autolytic in the cell envelope (YAO et al., 1999).

2.1 Penicillins

All penicillins are derivatives of 6-aminopenicillanic acid and contain a beta-lactam ring structure (Figure 1) that is essential for antibacterial activity. Peniciilin subclasses have additional chemical substituents that confer differences in antimicrobial activity, susceptibility to acid and enzymic hydrolysis, and biodisposition (Trevor et al., 2002).

Classification of penicillins

2.1.1 Penicillins (e.g., penicillin G)

These have the greatest activity against gram positive organisms, gram negative cocci and non beta-lactamase-producing anaerobes. However, they have little activity against gram negative rods.They are susceptible to hydrolysis by beta-lactamases.

2.1.2 Antistaphylococcal penicillins (e.g., nafcillin)

These penicillins are resistant to staphylococcal beta-lactamases. They are active against staphylococci and streptococci but inactive against enterococci, anaerobic bacteria and gram negative cocci and rods.

2.1.3 Extended-spectrum penicillins (ampicillin and the antipseudomonal penicillins)

These drugs retain the antibacterial spectrum of penicillin and have improved activity against gram negative organisms, but they are destroyed by beta-lactamases (Chambers, 2004).

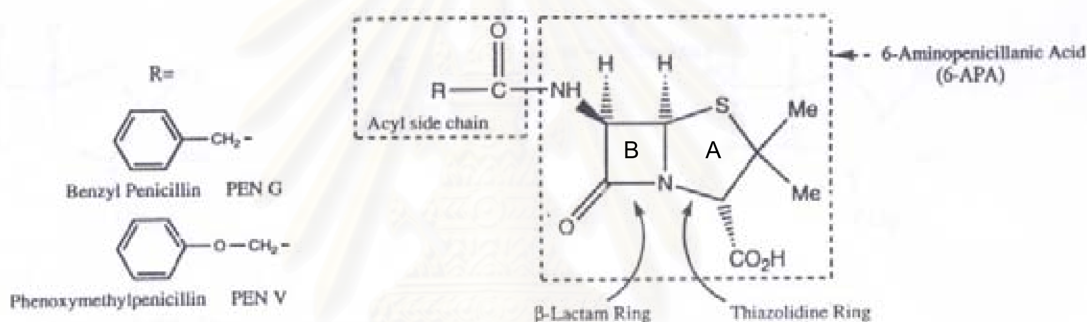

Figure 1: The basic nucleus of penicillin is 6-aminopenicillanic acid, which consists of a thiazolidine ring (A) linked to a beta-lactam ring (B). This latter ring carries a secondary amino group. The side chain substituents at R1 determine the main antibacterial and pharmacological characteristics of each particular penicillin (Patrick, 1995)

2.2 Cephalosporins

Some *Cephalosporium* fungi yield antimicrobial substance called cephalosporin. These are beta-lactam compounds with a nucleus of 7-aminocephalosporanic acid, instead of the penicillin 6-aminopenicillanic acid.

The structure of cephalosporin (Figure 2) has similarities to that of penicillin in that it has a bicyclic system containing a four-membered beta-lactam ring. However, this time the beta-lactam ring is fused with a six-membered dihydrothiazine ring (Patrick, 1995).

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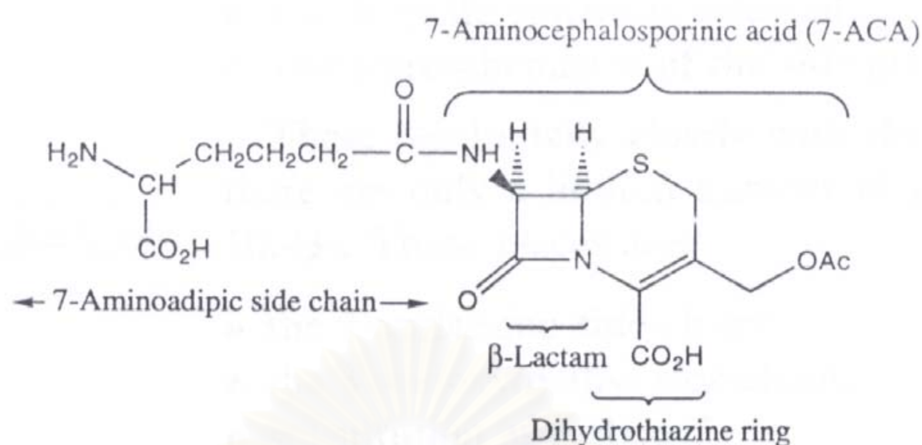

Figure 2: The structure of cephalosporin (Patrick, 1995)

Cephalosporins have been arranged into four major groups, or generations that are based on general features of their antibacterial activity as shown in Table 2 (Murray et al., 1999).

The only substitution which has been useful at position 7 has been the introduction of the 7-alpha-methoxy group to give a class of compounds known as the cephamycins (Figure 3).

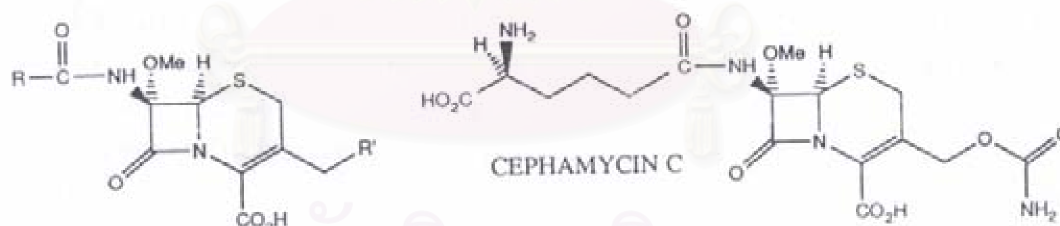

Figure 3: Cephamycin (Patrick, 1995)

The parent compound cephamycin was isolated from a culture of *Streptomyces clavuligerus* and was the first beta-lactam to be isolated from a bacterial source. Modification of the side-chain gave cefoxitin (Figure 4) which showed a broader spectrum of activity than most cephalosporins, due to greater resistance to penicillinase enzymes. This increased resistance is thought to be due to the steric hindrance provided by the extra methoxy group. However, it is interesting to note that introduction

of the methoxy group at the corresponding-6-alpha-position of penicillins results in loss of activity (Patrick, 1995; Rang etal., 2001)

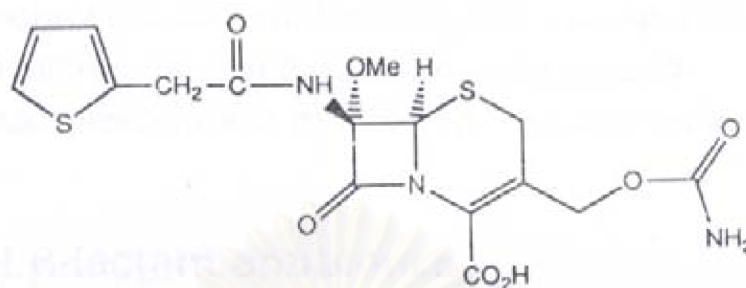

Figure 4: Cefoxitin (Patrick, 1995)

2.2.1 First Generation Cephalosporins

This group includes cefadroxil, cefazolin, cephalexin, cephalothin, cephapirin and cephradin. These drugs are very active against gram positive cocci, including staphylococci and common streptococci. These drugs have minimal activity against gram negative cocci, enterococci, methicillin-resistant staphylococci, and most gram negative rods.

2.2.2 Second Generation Cephalosporins

Members of this group include cefaclor, cefamandole, cefonicid, cefuroxime, cefprozil, loracarbef, and ceforanide (Chambers, 2004). Drugs in this subgroup usually have less activity against gram positive organisms than the first generation drugs but have an extended gram-negative coverage. Marked differences in activity occur among the drugs in this subgroup. Examples of clinical uses include infections caused and by *Moraxella catarrhalis* (cefuroxime, cefaclor) (Trevor et al., 2002).

2.2.3 Third Generation Cephalosporins

Third generation cephalosporins agents include cefoperazone, cefotaxime, ceftazidime, ceftizoxime, ceftriaxone, cefixime, cefpodoxime proxetil, cefditoren pivoxil, ceftibuten and moxalactam (Chambers, 2004). Most are active against gram negative

such as enterobacter, providencia, *Serratia marcescens*. Individual drugs (ceftazidime) also have activity against pseudomonas (Trevor et al., 2002).

Modification at side chain R1 of structure of cephalosporin is aminothizoyl-oxyimido side chain, and some compounds were modified at side chain R2. Modification of these cephalosporins have been large side chain to prevent beta-lactamases, called compounds were modified at R1 side chain that oxyimino cephalosporins (third generation cephalosporins), and called compounds were modified at both R1 and R2 side chain are extended spectrum cephalosporins (third and fourth generation cephalosporins) are shown Figure 5 (Foye et al., 1995; Patrick, 1995)

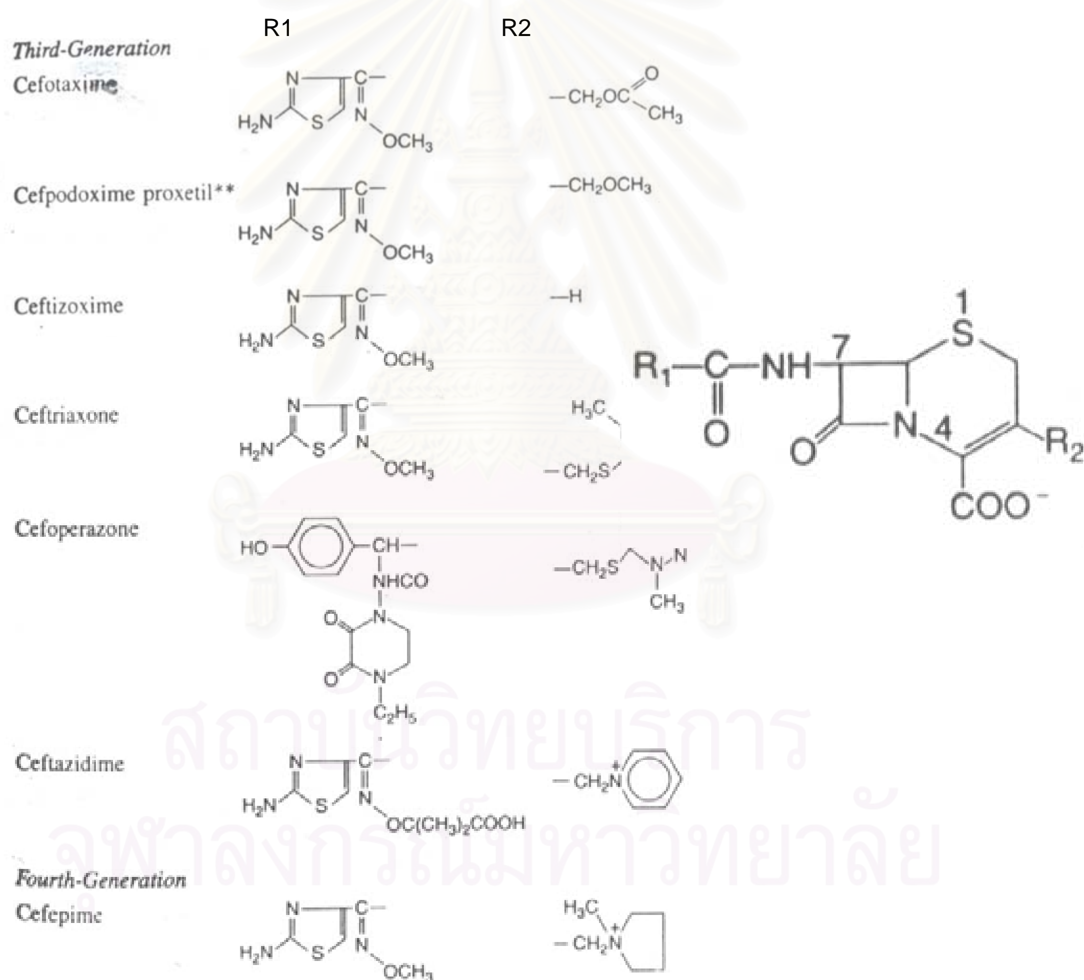

Figure 5: Modification of extended spectrum cephalosporins (Brooks et al., 1995)

2.2.4 Fourth Generation Cephalosporins

Some new cephalosporins (cefepime, cefpirome) are being classified as fourth generation drugs. The new agents have comparable or slightly enhanced activity against some Enterobacteriaceae that are resistant to third generation cephalosporins. They are not active against *P. aeruginosa* that are resistant to the third generation drugs. The activity against streptococci and nafcillin-susceptible staphylococci is comparable to that of the third generation compounds (Brooks et al., 1995).

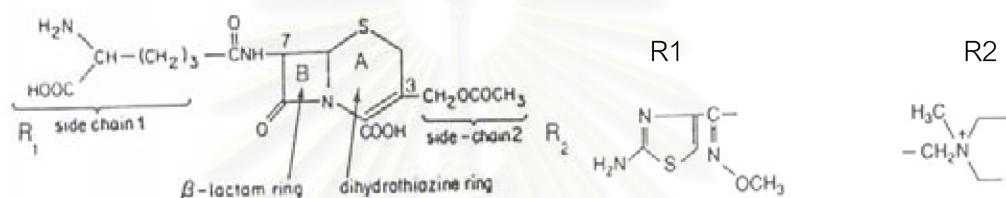

Figure 6: Cefepime

Table 2: Cephalosporins (Murray et al., 2004)

First generation cephalosporins	Second generation Cephalosporins	Third generation cephalosporins	Fourth generation cephalosporins
cefadroxil	cefaclor	cefdinir	cefepime
cefazolin	cefamandole	cefixime	cefpirome
cephaloridine	cefonicid	cefoperazone	
cephalothin	ceforanide	cefotaxime	
cephapirin	cefuroxime	cefpodoxime	
cephradine	cefprozil	ceftazidime	
	loracarbef	ceftibuten	
		ceftizoxime	
		ceftriaxone	

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2.3 Monobactams

Monobactams have a monocyclic beta-lactam ring and are resistant to beta-lactamases produced by certain gram negative rods. The drug has no activity against gram positive bacteria or anaerobes. It is an inhibitor of cell wall synthesis, preferentially binding to PBP3, and is synergistic with aminoglycosides (Trevor et al., 2002).

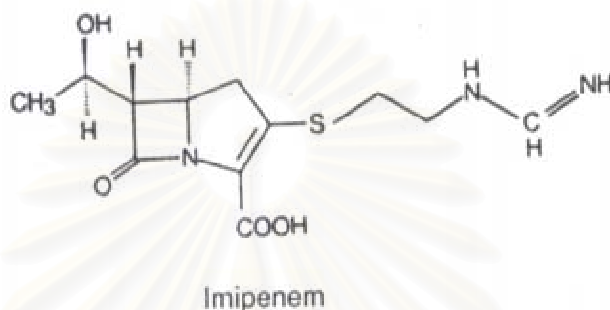

Figure 7: Imipenem (Trevor et al., 2002)

2.4 Carbapenems

These drugs are structurally related to beta-lactam antibiotics. Imipenem, the first such agent to become available, has good activity against many gram negative rods, gram positive organisms, and anaerobes. It is resistant to most beta-lactamases but is inactivated by dihydropeptidases in renal tubes (Brooks et al., 1995).

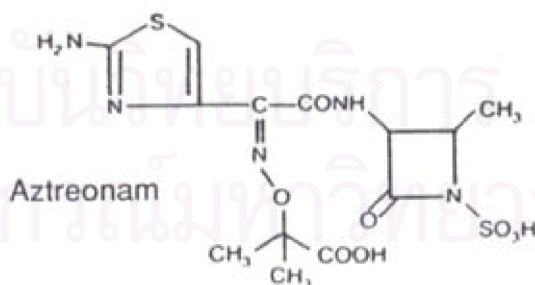

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Figure 8: Aztreonam (Brooks et al., 1995)

2.5 Beta – lactamase Inhibitors

Clavulanic acid, sulbactam, and tazobactam are used in fixed combinations with certain hydrolysable penicillins. They are most active against plasmid-encoded beta-lactamases such as those produced by gonococci, streptococci, *E. coli* and *H. influenzae*. They are not good inhibitors of inducible chromosomal beta-lactamases formed by enterobacter and pseudomonas (Trevor et al., 2002).

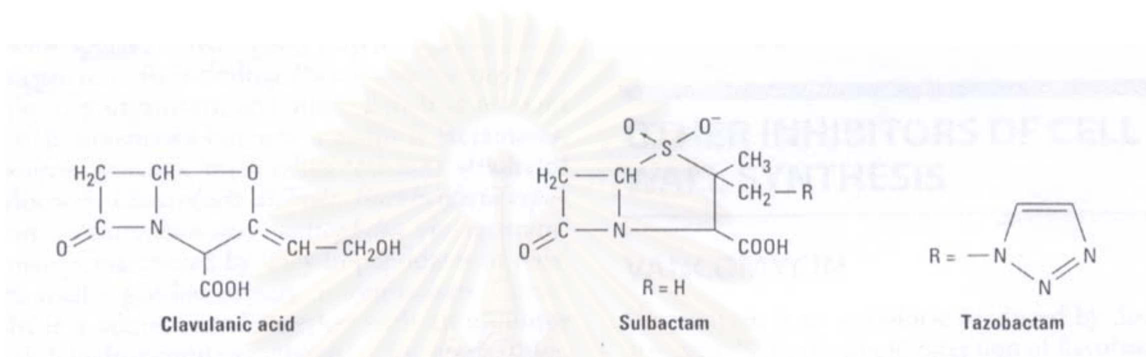

Figure 9: Beta-lactamase inhibitors (Trevor et al., 2002)

3. Mechanism of resistance to beta–lactam antibiotics

Beta-lactam antibiotics are the most frequently prescribed antibiotic worldwide. Therefore, the resistance to these agents has become a major problem for physicians. Beta-lactam antibiotics exert their antimicrobial effect by interfering with cell wall synthesis. This is accomplished by the drugs attaching covalently to their targets, the penicillin-binding proteins (PBPs). The PBPs are diverse enzymes involved in cell wall synthesis, and are anchored in the cytoplasmic membrane of the bacterium. The site at which beta-lactam drugs bind to PBPs is located on the portion of the PBP that extends into the periplasmic space of gram negative bacteria. Covalent binding to PBPs inferferes with synthesis of cell wall and ultimately leads to cell death (Georgopapadakou, 1993). Resistance to beta-lactam antibiotics arises through one or more of the following mechanisms:

3.1 Alteration of the target site

The target site of beta-lactam antibiotics is a group of enzymes (peptidoglycan transpeptidases) known as penicillin-binding protein (PBPs). These enzymes or PBPs

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are vital for synthesis and maintenance of the bacterial cell wall. Binding of a beta-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).

3.2 Decreased access to target site (Decreased uptake or Increased efflux)

Gram-negative bacteria have an outer phospholipids/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).

3.3 Enzymatic destruction (Beta-lactamases)

The best known example of enzymatic inactivation of antibiotics is that of the beta-lactamases. These enzymes render beta-lactam antibiotics inactive by cleaving the beta-lactam ring of susceptible antibiotics via an irreversible hydroxylation of the amide bond (Figure 10). Beta-lactamase production is a common resistance mechanism among gram positive and gram negative organisms, both aerobic and anaerobic. Gram-positive bacteria produce large quantities of beta-lactamase, which they must excrete into their external environment to inactivate the targeted antibiotic, before it reaches the organism. For gram-negative bacteria, the beta-lactamases are contained in periplasmic space, and may be produced in much smaller quantities (Bush, 1995).

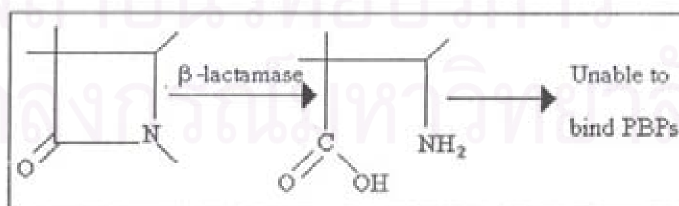

Figure 10: Mode of beta-lactamase enzyme activity. By cleaving the beta-lactam ring the molecule can no longer bind to penicillin binding protein (PBPs) and is no longer able to inhibit cell wall synthesis (Forbe et al., 1998).

4. Resistance to beta-lactam due to beta-lactamases

Of the various mechanisms of acquired resistance to beta-lactam antibiotics, resistance due to production of beta-lactamases by the cell is the most prevalent. Alteration in the preexisting PBP, acquisition of a novel PBP insensitive to beta-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).

Beta-lactamases comprise of a family of tremendous diversity. A number of classification schemes have been suggested according to their hydrolytic spectra, 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 beta-lactamases are placed into functional group based on substrate and inhibitor profile and molecular structure as shown in Table 3 (Bush and Jacoby, 1995).

Group 1: Beta-lactamase including the AmpC enzymes that are instrinsically resistance to beta-lactamase inhibitors. They are found in a variety of gram negative bacteria such as *Enterobacter* spp., *Citrobacter freundii*, *Serratia marcescens*, *P. aeruginosa*, *E. coli*, and *K. pneumoniae.* This group of enzymes are resistant to beta-lactamase inhibitor including when it is combined with penicillins, cephamycins, first-, second- and third generations cephalosporins and monobactams. However, they do not show their activities against cefepime and imipenem.

Group 2: Beta-lactamase including a variety of enzymes all of which are instrinsically susceptible to the beta-lactamase inhibitors. These enzymes are plasmid mediated, found in *E. coli* and *K. pneumoniae*, and responsible for resistance to ampicillin and first generation cephalosporins in these species. Mutant forms of these enzymes are now appearing in the *E. coli* and *K. pneumoniae*, which are responsible for the expanded spectrum of resistance to cephalosporins and aztreonam called extended spectrum beta-lactamases, ESBLs and also to beta-lactamase inhibitor/beta-lactam drug combinations.

Group 3: Beta-lactamase including the metallo-beta-lactamase that hydrolyze penicillins, cephalosporins and carbapenems, that are poorly inhibited by almost all beta-lactam-containing molecules.

Group 4: Penicillinases that are not well inhibited by clavulanic acid.

Groups 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. Class A, C and D gather evolutionarily distinct groups of serine enzymes, and class B is the zinc-dependent ("EDTA-inhibited") enzymes.

By using this scheme, ESBLs are defined as beta-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 beta-lactamases allows them to be classified in to one of the four evolutionary molecular classes (A, B, C and D). Beta - lactamases of class A, C and D act by a serine-ester-linked acyl enzyme. Molecular class A comprises penicillinases, cephalosporinases and broad-spectrum beta-lactamases that are generally inhibited by beta-lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam. This class includes the beta-lactamases of *S. aureus* (Bush group 2a) and many of the plasmid specified beta-lactamase of gram negative bacteria, such as the common TEM- and SHV-type enzymes (groups 2b and 2be). In gram negative bacteria, beta-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-beta-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 beta-lactam including carbapenems (e.g., imipenem and meropenem), and are not inhibited by penicillinase inhibitors.

Table 3: Functional and molecular characteristics of the major groups of beta-lactamases (Bush and Jacoby, 1995; Bush et al., 2001)

Functional Group	Major subgroup	Molecular Class	Attributes of beta-lactamases in functional group	Estimated number of enzymes, 2000
1		C	Often chromosomal enzymes in gram-negative bacteria but may be plasmid-encoded. Confer resistance to all classes Of beta-lactams, except carbapenems (unless combine with porin changes).Not inhibited by clavulanic acid	51
2		A, D	Most enzymes responsive to inhibition by clavulanic acid (unless otherwise noted).	256
	2a	A	Staphylococcal and enterococcal penicillinases included. Confer resistance to penicillins.	23
	2b	A	Broad spectrum beta-lactamases, including TEM-1 and SHV-1, primarily from gram-negative bacteria.	16
	2be	A	Extended-spectrum beta-lactamases conferring resistance to oxyimino-cephalosporins and monobactams.	150
	2br	A	Inhibitor-resistant TEM (IRT) beta-lactamases, one inhibitor resistant SHV derived enzymes	24
	2c	A	Carbenicillin-hydrolyzing enzymes.	19
	2d	D	Cloxacillin (oxacillin) hydrolyzing enzymes, modestly inhibited By clavulanic acid.	31
	2e	A	Cephalosporinases inhibited by clavulanic acid.	20
	2f	A	Carbapenem hydrolyzing enzymes with active site serine, inhibited by clavulanic acid.	4
3	3a,3b,3c	B	Metallo beta-lactamases conferring resistance to carbapenems and all beta-lactam classes except monobactams. Not inhibited by clavulanic acid.	24
4			Miscellaneous unsequenced enzymes that do not fit into the other groups.	9

Further dissemination of these potent enzymes should be expected since plasmid-mediated metallo beta-lactamases have now been found in *P. aeruginosa*, *B. fragilis*, *Serratia marcescens* and *K. pneumoniae* (Ito et al., 1995; Minami et al., 1996; Osano et al., 1991).

5. The spread of antibiotic resistance

Horizontal gene transfer is any process in which an organism transfers genetic material to another cell that is not its offspring. This process is thought to be a significant cause of increased drug resistance; when one bacterial cell acquires resistance, it can quickly transfer the resistance genes to many species. Also enteric bacteria appear to exchange genetic material with each other within the gut in which they live. By contrast, vertical transfer occurs when an organism receives genetic material from its ancestor such its parent or a species from which it evolved (Steven et al., 2001).

5.1 Mechanisms of genetic exchange

5.1.1 Transformation

The uptake of naked DNA is a common mode of horizontal gene transfer that can mediate the exchange of any part of a chromosome; this process is most common in bacteria that are naturally transformable; typically only short DNA fragments are exchanged.

5.1.2 Conjugation

The transfer of DNA mediated by conjugal plasmids or conjugal transposons; requires cell to cell contact but can occur between distantly related bacteria or even bacteria and eukaryotic cells; can transfer long fragments of DNA.

5.1.3 Transduction

The transfer of DNA by phage requires that the donor and recipient share cell surface receptors for phage binding and thus is usually limited to closely related

bacteria; the length of DNA transferred is limited by the size of the phage head (Maiden, 1995; Sowers and Schreier, 1996).

5.2 Vehicles for genetic exchange

5.2.1 Plasmids

Plasmid is extrachromosomal, circular, double stranded DNA molecules that carry the genes for a variety of enzymes that can degrade antibiotics and modify membrane transport systems. Plasmid–mediated resistance is very important clinically, because it occurs in many different species, especially in gram-negative rods. Plasmids frequently mediate resistance to multiple drugs (Levinson and Jawetz, 2000).

5.2.2 Transposons

Resistance genes may also occur on transposons, the so-called jumping genes, which by a replicative process are capable of generating copies which may integrate into the chromosome or into plasmids. The chromosome provides a more stable location for the genes, but they will be disseminated only as rapidly. Transposition can also occur between plasmids, for example from a non-transmissible to a transmissible plasmid, again accelerating dissemination (Mims et al., 2004).

5.2.4 Integrons

Integrons are DNA elements that can mediate the dissemination of antibiotic resistance genes by a site-specific recombination system (Stokes and Hall, 1989). They possess two conserved segments separated by a variable region, which includes integrated antibiotic resistance genes or cassettes of unknown function. The essential components of the integron are found within the 5’ conserved segment and include an integrase (*intI*1) gene and an adjacent recombination site, *attI* (Recchia et al., 1994; Rowe-Magaus et al., 2001). To date, five distinct integron classes have been found associated with cassettes that contain antibiotic resistance genes (Hall et al., 1991; Hochhut et al., 2001; Recchia et al., 1997; Stokes et al., 1989)

Class 1 integron (Figure 11) are the most widespread, especially among *Enterobacteriaceae*, with a 5' conserved segment that contains a promotor region from which integrated cassettes are expressed (Collis et al., 1993; Levesque et al., 1994). The 3' conserved segment contains a *qacE*Δ1 gene encoding resistance to quaternary ammonium compounds and most also contain a *sulI* gene encoding resistance to sulfonamides, an open reading frame (ORP5) of unknown function, and other sequences that differ from one integron to another (BIssonnette and Roy, 1992; Paulsen et al., 1993; Radstrom et al., 1991). The gene cassettes, which may be found within the variable region of integrons, are mobile, nonreplicating elements which comprise an open reading frame associated with an integrase-specific recombination site, *att*C, also known as the 59-base element (Hall et al., 1991; Hansson et al., 1997; Stokes et al., 1997).

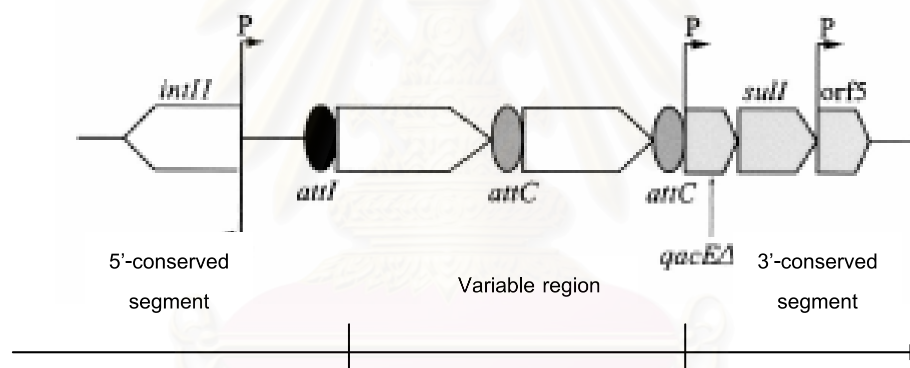

Figure 11: General structure of class 1 integron. Cassettes are inserted in the variable regions by the integrase using a site-specific recombination mechanism. The *attI* and *attC* sites are shown, by black and grey ovals respectively, and promoters are denoted by ''P", *intI*1, integrase gene; *qacE*Δ1, antiseptic resistance gene; *sulI*, sulfonamide resistance gene; *orf*5, gene of unknown function (Messier and Roy, 2001).

For class 2, the *intI*2 gene, which includes a termination codon, and presumably also *attI*2 and a promoter are found within transposons such as Tn*7* (Hall and Vockler, 1987; Hall et al., 1991; Sundstrom et al., 1991). The role of integron (class 1, class 2, and class 3) and gene cassette systems in the evolution of bacterial and plasmid genomes is now known to be much broader than their role in the dissemination of antibiotic resistance genes. Class 4 integron was recently found in the small

chromosome of several different *Vibrio cholerae* strains (Clark et al., 2000; Mazel et al., 1998), and in the *V. cholerae* strains that has been sequenced, the integron contains an array of 179 cassettes, only a few of which, e.g., the *catB9* gene cassette, contain genes that are likely to determine resistance to an antibiotic (Heidelberg et al., 2000).Furthermore, different *V. cholerae* strains contain different cassette arrays. Other *Vibrio* species also contain a chromosomally located integron (Clark et al., 2000). Some species of *Pseudomonas*, *Xanthomonas*, and various other bacteria whose genomes have been partially sequenced also contain integrons, as do unidentified bacteria from environmental soil samples (Row-Magnus et al., 2001; Vaisvila et al., 2001)

The IntI proteins, IntI1, IntI2, IntI3, etc., encoded by different integron types, are 34 to 94% identical or 57 to 96% similar in pairwise comparisons and form a distinct family of the tyrosine recombinase superfamily (Nield et al., 2001; Nunes-Düby et al., 1998). Most of the genes that encode IntI-type integrases identified to date have been found adjacent to gene cassettes, indicating that they are part of an integron. Each distinct IntI type (>98% identical) thus defines an integron class which encompasses all integrons with the same *intI* gene but different cassette arrays (Recchia and Hall, 1995).

6. History of extended spectrum beta–lactamases (ESBLs)

The first plasmid-mediated beta-lactamase in gram negative, TEM-1, was described in 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. It is capable of hydrolyzing penicillins and first generation cephalosporins, but is unable to attack the oxyimino cephalosporin (Datta and Kontomichalou, 1965).

The TEM-1 beta-lactamase spread worldwide through out the family *Enterobacteriaceae* within ten years. A close relative of TEM-1, TEM-2, was recorded in *Pseudomonas aeruginosa* in 1969, and by the 1970S, plasmid encoding the TEM-1 enzyme had spread in *Haemophilus influenzae* and *Neisseria gonorrhoeae* (Sirot et al., 1987). Another common plasmid-mediated beta-lactamase found in *Klebsiella pneumoniae* and *E. coli* is SHV-1 (for sulfydryl variable). The SHV-1 beta-lactamase is

chromosomally encoded in the majority of isolates of *K. pneumoniae* but is usually plasmid-mediated in *E. coli* (Champs et al., 1989).

Over the last 20 years, many new beta-lactam antibiotics have been developed that are specifically designed to be resistant to the hydrolytic action of beta-lactamses. However, increasing use of these agents has been associated with the emergence of resistant bacterial strains with mutated beta-lactamases (Medeiros, 1997). One of these new classes was the oxyimino cephalosporin, which became widely used for the treatment of serious infections due to gram negative bacteria in the 1980s. Because of their increased spectrum of activity, especially against the oxyimino cephalosporins, these enzymes were called extended-spectrum beta-lactamases (ESBLs) (Kliebe et al., 1985).

The first mutated form of beta-lactamase, SHV-2, was isolated from a clinical strain of *Klebsiella ozaenae* in the Federal Republic of Germany in 1983 (Knotche et al., 1983). The first ESBL observed at the teaching hospital of Clermont-Ferrand, France, in July 1984, was the cefotaximase TEM / CTX-1 (Sirot et al., 1987).

Most ESBLs are mutant enzymes derived from amino acid substitution in TEM-1, TEM-2 and SHV-1 enzymes (Matthew, 1979). At present, over 150 different ESBLs have been described. These beta-lactamases have been found worldwide in many different genera of Enterobacteriaceae (Ambler, 1980).

7. Types of ESBLs

Most ESBLs are derivatives of TEM or SHV enzymes (Bush et al., 1995; Jacoly et al., 1991). There are now 150 TEM-type beta-lactamases and 88 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*. and other genera of *Enterobacteriaceae* (Jacoby and Bush, 2006).

7.1 TEM

TEM-1, board spectrum beta-lactamase, is the most commonly encountered beta-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 beta-lactamase (Barthelemy 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 beta-lactamase that displayed the ESBL phenotype (Sougakoff et al., 1988). Since that first report, over 100 additional TEM derivatives have been described. Some of these beta-lactamases are inhibitor-resistant enzymes, but the majority of the new derivatives are ESBLs. The characteristics of TEM-type-beta-lactamases were shown in Table 4.

The amino acid substitutions within the TEM enzyme occurring at a limited number of positions are shown in Figure 12. 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 these positions (Bradford et al., 20001).

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Table 4: Characteristics of TEM-type-beta-lactamases (Bradford et al., 2001)

Isoelectric point (pI)	Enzymes	Enzyme type		
		Broad spectrum	ESBL	IRT
5.2	TEM-12,TEM-55-,TEM-57,TEM-58		X	
	TEM-30,TEM-31,TEM-35,TEM-36,TEM-37,TEM-38,TEM-41,TEM-45			X
	TEM-51,TEM-73,TEM-74			
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	TEM-15,TEM-47,TEM-48,TEM-49,TEM-52,TEM-66,TEM-92		X	
6.1	TEM-28,TEM-43		X	
6.3	TEM-3,TEM-16,TEM-21,TEM-22		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

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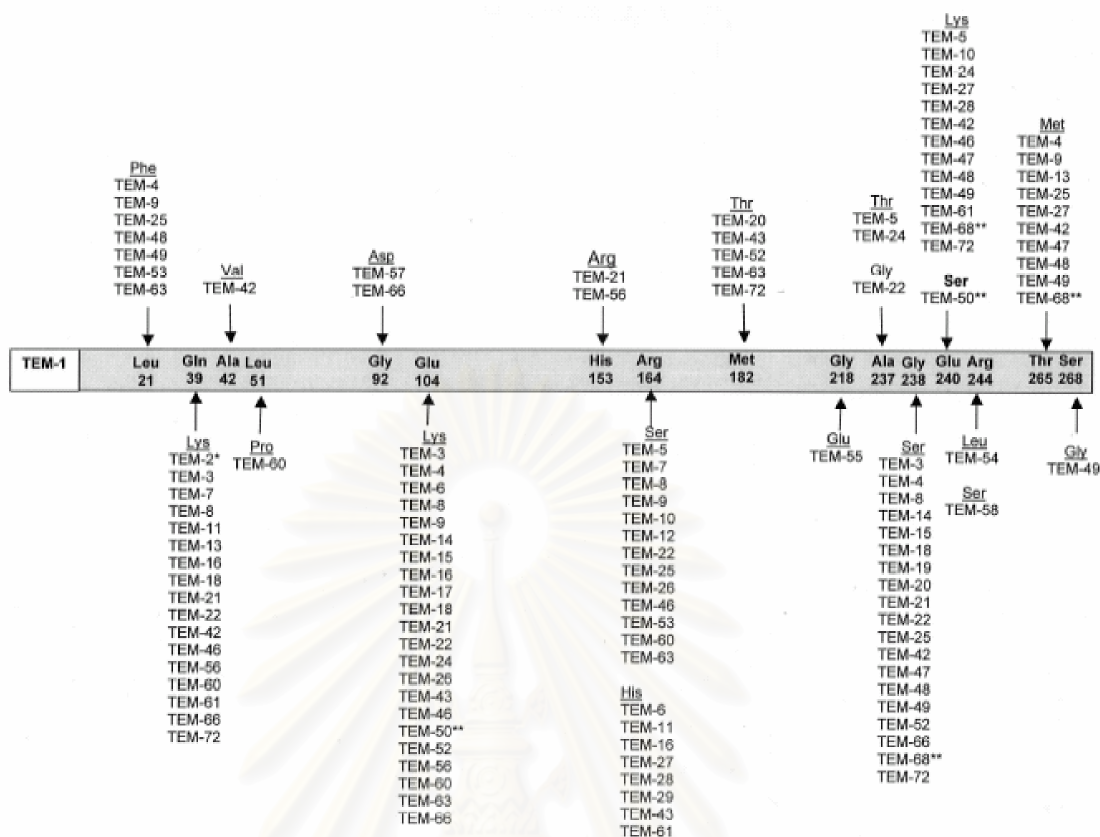

Figure 12: Amino acid substitutions in TEM ESBL derivatives. The amino acids listed within the bar are those found in the structural gene of TEM-1 beta-lactamase. Substitutions found in TEM-types ESBLderivatives are shown under the amino acids of TEM-1. TEM-types variants may contains more than one amino acid substitution. Only the amino acid substitutions that are common to TEM-type ESBLS are shown in this figure (Bradford et al., 2001).

7.2 Inhibitor-Resistant TEM Beta-Lactamases (IRT)

Although some of the inhibitor-resistant beta-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, Beta-lactamases resistant to inhibition by clavulanic acid were discovered. Nucleotide sequencing revealed that these enzymes are variants of the TEM-1 or TEM-2 beta-lactamases. These enzymes were at first given the designation IRT for inhibitor-resistant TEM beta-lactamases (Knox, 1995). However, all have subsequently been renamed with numerical TEM designations. There are at least 19 distinct inhibitor-resistant TEM beta-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 al., 1995). The amino acid substitutions in IRT are shown in Figure 13.

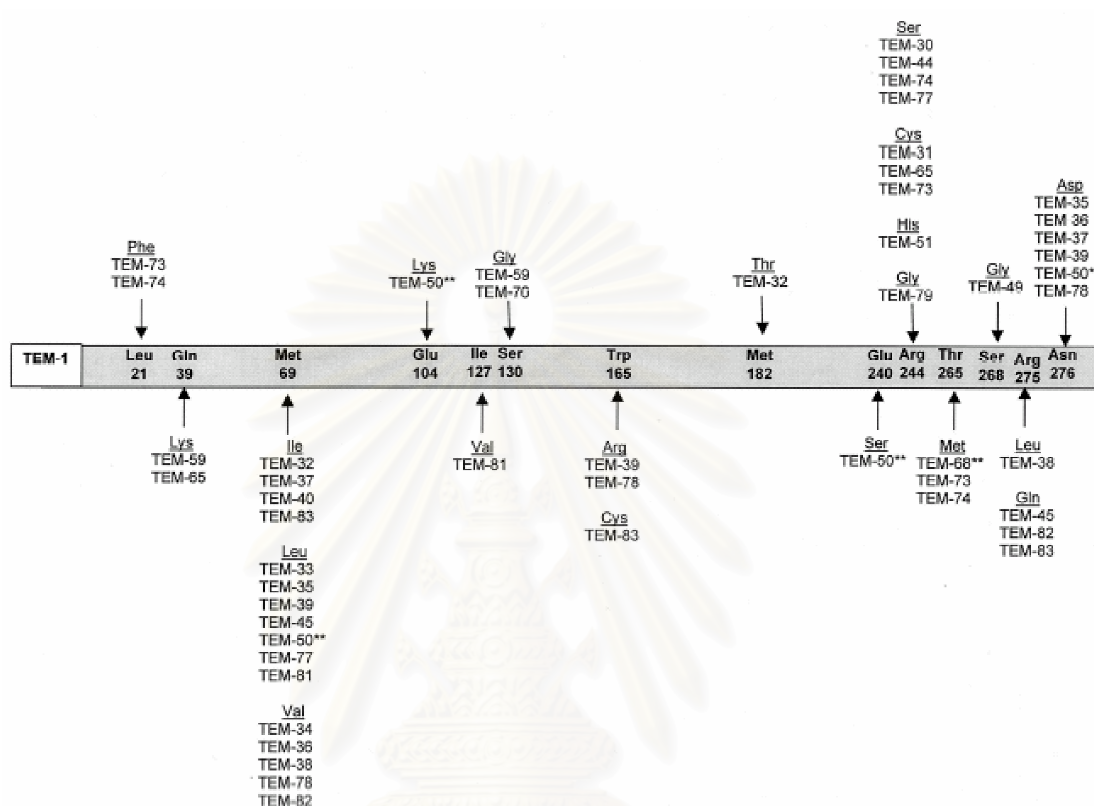

Figure 13: Amino acid substitutions in TEM IRT derivatives. The amino acids listed within the bar are those found in the structural gene of TEM-1 beta-lactamase. Substitutions found in TEM-types IRT derivatives are shown under the amino acids of TEM-1. TEM- types variants may contains more than one amino acid substitution. Only the amino acid substitutions that are common to TEM-type IRT are shown in this figure (Bradford et al., 2001).

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7.3 SHV

The SHV-1 (for sulphydryl variable) is most commonly found in *K. pneumoniae* and is responsible for up to 20% of the plasmid mediated ampicillin resistance in this species. Unlike the TEM-type beta-lactamases, there are relatively few derivatives of SHV-1 as shown in the Table 5 (Tzouvelekis and Bonomo, 1999). 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 as shown in Figure 14. 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). 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). 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)

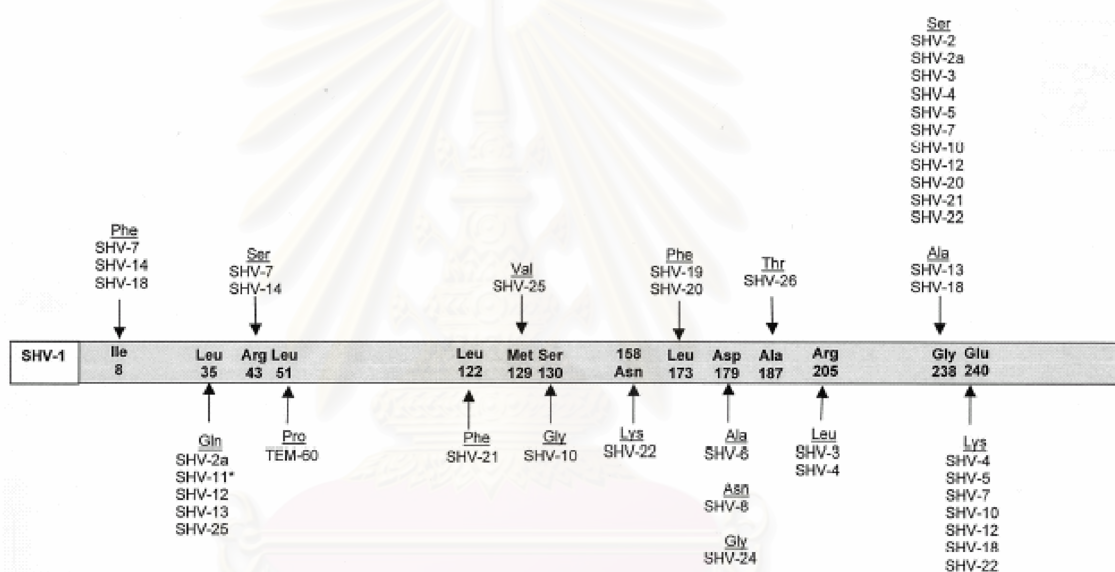

Figure 14: Amino acid substitutions in SHV-ESBL derivatives. The amino acids listed within the bar are those found in the structural gene of SHV-1 beta-lactamse. Substitutions found in SHV-types ESBL derivatives are shown under the amino acids of SHV-1. SHV-types variants may contains more than one amino acid substitution. SHV-11 is not an ESBL but is in included in the figure as a derivatives of SHV-1 (Bradford et al., 2001).

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Table 5: Characteristics of SHV-type-beta-lactamases (Bradford et al., 2001)

Isoelectric point (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-2a, shv-6, shv-8, shv-13,	X		
	SHV-19, SHV-20, SHV-21,SHV-22		X	
	SHV-4, SHV-7b, SHV-18			
7.8	SHV-5, SHV-9, SHV-12		X	
8.2	SHV-10		X	

7.4 CTX-M

In recent year, a new family of plasmid-mediated ESBLs, called cefotaximases (CTX-M), as 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 *in vitro* 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* as shown in Table 6 (Bauernfeind et al., 1990; Bonnet et al., 2000; Gazouli et al., 1998).

Strains expressing CTX-M type beta-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 with outbreaks have reported that the CTX-M type enzyme is the most frequently isolated ESBL among clinical isolates in their laboratories (Sabate et al., 2000).

Table 6: Characteristics of CTX-M type ESBLs (Bradford et al., 2001)

Beta-lactamase	Alternative name	Isoelectric point	Country of origin	Bacterial species
CTX-M-1	MEN-1	8.9	Germany, Italy	*E. coli*
CTX-M-2		7.9	Argentina	*S. enterica*
CTX-M-3		8.4	Poland	*C. freundii, E. coli*
CTX-M-4		8.4	Russia	*S. enterica*
CTX-M-5	CTX-M-3	8.8	Latvia	*S. enterica*
CTX-M-6		8.4	Greece	*S. enterica*
CTX-M-7	CTX-M-5	8.4	Greece	*S. enterica*
CTX-M-8		7.6	Brazil	*P. mirabilis, E. cloacae, E. aeruginasa, C. amalonaticus*
CTX-M-9		8	Spain	
CTX-M-10		8.1	Spain	

All strains of *S. enterica* were serovar Typhimurium

7.5 OXA

The OXA-type enzymes are another growing family of ESBLs. These beta-lactamases differ from the TEM and SHV enzymes in that they belong to molecular class D and functional group 2d. The OXA-type beta-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). The OXA beta-lactamase family was originally created as a phenotypic rather than a genotypic group for a few beta-lactamases that had a specific hydrolysis profile. Therefore, there is as little as 20% sequence homology among some of the members of this family. However, recent additions to this family show some degree of homology to one or more of the existing members of the OXA beta-lactamase family. While most ESBLs have been found in *E. coli*, *K. pneumoniae* and other *Enterobacteriaceae*, the OXA-type ESBLs have been found mainly in *P. aeruginosa* (Table 7). Several of the OXA-type ESBLs have been derived from OXA-10. OXA-14 differs from OXA-10 by only one amino acid residue, OXA-11 and OXA-16 differ by two,

and OXA-13 and OXA-19 differ by nine (Table 6) (Danel et al., 1999; Hall et al., 1993; Mugnier et al., 1998).

The majority of the OXA-type ESBLs, which confer resistance to ceftazidime, the OXA-17 beta-lactamase confers resistance to cefotaxime and ceftriaxone but provides only marginal protection against ceftazidime (Danel et al., 1999). With respect to beta-lactamase inhibitors, the original OXA enzymes were characterized by their lack of inhibition by clavulanic acid; however, the OXA-18 beta-lactamase was reported to be inhibited by this compound (Philippon et al., 1997).

Table 7: Characteristics of OXA – type ESBLs (Bradford et al., 2001)

Derivation	pI	Aminoacid substitution vs OXA-10	Country of origin	Bacterial species
OXA-10	6.4	Asn143Ser,Gly20Ser,Asp55N,Asn73Ser	Turkey	*P. aeruginasa*
OXA-10	8	Thr107Ser,Tyr174P?he,Glu229Gly, Ser245Asn,GLU259Ala	France	*P. aeruginasa*
OXA-10	6.2	Gly157Asp	Turkey	*P. aeruginasa*
OXA-2	8.7,8.9	NA	Turkey Turkey	*P. aeruginasa*
OXA-10	6.2	Ala124Thr,Gly157Asp	Turkey	*P. aeruginasa*
OXA-10	6.1	Asn73Ser	Turkey	*P. aeruginasa*
OXA-9, OXA-12	5.5	NA	France	*P. aeruginasa*
	7.6	Ile10ThR,Gly20Ser,Asp55Asn,Thr107Ser, Gly157Asp,Tyr174Phe,Glu229Gly,Ser245Asn, Glu259Ala	France	*P. aeruginasa*
OXA-10	7.6	Ile10ThR,Gly20Ser,Thr107Ser,Trp154Gly Trp154Gly,Fly157aSP,Tyr174Phe, Glu229Gly,Ser245Asn,Glu259Ala	France	*P. aeruginasa*

pI = Isoelectric point

NA, not applicable; these enzymes do not originate from OXA-10

7.6 Other ESBLs

While the majority of ESBLs are derived from TEM or SHV beta-lactamases and other can be categorized with one of the newer families of ESBLs, a few ESBLs have been reported that are not closely related to any of the established families of beta-lactamases (Table 8). The PER-1 beta-lactamase was first discovered in strains of *P.aeruginosa* isolated from patients in Turkey (Nordman et al., 1993). Later, it was also found among isolates of *S. enterica* serovar Typhimurium and *A. baumannii* (Vahaboglu et al., 1997). A related enzyme, PER-2, which has 86% amino acid homology with PER-1, was found among *S. enterica* serovar Typhimurium strains in Argentina (Bauernfeind et al., 1996). Another enzyme that is somewhat related to PER-1 is the VEB-1 beta-lactamase (Poirel et al., 1999).

Table 8: Characteristic of novel, unrelated ESBLs (Bradford et al., 2001)

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

pI = Isoelectric point

CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam

VEB-1 was first found in a single isolate of *E. coli* in a patient from Vietnam, but was subsequently also found in a *P. aeruginosa* isolates from a patient from Thailand (Naas et al., 1999). A third related enzyme is CME-1, which was isolated from

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Chryseobacterium meningosepticum (Rossolini et al., 1999). A fourth enzyme in this group is TLA-1, which was identified in an *E. coli* isolate from a patient in Mexico (Silva et al., 2000). These enzymes all confer resistance to oxyimino-cephalosporin, especially ceftazidime and aztreonam (Rossolini et al., 1999). An unusual feature of SFO-1, which is highly related to a class A beta-lactamase from *Serratia fonticola*, is that it is a transferable beta-lactamase that can be induced to high-level production of beta-lactamase by imipenem (Matsumoto et al., 1999). GES-1 is another uncommon ESBL enzyme that is not closely related to any other plasmid-mediated beta-lactamase, but does show 36% homology to a carbenicillinase from *Proteus mirabilis* and it has some carbapenemase activity (Poirel et al., 2000). BES-1 had only 51% identity with the most closely related beta-lactamase (chromosomal penicillinase of *Yersinia enterocolitica*). BES-1 is distantly related to either the CTX-M or GES-1 enzyme (Bonnet et al., 2000). FEC-1 was first found in *E. coli* from Japan, quite probably it comes from canine intestinal bacterial flora (Matsumoto et al., 1988).

8. ESBL detection methods

The increased prevalence of *Enterobacteriaceae*-producing ESBLs has created a great need for laboratory methods that will accurately identify the presence of these enzymes in clinical isolates (Katsanis et al., 1994). Various tests have, therefore, been developed to detect ESBLs in *K. pneumoniae*, the main host genus and are equally applicable to other *Enterobacteriaceae* with little or no chromosomal beta-lactamase activity, e.g., *E. coli* and *P. mirabilis* (Livermore, 1995).

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-cephalosporins are less reliable indicators. 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 is used (Tenover et al., 2001).

Nevertheless, one has to bear in mind that the ESBL tests described above only confirm whether an ESBL is produced. Definitive identification is very demanding and only possible by molecular detection methods. The techniques, which are necessary for the task of identifying the exact ESBL subtype. Clinical microbiology techniques and molecular detection methods were summarized in the Table 10 and 11, respectively (Bradford, 2001).

8.1 Clinical Microbiology Techniques

8.1.1 NCCLS Initial and Confirmatory test

Currently, the NCCLS recommends an initial screening by disk diffusion test. Diminished zones of inhibition around third-generation beta-lactam disk were interpreted as positive results. The beta-lactams and zone diameter breakpoints used were; cefpodoxime ≤ 22 mm; ceftazidime ≤ 22 mm; cefotaxime ≤ 25 mm; ceftriaxone ≤ 25 mm; aztreonam ≤ 27 mm as listed in Table 9. A positive result is reported as suspicious for the presence of an ESBL. This screen is then followed by a phenotypic confirmatory that the NCCLS recommends the combination disk method and the MIC method for ESBL confirmation.

The combination disc method depended 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 ≥ 5 mm. large than those without the inhibitor. The MIC method depend on MIC of either ceftazidime or cefotaxime with and without the presence of clavulanic acid (4 μg/ml), decrease in the MIC ≥ 3 twofold dilutions in the presence of clavulanic acid 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, 2004).

Table 9: NCCLS screening criteria for ESBLs (NCCLS, 2004)

Antimicrobial agent	Disc diffusion zone (mm.)
cefpodoxime	≤ 22 mm
cetazidime	≤ 22 mm
cefotaxime	≤ 25 mm
ceftriaxone	≤ 25 mm
aztreonam	≤ 27 mm

8.1.2 Double-Disk Synergy Test (DDST)

This test is most widely used due to its simplicity and ease of interpretation. Extended-spectrum beta-lactam disks (ceftazidime, cefotaxime, ceftriaxone and aztreonam) were placed 25-30 mm (center to center) away from a disk containing a beta-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., 1998).

8.1.3 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 disk 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).

8.1.4 Three-Dimensional Test (3 – D)

Three-dimensional test is based on the Kirby-Bauer disc 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 dusks 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).

8.1.5 E-test ESBL Strips

This strip contains a ceftazidime gradient on one end and a ceftazidime plus clavulanic acid gradient on the other end. After incubation, the intersections of the growth ellipses and the strip gives the MICs by direct reading. The presence of an ESBL is confirmed by the appearance of a phantom zone or deformation of the ceftazidime ellipse or when the MIC is reduced by $\geq 3 \log_2$ dilutions in the presence of clavulanic acid. Similar strips containing cefotaxime/clavulanic acid gradients are also available. In order to detect various substrate affinities, testing must be done with both ceftazidime/ clavulanic acid and cefotaxime/clavulanic acid strips (Brown et al., 2000; Cormican et al., 1996).

8.1.6 Vitek ESBL Test

The automated microbial susceptibility test system Vitek has also produce an ESBL test that utilizes either ceftazidime or cefotaxime alone and in combination with clavulanic acid (4μg/ml). A predetermined reduction in growth in wells containing clavulanate compared to those containing drug alone indicates the presence of an ESBL. In a study of *Klebsiella* spp. and *E.coli* expressing well-characterized beta-lactamases, Sanders et al. showed that the Vitek ESBL test was 99% sensitive and specific for the detection of ESBLs (Sanders et al., 1996). Furthermore, updated computer algorithms in the new Vitek system have also been shown to categorize beta-lactamases present in many gram negative clinical isolates based on the phenotype of susceptibility patterns with various beta-lactam antibiotic (Sanders et al., 2000).

8.2 Molecular Detection Methods

8.2.1 Isoelectric Focusing

In the early of studying ESBLs, determination of the isoelectric point was usually sufficient to identify the presence of ESBL. However, with > 90 TEM-type beta-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).

8.2.2 DNA Probe

Early detection of beta-lactamase gene 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.

8.2.3 Polymerase Chain Reaction (PCR)

PCR is easiest and most common molecular method used to detect the presence of a beta-lactamase gene. Oligonucleotide primers can be chosen from sequence 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.

8.2.4 Oligonucleotide Probe

The first molecular method for the identification of beta-lactamase was the oligotyping method developed by Ouellete 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 $\mathit{bla}_{\mathrm{TEM}}$ gene. Using this method, several new TEM variants were identified within a set of clinical isolates. The probes used in oligotyping tests for TEM beta-lactamases have been labeled either with a radioisotope or with biotin (Mabilat and Courvalin, 1990; Tham et al., 1990).

8.2.5 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 fragment 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 (Nuesch-Inderbinen and Hachler, 1996).

8.2.6 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% polyacrlamide gel. Gene for SHV-1, -2, -3, -4, -5 and –7 beta-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 was 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).

8.2.7 Ligase Chain Reaction (LCR)

LCR allows the discrimination of DNA sequence 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).

8.2.8 Nucleotide Sequencing

Nucleotide sequencing remains the standard for determination of the specific beta-lactamase gene present in a strain. However, the variability can be seen in the sequences for some of the SHV beta-lactamases due to compressions and difficulty in reading traditional sequencing autoradiographs, rather than actual differences in the sequence. Amino acid sequences of beta-lactamases were deduced and compared with other on website www.lahey.org/studies/web.htm (Jacoby and Bush, 2001).

Table 10: Clinical Microbiology for ESBL detection techniques (Bradford, 2001)

Test	Advantages	Disadvantages
Standard NCCLS interpretive criteria	Easy to use, performed	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 placment for optimal sensitivity not standardized
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
E test 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

Table 11: 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 method

9. Epidemiology

ESBLs are now a problem in hospitalized patients worldwide. The ESBL phenomenon began in Western Europe, most likely because expanded-spectrum beta-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/SURVEILL/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 beta-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% *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 beta-lactamses are the most common ESBLs found in Korea (Kim et al., 1998). In contrast, the SHV-5 beta-lactamase is commonly encountered worldwide and has been reported

in Croatia, France, Greece, Hungary, Pland, 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).

Many investigators are using molecular methods such as pulsed-field gel electrophoresis (PFGE) to examine epidemiology with the strains involved in outbreaks of infections caused by ESBLs (Branger et al., 1998; Cotton et al., 2000; Gaillot et al., 1998). Other methods for studying the epidemiology of these strains include plasmid profiles, ribotyping, random amplified polymorphic DNA (RAPD), and arbitrarily primed PCR (Bermudes et al., 1996; D'Agata et al., 1998; Shannon et al., 1998; Villari et al., 1998). These outbreaks often start in an ICU and then spread to other parts of the hospital by the usual transmission routes (Bermudes *et al*., 1996). Very often, the exact source of outbreaks caused by ESBL-producing organisms is never identified. However, some interesting epidemiology of these resistant bacteria has been reported. In one hospital in France, ceftazidime-resistant *K. pneumoniae* expressing SHV-5 was isolated from six peripartum women and two neonates. Plasmid and PFGE profiles of the strains revealed that all of the strains were identical to a strain that was cultured from contaminated ultrasonography coupling gel (Gaillot et al., 1998). Another study demonstrated that cockroaches infesting a neonatal ICU in South Africa carried the same PFGE strain types of ESBL-producing *K. pneumoniae* that were responsible for an outbreak of infections and high mortality rate among neonates in that institution (Cotton et al., 2000).

Pagani et al. studied multiple CTX-M type extended spectrum beta-lactamases in nosocomial isolates of *Enterobacteriaceae* from a hospital in Northern Italy during the period of January 2001 to July 2002. Twelve isolates of Enterobacteriaceae (1 of *K. pneumoniae*, 8 of *E. coli*, 1 of *P. mirabilis*, and 2 of *Proteus vulgaris*) classified as extended-spectrum beta-lactamase (ESBL) producers according to the ESBL screen flow application of the BD-Phoenix automatic system and for which the cefotaxime MICs were higher than those of ceftazidime. By PCR and sequencing, a CTX-M-type determinant was detected in six isolates, including three of *E. coli* (carrying $bla_{CTX\text{-}M\text{-}1}$), two of *P. vulgaris* (carrying $bla_{CTX\text{-}M\text{-}2}$), and one of *K. pneumoniae* (carrying $bla_{CTX\text{-}M\text{-}15}$),.

The three CTX-M–1-producing *E. coli* isolates were clonally unrelated to each other. The two CTX-M-2-producing *P. vulgaris* isolates were from the same ward (although isolated several months apart), and PFGE analysis revealed probable clonal relatedness. This is also the first report of CTX-M-2 in *P. vulgaris* (Pagani et al., 2003).

Eckert et al. studied 19 clinical isolates of the family *Enterobacteriaceae* (16 *E. coli* isolates and 3 *K. pneumoniae* isolates) collected from four different hospitals in Paris, France, from 2000 to 2002. These strains had a particular extended-spectrum cephalosporin resistance profile characterized by a higher level of resistance to cefotaxime and aztreonam than to ceftazidime. The $bla_{CTX\text{-}M}$ genes encoding these beta-lactamases were involved in this resistance, with a predominance of $bla_{CTX\text{-}M\text{-}15}$. Ten of the 19 isolates produced both TEM-1 and CTX-M-type enzymes. One strain (*E. coli* TN13) expressed CMY-2, TEM-1 and CTX-M-14, $bla_{CTX\text{-}M}$ genes were found on large plasmids. In 15 cases, the same insertion sequence, IS*Ecp*1, was located upstream of the 5' end of the $bla_{CTX\text{-}M}$ genes. They identified an insertion sequence designated IS26 in one case. Examination of the other three $bla_{CTX\text{-}M}$ genes by cloning, sequence and PCR analysis revealed the presence of a complex *sulI*-type integron that included open reading frame ORF513, which carried the *bla* gene and the surrounding DNA. Five isolates had the same plasmid DNA fingerprint, suggesting clonal dissemination of CTX-M-15-producing strains in the Paris area (Eckert et al., 2004).

Cao et al. studied the distribution of extended-spectrum beta-lactamases in clinical isolates of *Enterobacteriaceae* in seven hospitals in Ho Chi Minh city, Vietnam during the period September 2000 to September 2001. Among 730 *E. coli*, 32% were resistant to ceftazidime, 30% were resistant to cefotaxime, 30% were resistant to ceftriaxone, 15% were resistant to cefoperazone, 3% were resistant to cefepime, and 3% were resistant to imipenem. Four hundred and thirty-eight *K. pneumoniae*, 17% were resistant to ceftazidime, 32% were resistant to cefotaxime, 35% were resistant to ceftriaxone, 19% were resistant to cefoperazone, 10% were resistant to cefepime, and 10% were resistant to imipenem. One hundred and forty-one *P. mirabilis* isolates, 30% were resistant to ceftazidime, 25% were resistant to cefotaxime, 28% were resistant to ceftriaxone, 11% were resistant to cefoperazone, 9% were resistant to cefepime, and

only 4% were resistant to imipenem. In 55 strains producing extend-spectrum beta-lactamases (32 *E. coli* isolates, 13 *K. pneumoniae* isolates and 10 *P. mirabilis* isolates), structural genes for VEB-1 (25.5%), CTX-M (25.5%), SHV (38.1%) and TEM (76.3%) enzymes were detected alone or in combination. Sequencing of the PCR products obtained from the *K. pneumoniae* isolates revealed the presence of bla_{VEB-1}, $bla_{CTX-M-14}$, $bla_{CTX-M-17}$, bla_{SHV-2} and bla_{TEM-1}. Molecular typing of the strains with a similar resistance phenotype to broad-spectrum cephalosporins indicated polyclonal spread (Cao et al., 2002).

Mulvey et al. studied Ambler class A extended-spectrum beta-lactamase-pruducing *E. coli* and *Klebsiella* spp. in Canadian hospital during the period October 1999 to September 2000. A total of 29,323 *E. coli* and 5,156 *Klebsiella* spp. isolates were screened at 12 participating sites. Of these, 505 clinically significant, nonrepeated isolates displaying reduced susceptibility to the NCCLS-recommened beta-lactams were submitted to a central laboratory over a 1-year period ending on 30 September 2000. A total of 116 isolates were confirmed to be ESBL producers. PCR and sequence analysis revealed the presence of TEM-11 (n=1), TEM-12 (n=1), TEM-29 (n=1), TEM-52 (n=4). CTX-M-13 (n=1), CTX-M-14 (n=15), CTX-M-15 (n=11), SHV-2 (n=2), SHV-2a (n=12), SHV-5 (n=6), SHV-12 (n=45) and SHV-30 (n=2). Five novel beta-lactamases were identified and designated TEM-115 (n=2), TEM (n=1), SHV-40 (n=2), SHV-41 (n=4) and SHV-42 (n=1). In addition, no molecular mechanism was identified for five isolates displaying an ESBL phenotype. Macrorestriction analysis of all ESBL isolates was conducted, as was restriction fragment length polymorphism analysis of plasmids harboring ESBLs. Although a clonal distribution of isolates was observed at some individual sites, there was very little evidence suggesting intrahospital spread. In addition, examples of identical or closely related plasmids that were identified at geographically distinct sites across Canada are given (Mulvey et al., 2004).

Girlich et al. studied molecular epidemiology of the Integron-located VEB-1 extened-spectrum beta-lactamase in nosocomial enterobacteriaceae isolates at Siriraj hospital in Bangkok, Thailand from June to August 1999. Thirty seven ceftazidime resistant nonrepetitive enterobacterial isolates were collected from 37 patients. Eighty

one percent of these strains expressed a clavulanic acid-inhibited extended-cephalosporin resistance profile. An identical extended-spectrum beta-lactamase (ESBL), VEB-1, was found in 16 unrelated enterobacterial isolates (*E. coli*, n= 10; *Enterobacter cloacae*, n= 2; *Enterobacter sakazakii*, n = 1; *K. pneumoniae* = 3) and in two clonally related *E. cloacae* isolates. The bla_{VEB-1} gene was located on mostly self-conjugative plasmid that conferred additional non beta-lactam antibiotic resistance patterns. Additionally, the bla_{VEB-1} gene cassette was part of class 1 integrons varying in size and structure. The bla_{VEB-1}-containing integrons were mostly associated with bla_{OXA-10} -like and *arr*-2-like gene cassettes, the latter conferring resistance to rifampin. These data indicated the spread of bla_{VEB-1} in Bangkok due to frequent transfer of different plasmids and class 1 integrons and rarely to clonally related strains. Plasmid- and integron-mediated resistance to rifampin was also found in enterobacterial isolates (Girlich et al., 2001).

Hadziyannis et al. performed the screening and confirmatory testing for extended-spectrum beta-lactamases in *E. coli*, *K. pneumoniae* and *K. oxytoca* from clinical microbiology laboratory during the period June 1998 to February 1999. By using the NCCLS proposed ESBL confirmatory method, they tested 61 screen-positive isolates from 42 patients, 30 randomly selected susceptible isolates, and 12 isolates with previously characterized beta-lactamases. Ceftazidime contributed to 97% of screen-positive isolates, whereas only one isolate was susceptible to ceftazidime but resistant to aztreonam. None of the susceptible isolates were shown to produce ESBL. Based on these findings a comment regarding the presence of ESBL seems sufficient for *Klebsiella* spp. but confirmatory testing is indicated for *E. coli*. There was 85% agreement between the type of beta-lactamase and the result of the ESBL confirmatory test. When a cefotaxime MIC > 0.5 µg/ml was used to indicate the presence of ESBL, the specificity of the assay increased to 100%. The NCCLS ESBL phenotypic confirmatory method was reproducible and accurate enough to be used in the clinical laboratory (Hadziyannis et al., 2000).

Kim et al. studied the epidemiologic features and clinical outcomes of bloodstream infections caused by extended-spectrum beta-lactamase-producing *E. coli*

and *K. pneumoniae* isolates, cases of bacteremia caused by these organisms in children were analyzed retrospectively. Among the 157 blood isolates recovered from 1993 to 1998 at the Seoul National University Children's Hospital, the prevalence of ESBL production was 17.9% among the *E. coli* isolates and 52.9% among the *K. pneumoniae* isolates. The commonest ESBLs were SHV-2a and TEM-52. A novel ESBL, TEM-88, was identified. Pulsed-field gel electrophoresis analysis of the ESBL-producing organisms showed extensive diversity in clonality. The medical records of 142 episodes were reviewed. The risk factors for bloodstream infection with ESBL-producing organisms were prior hospitalization, prior use of oxyimino-cephalosporins, and admission to an intensive care unit within the previous month. There was no difference in clinical severity between patients infected with ESBL-producing strains (the ESBL group) and those infected with ESBL-nonproducing strains (the non-ESBL group) at the time of presentation. However, the overall fatality rate for the ESBL group was significantly higher than that for the non-ESBL group: 12 of 45(26.7%) versus 5 of 87 (5.7%) (*P*=0.001). In a subset analysis of patients treated with extended-spectrum cephalosporins with or without an aminoglycoside, favorable response rates were significantly higher in the non-ESBL group at the 3rd day (6 of 17 versus 33 of 51; *P*=0.035), the 5th day (6 of 17 versus 36 of 50; *P*<0.05), and the end of therapy (9 of 17 versus 47 of 50; *P*<0.001). In conclusion, the ESBL production of the infecting organisms has a significant impact on the clinical course and survival of pediatric patients with bacteremia caused by *E. coli* and *K. pneumoniae* (Kim et al, 2002).

Saurina et al. studied epidemiology of antimicrobial resistance and relation to antibiotic usage pattern in Enterobacteriaceae at 15 hospitals in Brooklyn, New York during the month of November 1997. Extended-spectrum beta-lactamases were present in 44% of 409 *K. pneumoniae* isolates. Six isolates had reduced susceptibility to carbapenems, including two that were not susceptible to any of the antibiotics tested. PFGE revealed a commonality of resistant isolates within and between hospitals. The occurrence of ESBL-containing isolates was associated with cephalosporin usage (*P*=0.055). ESBLs were present in 4.7% of *E. coli* and 9.5% of *P. mirabis* isolates. It is concluded that ESBL-producing *Enterobacteriaceae* are endemic in Brooklyn, are

spread between hospitals, and may be associated with cephalosporin usage (Saurina et al., 2000).

Siu et al. studied bacteremia due to extended-spectrum beta-lactamase-producing *E. coli* and *K. pneumoniae* in a pediatric oncology ward. Thirteen patients who had 16 episodes of bacteremia were observed during the periods 1993 to 1997 with a high background isolation rate of cefotaxime- or aztreonam-resistant gram-negative bacteria. Four blood isolates were *E. coli* and 12 were *K. pneumoniae*, and these isolates harbored extended-spectrum beta-lactamases. All episodes of bacteremia were nosocomial, all except one of the episodes occurred in neutropenic patients, and all patients were treated with piperacillin or ceftazidime with amikacin and cefazolin prior to the onset of bacteremia. Nine of 13 patients were receiving extended-spectrum beta-lactam treatment when the bacteremias caused by ESBL producers occurred. Molecular studies revealed that four *K. pneumoniae* SHV-2 producing isolates from 1994 were of the same clone. Other ESBL producers, including six that carried both TEM-1 and SHV-5, five that carried SHV-5, and one that carried SHV-2 alone, were unrelated. In conclusion, SHV-5 was present in 11 of the 16 isolates and coexisted with TEM-1 in 6 isolates. Acquisition of resistance genes probably occurred under antibiotic selection pressure (Siu et al., 1999).

In Thailand, the data of ESBL-producing strains and prevalence of class 1 integron is limited. In this study, disc diffusion and combination disc were used to detect ESBL producer. The positive results were subjected to PCR amplification of ESBL gene (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{VEB} genes) and the spread of antibiotic resistance was assessed from the pulsotyping of ESBL producer. The integrase (*intI*1) gene detection of all clinical isolates in order to assess prevalence of class 1 integron elements from PCR and southern blot hybridization. The data will be useful for the treatment of the patients who are infected with multidrug resistant *E. coli* strains.

CHAPTER IV

MATERIALS AND METHODS

1. Microorganisms

1.1 Clinical strains

The total of 120 *E. coli* isolates from clinical specimens from patients admitted at Siriraj hospital during the period of June to August 2004. Among these isolates, 60 (50%) isolates were isolated from urine, 30 (25%) isolates from blood, and 30 (25%) isolates from pus, as shown in the Table 12

Table 12: The total number of *E. coli* strains from various clinical specimens in this study

Specimens	Number of *E. Coli* strains (%)
Blood	30* (25)**
Pus	30 (25)
Urine	60 (50)
Total	120 (100)

* The number of *E. coli* strains in each specimen

** Percent of *E. coli* strains in each specimen

1.2 Control strains

E. coli ATCC 25922 was also included in the study as the control strain.

2. Confirmatory identification of *E. coli*

All the isolates obtained were reidentified according to the methods described in by Mahon and Manuselis (Mahon and Manuselis, 2000).

The identification steps were as followed:

2.1 Gram staining

All the isolates were gram stained. *E. coli* is gram-negative, straight rods, 0.3 – 1.3 μm in length, arranged singly, in pairs or short chains

2.2 Biochemical tests

The strains were tested for the biochemical characteristics as followed:

Biochemical tests	Characteristics of *E. coli*
Indole	positive
Methyl red	positive
Voges-Proskauer test	negative
Citrate utilization	negative
Urease	negative
Motility test	positive
H_2S	negative
Gas	positive
Malonate	negative
Mannitol	positive
Lysine decarboxylase	positive
Triple Sugar Iron (butt/slant)	acid/acid or alkaline/acid

3. Detection of Extended spectrum beta-lactamase (ESBL) producing *E. coli*

The ESBL producing *E. coli* were detected using the initial screen test and phenotypic confirmatory test as recommended in NCCLS (NCCLS, 2004). The methods were briefly described as followed.

3.1 Initial screen test (NCCLS, 2004)

3.1.1 Inoculum preparation

At least 3 to 5 well-isolated colonies from 18-24 hours agar plate of all *E. coli* isolates including the control strain *E. coli* ATCC 25922 were inoculated into the tubes

containing approximately 5 ml of normal saline. The suspension was adjusted to match the 0.5 McFarland turbidity standard.

3.1.2 Inoculation of test plates

(a) Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a steriled cotton swab was dipped into the adjusted suspension. The swab should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This step would remove the excess inoculum from the swab.

(b) The dried surface of a Mueller–Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, the plate was rotated approximately 60º each time to ensure an even distrilbution of inoculum. As the final step, the rim of the agar was swabbed.

(c) The plate lid was left a jar for 3 minutes to allow for any excess surface moisture to be absorbed before applying the drug impregnated disks.

3.1.3 Application of disks to inoculated agar plate

The five antimicrobial discs (cefpodoxime 10 μg, ceftazidime 30 μg, ceftriaxone 30 μg, cefotaxime 30 μg and aztreonam 30 μg) were placed on each plate. Each disc must be pressed down to ensure complete contact with the agar surface. The plates were inverted and placed in an incubator set 37 ºC for 24 hours, within 15 minutes after the disks were applied.

3.1.4 Reading plates and Interpreting results

After 18 hours of incubation, each plate was examined. The diameter of the zone of inhibition was measured. Zone was measured to the nearest whole millimeter, using a sliding caliper, which was held on the back of the plate. The sizes of the zones of inhibition were interpreted by referring to the table of zone diameter standard of national committee of Clinical Laboratory Standards (NCCLS, 2004) for at least one antimicrobial agents indicates suspicious ESBL producing strain, as shown in the Table 13.

Table13: The inhibition zone of antimicrobial agents to interpreted suspicious ESBL producing organisms (NCCLS, 2004).

Antimicrobial agents	Inhibition zone of suspicious ESBL Producing organisms (mm.)
cefpodoxime (10 μg)	≤ 22
ceftazidime (30 μg)	≤ 22
ceftriaxine (30 μg)	≤ 25
cefotaxime (30 μg)	≤ 27
aztreonam (30 μg)	≤ 27

3.2 Phenotypic confirmatory test of ESBL producing *E. coli* (The combination disc method)

3.2.1 The inoculum preparation and inoculation of test plates

The inoculum preparation and the inoculation of the test plates were done in the similar way as in the initial screen test method.

3.2.2 Application plates and Interpreting results

The antimicrobial disc and combination discs (cefpodoxime / cefpodoxime + clavulanic acid, ceftazidime / ceftazidime + clavulanic acid, cefotaxime / cefotaxime + clavulanic acid) were placed on each plate. Each disc must be pressed down to ensure complete contact with the agar surface. The plates were inverted and placed in an incubator set to 37 ºC for 18 hours, within 15 minutes after the discs were applied.

3.2.3 Reading plates and Interpreting Results

After 18 hours of incubation, each plate was examined. The diameter of the zone of the complete inhibition were measured. Zone was measured to the nearest whole millimeter, using a sliding caliper, which was held on the back of the plate. The sizes of

the zones of inhibition were interpreted by referring to the table of zone diameter standard of National Committee for Clinical Laboratory Standards (NCCLS, 2000). An organism was interpreted as the ESBL producer if there is an increase of ≥ 5 mm. in the inhibition zone of combination disc when compared to that of the cephalosporin. A ≥ 5 mm. increase in zone diameter for at least one antimicrobial agent its combination indicates ESBL production.

4. Antimicrobial susceptibility test

Paper disc susceptibility test was performed according to disc diffusion method by Kirby-Bauer (Bauer et al., 1966) and NCCLS (NCCLS, 2004). *E. coli* ATCC 25922 was also included in this test as the control strain.

All the *E. coli* strains were tested for antimicrobial susceptibility against 13 antimicrobial agents which have been commonly used in the treatment of infections due to *E. coli* (Brooks et al., 1995; Murray et al., 1999; Trevor et al., 2002).

The antimicrobial discs were ampicillin 10 μg (AM) , amoxicillin/clavulanic acid 20/10 μg (AMC), cefazolin 30 μg (CZ), cefoxitin 30 μg (FOX), cefpodoxime 10 μg (CPD), cetazidime 30 μg (CAZ), ceftriaxone 30 μg (CRO), cefotaxime 30 μg (CTX), aztreonam 30 μg (ATM), imipenem 10 μg (IMP), gentamicin 10 μg (GN), norfloxacin 10 μg (NOR), trimethoprim/sulfamethoxazole 1.25/23.75 μg (SXT).

4.1 Preparation of media

Twenty-five millimeters of Mueller-Hinton agar (Difco, USA) were poured into each 10 cm-diameter petri dish to yield an agar depth of 4 mm. The media were then stored at 4 ºC and used within 2 weeks. Before performing the test, the petri disces were placed in an incubator at 35 ºC for 30 minutes with their lids slightly open to permit the evaporation of surface media.

4.2 Preparation of inoculum and standardization of inoculum

The well-isolated colonies of each 18 hours *E. coli* isolated from clinical specimen and *E. coli* ATCC 25922 were selected from Tryptic soy agar plates and

transferred to a tube containing 5 ml normal saline sulution (NSS). The turbidity of culture was adjusted to 0.5 McFarland standard solution to obtain approximately 1.5×10^8 cells/ml.

4.3 Inoculation of standard inoculum

A sterile cotton swab was dipped in the adjusted inoculum and rotated several times against the inside wall of the tube to remove excess liquid. The entire surface of the Mueller-Hinton agar plate was inoculated by streaking the swab over the surface. Streaking was repeated 3 times and for each time the plates were rotated 60º to ensure an even distribution of inoculum.

4.4 Application of discs

Immediately or not later than 15 minutes after the inoculation of the plates, the antibiotic discs were applied to the surface of the medium with sterile forceps in order that diffusion and growth proceeded stimulously. The discs were then slightly pressed down to ensure complete contact of the discs to the agar surface.

The discs were arranged at least 15 mm. from the edge of the plate and aparted from each other by distance of 15 to 20 mm. This arrangement reduced the likelihood of zones overlapping each other, which made interpretation difficult.

4.5 Incubation of plates

The inoculated plates were incubated aerobically at 35 ºC for 18 hours in an inverted position.

4.6 Interpretation of the disc susceptibility test

The diameter of each zone of inhibition was measured with digital sliding venier caliper. The size of the zone of inhibition was interpreted by referring to table of zone diameter standard of National Committee of Clinical Laboratory Standards (NCCLS, 2004) as shown in the Table 14. The organisms were reported as either susceptibility, intermediate susceptible, or resistant to the agents tested.

Table 14: Zone diameter interpretive standards antimicrobial agents of *E. coli* (NCCLS, 2004)

Zone diameter interpretive standards (mm.)			
Antimicrobial disc/Concentration	Resistance	Intermediate	Susceptible
ampicillin 10 μg	≤ 13	14 – 16	≥ 17
amoxicillin/clavulanic acid 20/10 μg	≤ 13	14 – 17	≥ 18
cefazolin 30 μg	≤ 14	15 – 17	≥ 18
cefoxitin 30 μg	≤ 14	15 – 17	≥ 18
cefpodoxime 10 μg	≤ 17	18 – 20	≥ 21
ceftazidime 30 μg	≤ 14	15 – 17	≥ 18
ceftriaxone 30 μg	≤ 13	14 – 20	≥ 21
cefotaxime 30 μg	≤ 14	15 – 22	≥ 23
aztreonam 30 μg	≤ 15	16 – 21	≥ 22
imipenem 10 μg	≤ 13	14 – 15	≥ 16
gentamicin 30 μg	≤ 12	13 – 14	≥ 15
norfloxacin 10 μg	≤ 12	13 – 16	≥ 17
Trimethoprim/sulfamethoxazole 1.25 / 23.75 μg	≤ 10	11 – 15	≥ 16

5. Detection of beta-lactam antibiotic resistant genes by Polymerase Chain Reaction (PCR) technique

5.1 DNA Extraction

Total extracted DNA from *E. coli* strains were purified by Microbial DNA Isolation Kit as described by Mo Bio Laboratories, Inc briefly as followed:

A 1.8 ml of each bacterial culture in 1.9 ml microcentrifuge tube was centrifuged for 30 seconds at 10,000xg, and then resuspended in 300 μl of microbead solution. The resuspended cells were then placed in a microbead tube which was added 50 μl of solution MD1 and heat at 65 ºC for 10 minutes before vortex at maximum speed for 10 minutes. Each sample was centrifuged for 30 seconds at 10,000xg, and the 350 μl supernatant was then transferred to a clean microcentrifuge tube, which 100 μl of solution MD2 was added to the supernatant. The mixture was then vortexed for 5 seconds and incubated at 4 ºC for 5 minutes. Each sample was centrifuged for 1 minute at 10,000xg, and then the 450 μl supernatant was then transferred to a clean 1.9 ml tube. Nine hundred microlitre of solution MD3 was added and vortexed for 5 seconds. Seven hundred microlitre of each sample was loaded into the spin filter and centrifuged at 10,000xg for 30 seconds. The flow through was discarded. The remaining supernatant of each sample was added to the spin filter and centrifuged for 30 seconds at 10,000xg. All flow through liquid was discarded. The 300 μl of solution MD4 was added and centrifuged for 30 seconds at 10,000xg. The flow through was discarded. The filter was centrifuged again for 1 minute and then placed in a new 1.9 ml tube. The 50 μl of solution MD5 was added to the center of the white filter membrane and centrifuged for 30 seconds. The flow through containing extracted DNA were stored at -20 ºC.

5.2 DNA amplification (Sambrook and Russel, 2001)

Antibiotic resistant genes including bla_{TEM}, bla_{SHV}, $bla_{\mathrm{CTX\text{-}M}}$, and bla_{VEB} as well as gene encoded for a site specific recombinate enzyme (integrase gene) were amplified by Polymerase Chain Reaction.

Primer specific for Integrase (*intI*1) gene, bla_{TEM}, bla_{SHV}, $bla_{\mathrm{CTX\text{-}M}}$, and bla_{VEB} used in this study were shown in Table 15.

Table 15: Sequence of primer and size of PCR product

Specific for	Primer name	Primer sequence	Product size (base)	Reference
*Intl*1	INT1F	5-'AAGGATCGGGCCTTGATGTT-3'	472	Levesque et al., 1995
	INT1R	5'-CAGCGCATCAAGCGGTGAGC-3'		
bla_{TEM}	TEM-for	5'-ATGAGTATTCAACATTTCCG-3'	863	Rasheed et al., 1997
	TEM-rev	5'-CTGACAGTTACCAATGCTTA-3'		
bla_{SHV}	SHV-for	5'-GGTTATGCGTTATATTCGCC-3'	867	Rasheed et al., 1997
	SHV-rev	5'-TTAGCGTTGCCAGTGCTC-3'		
bla_{CTX-M}	CTXM-MA1	5'-SCSATGTGCAGYACCAGTAA-3'	544	Eckert et al., 2004
	CTXM-MA2	5'-CCGCRATATGRTTGGTGGTG-3'		
bla_{VEB-1}	VEB1F	5'-CGACTTCCATTTCCCGATGC-3'	643	Cao et al., 2002
	VEB1R	5'-GGACTCTGCAACAAATACGC-3'		

*Intl*1, integrase gene of class 1 integron

The DNA amplifications were performed as followed; the DNA 0.5 μl was used as the template for PCR. The total volume of 50 μl contained 0.5 μl of the bacterial DNA preparation, 38 μl distilled water, 5 μl 1.5 mM $MgCl_2$, 5 μl 10x the four deoxynucleotide triphosphates (100 mM each), 0.5 μl of each primer and 0.5 μl *Taq* DNA polymerase (Finnzymes, Finland)

The reaction were run for 30 cycles through a temperature profile of 94 ºC for 60 seconds (denaturation), 55 ºC for 60 seconds (annealing) and 72 ºC for 60 seconds (extension). A final extension was performed at 72 ºC for 10 minutes.

5.3 Analysis of PCR Product

Amplification products were analyzed by running 10 μl of the product (PCR product 7 μl, loading dye 3 μl) in a 2% agarose gel in 0.5 x TBE buffer. A 1 Kb Plus DNA Ladder (Invitrogen, USA) was used as a DNA size marker. Gels were run in TBE buffer at a constant 100 volts for 60 minutes.

The gel was stained with 1 mg/ml of ethidium bromide in distilled water for 20 minutes. After that it was rinsed and destained with deionized water for 40 minutes. The gel was then photographed under UV transluminator.

6. Confirmation of Integrase (*IntI*1) gene by Dot Blot Hybridization

Detection of an *intI*1 was performed using the dot blot hybridization as described by Gene Images Random Prime Labelling Module and CDP-Star Detection Module (Amersham Biosciences, 2002). The methods were briefly described as followed.

6.1 Preparation of labeled probe

The specific *intI*1 (DNA template) of approximately 472 base pair was the cloned integrase gene in plasmid pCTF202 (Tribuddharat, 1999), in order to determine the results for the dot blot hybridization. The DNA template was denatured by heating for 5 minutes in a boiling water bath and then was chilled on ice. The 1.5 ml microcentrifuge tube was placed in an ice bath and the appropriate volume of each reagent was added in the following order; 29 μl water, 5 μl (50 ng) DNA template, 10 μl nucleotide mixture (5x stock solution of fluorescein-11-dUTP, dATP, dCTP, dGTP and dTTP), 5 μl primer (random nonamer), 1 μl (5 units/μl) Klenow. The mixture was then incubated for 4 hours at 37 ºC. The specific *intI*1 probe was stored -20 ºC.

6.2 Preparation of pre-hybridization and hybridization

Total extracted DNA 2 μl from each *E. coli* strains and 1 μl loading dye were mixed before which were blotted on to nylon membranes (Schleicher and Schuell, UK). The DNA samples were denatured for 5 minutes in 0.4M NaOH and were neutralized for

5 minutes in 2xSSC (1.5M NaCl, 0.5M Tris-HCl, pH adjusted to pH7.5). The membrane was prehybridized for 30 minutes at 58 ºC in 5xSSC (20xSSC, 0.1%w/v SDS, 5%w/v dextran sulphate, 20-fold dilution of liquid block). Hybridization was carried out overnight at 58 ºC with the specific integrase (*intI*1) gene probe. The stringency washes were carried out at 58 ºC as followed: the first stringency wash (10%SSC, 0.1%SDS) was pre-heated at 58 ºC for 15 minutes before the membrane was washed at 58 ºC for 15 minutes, the second stringency wash (0.5%SSC, 0.1%SDS) was pre-heated and washed at the same time and temparature as the first stringency wash.

6.3 Blocking, antibody incubation and washes

Signal was then detected using the CDP-Star Detection Module (Amersham Biosciences, UK) as directed by the manufacturer. After the membrane was washed with the stringency washes, which was blocked with a 1 in 10 dilution of liquid blocking agent (CDP-Star detection kit) in buffer A (100mM Tris-HCl, 300mM NaCl, pH 9.5) at room temparature for 1 hour. After a 1-hour blocking step, the membrane was incubated with an anti-fluorescein alkaline phosphatase conjugate diluted 5000-fold in freshly-prepared 0.5%w/v bovine serum albumin in buffer A at room temparature for 1 hour. The excess conjugate was removed from the membrane by three 10-minutes washes in 0.3% Tween 20 (USB, USA) in buffer A, after which the membrane was briefly washed in buffer A.

6.4 Detection of signal

The membrane was then placed in a clear plastic bag followed by hybridized blots were detected with the CDP-Star reagent (30-40 $\mu l/cm^2$). The probe-bound alkaline phosphatase cleaves the CDP-Star substrate, yielding a highly fluorescent product. Plastic bag was placed in the Hyberfilm MP (film cassette) that was took to a dark-room and was exposed for 1 hour.

7. Analysis of restriction fragments of chromosomal DNA by Pulsed–Field Gel Electrophoresis (PFGE)

Chromosomal DNA analysis by Pulsed-Field Gel Electrophoresis was performed according to the method recommended by Maslow et al. (Maslow et al., 2000)

The methods were briefly described as followed.

7.1 Sample preparation

Each strains of *E. coli* was streaked onto Tryptic Soy Agar (TSA) to yield a single colony which was then inoculated into 0.5 ml Tryptic Soy Broth (TSB) and incubated for 2 hours at 37 ºC. The culture was streaked out onto a TSA plate and incubated for 20 hours at 37 ºC. A single colony was picked and inoculated into 5 ml TSB and then incubated for 20 hours at 37 ºC. Dispensed 1.5 ml of the culture into 5 ml cold PIV buffer and then centrifuged at 1100xg for 15 minutes at 4 ºC. The PIV buffer was removed from the cell pellet. The cell was resuspended and mixed thoroughly in 1.5 ml cold PIV buffer and was placed on ice.

The 1.3% of low melting point agarose (Promega, USA) in PIV buffer was prepared. The agarose was melted by placing the flask into the beaker of boiling water. One ml of melted agarose was dispensed into 5 ml snap-top tube and was then placed in 50 ºC waterbath. One ml of *E.* coli cell in PIV buffer was added into each tube then slightly vortexed. Three hundred microliters of the mixture was immediately dispensed into each well of the plug molds that had already placed in the ice-tray for 15 minutes before used. The molds were then placed at 4 ºC for 30 minutes to solidify the agarose plug.

Fresh lysis solution was prepared by adding 80 μl RNase (10 mg/ml) and 800 μl lysozyme (50 mg/ml) into 40 ml lysis buffer. Four ml lysis solution was dispensed into each 15 ml snap-top tube. When the plug was solidified, each of them were pushed out from the molds into each lysis solution tube and then incubated for 20 hours at 37 ºC on a tube roller. The tubes were then chilled on ice for at least 15 minutes to harden the plugs. The lysis solution was carefully aspirated, then 4 ml of ESP solution was

dispensed into each tube. Each plug was incubated overnight at 50 ºC with gently shaking. The tubes were again chilled. The ESP solution was changed one more and then stored at 4 ºC.

7.2 Restriction enzyme digestion

The plugs were washed in 7 ml 1xTE buffer at 37 ºC on a tube roller four times at 2 hours, 2 hours, 1 hour, and overnight, respectively. A labeled microcentrifuged tube containing restriction enzyme *Xba*I (Invitrogen, USA), 10x restriction enzyme buffer, bovine serum albumin (final concentration, 100 μl/ml) and water to a final volume of 250 μl were prepared for each strain. Each washed plug was sliced into a small piece about 1 mm thick using a glass coverslip. A sliced plug was added to the labeled microcentrifuged tube. The restriction enzyme solution was added and then incubated overnight at 37 ºC in waterbath. Each sliced plug was washed using 1000 μl 1xTE buffer and placed on ice for 30 minutes. The 1xTE was removed and 1000 μl 0.5xTBE was dispensed into each plug. The plugs were placed on ice for 15 minutes.

7.3 Gel preparation and pre-electrophoresis

The running gel was prepared by dissolving 0.9 gram of ultrapure high melting temperature agarose (1% wt/vol) in 90 ml 0.5xTBE buffer. The agarose was melted until completely dissolved and was cooled down to approximately 50 ºC, and then poured into the gel casting. The 15-well comb was placed in the gel to make 15-well running agarose gel. The gel was placed in the gel casting until solidified. The gel was then transferred to the electrophoresis tank (CHEF-DRIII system, BioRad, USA). The running gel was preelectrophoresed for 0.5 hour in 0.5xTBE buffer to improve the clarity and resolution of the gel using the following condition; V = 6 v/cm, initial switch time = 5 s, final switch time = 50 s, and the temperature was 14 ºC. The running gel was removed from the tank.

7.4 Sample loading and electrophoresis

Each sliced plug sample including a plug of λ ladder marker was loaded into each well of the preelectrophoresed gel. All the wells of the gel were filled with 1% low-

melting point agarose to protect the sliced plug from floating out of the well. The gel was then placed in the PFGE tank with 0.5xTBE buffer and electrophoresed using the same condition as the preelectrophoresis condition except that the running time was 22 hours

<u>7.5 Gel visualization</u>

The gel was stained with 300 μl ethidium bromide (1mg/ml) in 300 ml ultrapure water for 20 minutes. After that it was rinsed and destained with 300 ml ultrapure water for 40 minutes. The gel was then photographed under UV transluminator. Result was interpreted by according to Tenover et al (Tenover et al., 1995).

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CHAPTER V

RESULTS

1. The suspicious extended spectrum beta-lactamase (ESBL) producing *E. coli* from initial screen test (NCCLS, 2004)

All of 120 *E. coli* isolates were examined for extended spectrum beta-lactamase producing strains by initial screen test according to NCCLS, 2004. Forty-six isolates were accepted as suspicious ESBL producing *E. coli* strains. Among them, 58.7% were isolated from urine, 15.2% were from blood, and 26.1% were from pus. The results were shown in the Table 16.

There were 46 suspicious ESBL producing *E. coli* strains according to NCCLS criteria for detection using either one of the five recommended cephalosporins. The number of suspicious ESBL producing *E. coli* strains were isolated from the clinical specimens in relation to the number of the antimicrobial agents used in the test as shown in the Table 17.

2. The extended spectrum beta-lactamase (ESBL) producing *E. coli* from phenotypic confirmatory test

The 46 suspicious ESBL producing *E. coli* strains were confirmed for the ESBL producing strains using the NCCLS phenotypic confirmatory test (the combination disc method). Thirty-seven strains of 120 strains (30.8%) were accepted as ESBL producing *E. coli* strains. Among them 38.3% were isolated from urine, 23.3% from blood, and 23.3% from pus as shown in the Table 18.

One strain which was screened by one antimicrobial agent for the suspicious ESBL producing *E. coli* strain but it was not ESBL producing *E. coli* strain for confirmatory test. On the other hand, one isolate which was screened by three antimicrobial agents as the suspicious ESBL *E. coli* strain was confirmed to be ESBL producer as well as all 13 strains which were screened by four antimicrobial agents and

23 of 31 strains which were screened by five antimicrobial agents were also confirmed as ESBL producers.

Therefore, the concordant results were obtained between the initial screen test using one to five antimicrobial agents and the phenotypic confirmatory test as shown in the Table 19. There were 1 suspicious ESBL producing strain from initial screen test using only one antimicrobial agent and 8 strains using all five antimicrobial agents, respectively, were not confirmed as ESBL producing *E. coli* strains.

3. The antimicrobial susceptibility patterns

3.1 The antimicrobial susceptibility patterns of 120 *E. coli* strains from various clinical specimens

The 120 *E. coli* strains were tested against 13 antimicrobial agents in order to study the susceptibility patterns of the organisms. The results were summarized in Table 20 which showed that there were 56 susceptibility patterns or antibiograms found among all of the tested isolates.

Ten strains of *E. coli* were in antibiogram pattern 12 which were susceptible to amoxicillin/clavulanic acid, cefazolin, cefoxitin, cefpodoxime, ceftazidime, ceftriaxone, cefotaxime, aztreonam, imipenem, gentamicin, and norfloxacin. Seven *E. coli* strains were in antibiogram pattern 1 and were susceptible to all of 13 antimicrobial agents while seven strains were in pattern 9 but were susceptible to 12 antimicrobial agents except ampicillin.

Six *E. coli* strains were in antibiogram pattern 39 and were susceptible to cefoxitin, ceftazidime, and imipenem while six *E. coli* strains were in antibiogram pattern 52 and were susceptible to only imipenem. Five *E. coli* strains were antibiogram pattern 11 and were susceptible to amoxicillin/clavulanic acid, cefazolin, cefoxitin, cefpodoxime, ceftazidime, ceftriaxone, cefotaxime, aztreonam, imipenem, and norfloxacin. There were only few *E. coli* strains in each of the other antibiogram patterns.

The result in the Table 21 showed that 10.9% of 120 *E. coli* strains were susceptible to ampicillin, 45.0% to amoxicillin/clavulanic acid, 49.2% to cefazolin, 80.0% to cefoxitin, 62.5% to cefpodoxime, 75.4% to ceftazidime, 64.2% to ceftriaxone, 61.7% to cefotaxime, 69.2% to aztreonam, 99.2% to imipenem, 55.0% to gentamicin, 56.7% to norfloxacin, and 30.4% to trimethoprim/sulfamethoxazole.

3.2 The antimicrobial susceptibility pattern of 37 ESBL producing *E. coli* strains from various clinical specimens

There were 21 antibiograms among 37 ESBL producing *E. coli* strains as shown in the Table 22.

Five strains of ESBL producers were in antibiogram pattern 8 and 20. ESBL producing *E. coli* strain were pattern 8 and were susceptible to cefoxitin, ceftazidime, and imipenem ; however, five strains in pattern 20 were susceptible to imipenem and norfloxacin. Four strains were patterns 12 and were susceptible to cefoxitin and imipenem. While there were only few *E. coli* strains in each of the other antibiogram patterns.

The percentage of the susceptibility of ESBL producing *E. coli* strains against 13 antimicrobial agents were shown in the Table 23. There were 2.7% of ESBL producing *E. coli* strains which were susceptible to ampicillin, 10.8% to amoxicillin/clavulanic acid, 2.7% to cefazolin, 67.6% to cefoxitin, 46.0% to ceftazidime, 5.4% to ceftriaxone, 3.0% to cefotaxime, 19.0% to aztreonam, 97.3% to imipenem, 13.5% to gentamicin, 32.4% to norfloxacin, and 27.0% to trimethoprim/sulfamethoxazole. None of strains was susceptible to cefpodoxime.

4. The integrase (*intI*1) gene and ESBL genes

4.1 Percentage of Integrase (*intI*1) gene and ESBL genes

The presence of an *intI*1 and ESBL gene (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{VEB} genes) were detected using the Polymerase Chain Reaction (PCR) technique.

The purified DNA from all 120 *E. coli* strains were subjected for PCR amplification specific of an *intI*1. Percent of *intI* 1 PCR positive were 99.2% (119 of 120), as shown in the Table 24. The purified DNA from 37 ESBL producing *E. coli* strains from the phenotypic confirmatory test and the other 9 suspicious ESBL *E. coli* strains that were negative in the confirmatort test were subjected for the PCR amplification of ESBL genes (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{VEB} genes). The ESBL genes of 37 ESBL producers were summarized in the Table 25. It was shown that 78.4% of ESBL producing *E. coli* strains were bla_{TEM} positive, 8.1% were bla_{SHV} positive, 78.4% were bla_{CTX-M} positive, 8.1% were bla_{VEB} positive, only 2.7% were coharboured with bla_{TEM} and bla_{SHV}, 2.7% were coharboured with bla_{TEM} and bla_{VEB}, 62.2% were coharboured with bla_{TEM} and bla_{CTX-M}, only 2.7% were coharboured with bla_{VEB} and bla_{CTX-M}, and 5.4% were coharboured with bla_{TEM}, bla_{SHV}, and bla_{CTX-M}. It was remarkable that 75.7% (28 of 37) were coharboured at least 2 different *bla* genes; however, 8.1% of ESBL producer were negative for all 4 ESBL genes. For nine suspicious ESBL producers gave positive results only for bla_{TEM} as shown in the Table 26.

For all 37 ESBL producing isolates, *intI*1 PCR positive were 97.3%, as shown in the Table 27. The result of coharbouring between *intI*1 and various ESBL gene were showed that majority of ESBL producing *E.coli* isolates (62.2%) were coharboured with *intI*1, bla_{TEM} and bla_{CTX-M} were the most common, whereas only 2.7% were negative for *intI* 1 and all ESBL genes.

4.2 The *IntI*1 and ESBL genes patterns of 37 ESBL producing *E. coli* strains

The *intI*1 and ESBL genes patterns of 37 ESBL producers were summarized in the Table 28. There were 10 patterns found among all of the tested strains.

Most of ESBL producing *E. coli* strains (23 strains) were in pattern 4 which were *intI*1 positive, bla_{TEM} positive, and bla_{CTX-M} positive. There were only few ESBL producing *E. coli* strains in each of the other patterns.

4.3 Comparison between the genes patterns and the antibiograms of 37 ESBL producing *E. coli* strains

The correlation between the genes patterns and the antibiograms were summarized in the Table 29. It was showed that gene pattern 4 which was the most prevalent type in this study was divided into various different antibiogram patterns. There was no correlation between the gene patterns and the antibiograms.

5. Southern blot hybridization (Dot blot hybridization) for detection of an *intI*1

Southern blot analysis using *intI*1 as a probe was also performed for all isolated DNA. The *intI*1 hybridizations showed that 99.2% 120 *E. coli* isolates were positive for *intI*1. Percent of *intI*1 positive PCR were similar to percent of *intI*1 positive hybridization, as shown in the Table 30.

6. Genotypes of extended spectrum beta-lactamase producing *E. coli* from the pulsed-field gel electrophoresis (PFGE) technique

All 37 ESBL producing *E. coli* strains were typed by PFGE as shown in the Table 31. There were as many as 32 different pulsotypes designated as type 1 to type 32 but 2 isolates were untypeable. Almost all strains were sporadic types because only 3 and 2 strains were in pulsotype 5 and 14, respectively.

The correlation between the pulsotypes and the antibiograms were summarized in Table 32. It was shown that ESBL producing *E. coli* strains with pulsotype 5 which was the most prevalent type in this study were divided into various different antibiogram patterns. It was found that there was no correlation between the pulsotype and the antibiogram.

The correlation between the pulsotypes and the genes patterns were summaried in Table 33. It was shown that most strains (23 of 37) carried genes patterns 4 which was divided into various different pulsotypes. It was found that there was no correlation between the pulsotype and the genes patterns.

Table 16: Number of suspicious extended spectrum beta-lactamase (ESBL) producing *E. coli* strains from various clinical specimens based on NCCLS initial screen test

Specimens	Suspicious ESBL producing *E. coli* strains (% of total positive strains)
Urine	27* (58.7%)**
Blood	7 (15.2%)
Pus	12 (26.1%)
Total	46

* ESBL screening test using either one of five drugs; cefpodoxime or ceftazidime or ceftriaxone or cefotaxime or aztreonam

** Percent from the total *E. coli* strains from each type of specimens

Table 17: Number of suspicious extended spectrum beta-lactamase (ESBL) producing *E. coli* strains from various clinical specimens in relation to the number of antimicrobial agents tests by initial screen test

Specimen	Number of suspicious ESBL producing *E. coli* strains					
	One antimicrobial agent test	Two antimicrobial agents test	Three Antimicrobial agents test	Four antimicrobial Agents test	All five antimicrobial agents test	Total
Urine	1*	0**	0***	8****	18*****	27
Blood	0	0	1	2	4	7
Pus	0	0	0	3	9	12
Total	1	0	1	13	31	46

*One agent: cefpodoxime or ceftazidime or ceftriaxone or cefotaxime or aztreonam

**Two agents: cefpodoxime and ceftazidime or cefpodoxime and ceftriaxone or cefpodoxime and cefotaxime or cefpodoxime and aztreonam or ceftazidime and ceftriaxone or ceftazidime and cefotaxime or ceftazidime and aztreonam or ceftriaxone and cefotaxime or ceftriaxone and aztreonam or cefotaxime and aztreonam

***Three agents: cefpodoxime, ceftazidime and ceftriaxone or cefpodoxime, ceftazidime and cefotaxime or cefpodoxime, ceftazidime and aztreonam or cefpodoxime, ceftriaxone and cefotaxime or cefpodoxime, ceftriaxone and aztreonam or cefpodoxime, cefotaxime and aztreonam

****Four agents: cefpodoxime, ceftazidime, ceftriaxone and cefotaxime or cefpodoxime, ceftazidime, ceftriaxone and aztreonam or cefpodoxime, ceftazidime, cefotaxime and aztreonam or cefpodoxime, ceftriaxone, cefotaxime and aztreonam or ceftazidime, ceftriaxone, cefotaxime and aztreonam

*****Five agents: cefpodoxime, ceftazidime, ceftriaxone, cefotaxime and aztreonam

Table 18: Number of extended spectrum beta-lactamase (ESBL) producing *E. coli* strains from various clinical specimens determined by phenotypic confirmatory test as compared with the initial screen test

Specimen	Number of suspicious ESBL producing *E. coli* strains	Comparative number of ESBL producing *E. coli* strains determined by two different tests	
		Initial screen test	Phenotypic confirmatory test
Urine	60	27*	23**
Blood	30	7	7
Pus	30	12	7
Total	120	46	37

*Number of positive test determine by initial screen test

**Number of ESBL producer determined by phenotypic confirmatory test

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Table 19: Number of ESBL producing *E. coli* strains from various clinical specimens determined by phenotypic confirmatory test as compared with the initial screen test

Specimen	Number of suspicious ESBL producing *E. coli* strains	Comparative number of ESBL producing strains determined by 2 different tests				
		Initial screen by only one antimicrobial agents	Initial screen by two antimicrobial agents	Initial screen by three antimicrobial agents	Initial screen by four antimicrobial agents	Initial screen by all five antimicrobial agents
Urine	27	0/0*	0/0	1/1	2/2	4/4
Blood	7	0/0	0/0	0/0	3/3	4/9
Pus	12	0/1	0/0	0/0	8/8	15/18
Total	46	0/1	0/0	1/1	13/13	23/31

*Number of ESBL producer determined by phenotypic confirmatory test / Number of positive test determined by initial screen test

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Table 20: The antimicrobial susceptibility patterns (antibiogram) and frequency in each pattern among 120 *E. coli* strains from clinical specimens

Type	AM	AMC	CZ	FOX	CPD	CAZ	CRO	CTX	ATM	IMP	GN	NOR	SXT	No. of strains	Strain Code Number
1	S	S	S	S	S	S	S	S	S	S	S	S	S	8	EB2, EB21, EB28, EP10, EP21, EU4, EU14, EU18
2	S	S	S	S	S	S	S	S	S	S	S	S	R	1	EP4
3	S	S	S	S	S	S	S	S	S	S	S	R	R	2	EU4, EU37
4	S	S	S	S	S	S	S	S	S	S	R	S	S	1	EU30
5	S	S	R	S	S	S	S	S	S	S	S	S	R	1	EU47
6	S	R	R	S	S	S	S	S	S	S	S	S	R	1	EU54
7	S	R	R	S	R	S	R	R	R	S	R	R	S	1	EU20
8	S	R	R	R	R	R	R	R	S	S	S	S	S	1	EP14
9	R	S	S	S	S	S	S	S	S	S	S	S	S	8	EB26, EP19, EP27, EP28, EU28, EU32, EU36, EU39
10	R	S	S	S	S	S	S	S	S	S	S	R	S	1	EU49
11	R	S	S	S	S	S	S	S	S	S	R	S	R	5	EB8, EB20, EB29, EB30, EP23
12	R	S	S	S	S	S	S	S	S	S	S	S	R	11	EB1, EB5, EB16, EB22, EP1, EP16, EP20, EP30, EU2, EU53, EU58
13	R	S	S	S	S	S	S	S	S	S	R	R	R	2	EP8, EU31
14	R	S	R	S	S	S	S	S	S	S	S	R	R	1	EU44
15	R	S	R	S	R	S	R	R	S	S	R	S	S	1	EU48
16	R	S	S	S	S	S	S	S	S	S	R	S	S	2	EB24, EU52

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Table 20 (cont.)

Type	AM	AMC	CZ	FOX	CPD	CAZ	CRO	CTX	ATM	IMP	GN	NOR	SXT	No. of strains	Strain Code Number
17	R	S	R	S	R	S	R	R	R	S	S	S	S	1	EU59
18	R	S	R	S	R	S	R	R	S	S	R	S	R	1	EB9
19	R	S	R	S	S	S	S	R	S	S	R	S	R	1	EB13
20	R	S	R	S	S	R	S	R	S	S	S	R	R	1	EB11
21	R	S	R	S	S	S	S	S	S	S	S	S	R	1	EB15
22	R	S	R	S	S	R	S	R	S	S	S	S	R	1	EB17
23	R	S	S	S	S	S	S	S	S	S	R	R	R	3	EU7, EU23, EU40
24	R	S	R	S	S	R	S	R	R	S	R	R	R	1	EU22
25	R	R	S	R	R	R	R	S	R	R	R	R	R	1	EU9
26	R	R	S	S	S	S	S	S	S	S	S	S	S	3	EB19, EP3, EU11
27	R	R	S	R	S	S	S	S	S	S	S	R	R	1	EU13
28	R	R	S	S	S	S	S	S	S	S	S	S	R	3	EP24, EP29, EU17
29	R	R	S	S	S	S	S	S	S	S	R	R	R	2	EU27, EU57
30	R	R	S	S	S	S	S	S	S	S	S	R	R	2	EU45, EU55
31	R	R	S	S	S	S	S	S	S	S	S	R	S	1	EU60
32	R	R	S	S	S	S	S	S	S	S	R	S	R	2	EB7, EP6
33	R	R	R	S	R	R	R	R	R	S	R	S	S	3	EB27, EP7, EU5

Table 20 (cont.)

Type	AM	AMC	CZ	FOX	CPD	CAZ	CRO	CTX	ATM	IMP	GN	NOR	SXT	No. of strains	Strain Code Number
34	R	R	R	S	S	R	S	R	S	S	R	S	S	1	EB14
35	R	R	R	S	S	S	S	S	S	S	S	R	R	2	EU15, EU50
36	R	R	R	S	R	S	R	R	S	S	R	S	R	2	EB3, EP13
37	R	R	R	S	R	R	R	R	R	S	R	R	R	4	EB23, EP17, EU19, EU34
38	R	R	R	S	R	S	R	R	S	S	R	R	R	2	EP18, EU21
39	R	R	R	S	R	S	R	R	R	S	R	R	R	6	EB12, EB25, EP12, EU33, EU43, EU51
40	R	R	R	S	S	S	S	S	S	S	R	R	R	3	EP15, EU24, EU46
41	R	R	R	S	R	R	R	R	R	S	R	R	S	2	EP2, EU29
42	R	R	R	S	R	R	R	R	R	S	R	S	R	1	EU42
43	R	R	R	S	R	R	S	S	R	S	R	S	S	1	EP9
44	R	R	R	S	S	S	S	R	S	S	S	S	R	1	EB18
45	R	R	R	S	S	S	S	S	S	S	S	S	R	2	EB10, EP26
46	R	R	R	R	R	S	S	S	S	S	S	R	R	1	EU25
47	R	R	R	R	R	S	R	R	R	S	S	R	R	1	EU35
48	R	R	R	R	R	S	R	R	R	S	R	R	R	1	EU56
49	R	R	R	R	R	S	S	S	R	S	R	S	R	1	EU3
50	R	R	R	R	R	S	R	S	S	S	S	S	R	1	EB6

Table 20 (cont.)

Type	AM	AMC	CZ	FOX	CPD	CAZ	CRO	CTX	ATM	IMP	GN	NOR	SXT	No. of strains	Strain Code Number
51	R	R	R	R	R	R	R	R	R	S	S	R	S	1	EU26
52	R	R	R	R	R	R	R	R	R	S	R	R	R	6	EP22, EU1, EU10, EU12, EU16, EU38
53	R	R	R	R	R	R	R	R	R	S	S	R	R	3	EP5, EP11, EU8
54	R	R	R	R	R	R	R	R	R	S	R	S	R	1	EU41
55	R	R	R	R	R	R	R	R	R	S	S	S	S	1	EP25
56	R	R	R	R	R	R	R	R	R	S	S	S	R	1	EU6

AM = ampicillin , AMC = amoxycillin/clavulanic acid , CZ = cefazolin , FOX = cefoxitin , CPD = cefpodoxime , CAZ = ceftazidime , CRO= ceftriaxone , CTX = cefotaxime ,

ATM = aztreonam , IMP = imipenem , GN = gentamicin , NOR = norfloxacin , SXT = trimethoprim/sulfamethoxazole

S = susceptible, R = resistant (Intermediate counts as the resistance)

EB = *E. coli* isolates from blood , EP = *E. coli* isolates from pus, EU = *E. coli* isolates from urine

No. of strains = Number of strains

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Table 21: Antimicrobial susceptibility of 120 *E. coli* strains from each clinical specimens against antimicrobial agents (agar disc diffusion method)

Specimen (No. of *E. coli*)	Number of susceptible *E.coli* strains (percent)												
	AM	AMC	CZ	FXO	CPD	CAZ	CRO	CTX	ATM	IMP	GN	NOR	SXT
Urine (60)	7 (11.7) *	23 (38.3)	27 (45.0)	44 (73.3)	34 (56.7)	45 (75.0)	35 (58.3)	36 (60.0)	36 (60.0)	59 (98.3)	30 (50.0)	22 (36.7)	17 (28.3)
Blood (30)	3 (10.0)	19 (63.3)	16 (53.3)	28 (93.3)	23 (76.7)	25 (83.3)	23 (76.7)	19 (63.3)	26 (86.7)	30 (100.0)	17 (56.7)	25 (83.3)	9 (30.0)
Pus (30)	3 (10.0)	12 (40.0)	16 (53.3)	24 (80.0)	18 (60.0)	21 (70.0)	19 (63.3)	19 (63.3)	21 (70.0)	30 (100.0)	19 (63.3)	21 (70.0)	11 (36.7)
Total (120)	13 (10.9)	54 (45.0)	59 (49.2)	96 (80.0)	75 (62.5)	91 (75.4)	77 (64.2)	74 (61.7)	83 (69.2)	119 (99.2)	66 (55.0)	68 (56.7)	37 (30.9)

AM = ampicillin , AMC = amoxycillin/clavulanic acid , CZ = cefazolin , FOX = cefoxitin , CPD = cefpodoxime , CAZ = ceftazidime , CRO = ceftriaxone , CTX = cefotaxime, ATM = aztreonam , IMP = imipenem , GN = gentamicin , NOR = norfloxacin , SXT = trimethoprim/sulfamethoxazole

No. of *E. coli* = Number of *E. coli*

*Percent of susceptible *E. coli* strains

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Table 22: The antimicrobial susceptibility patterns (antibiogram) and frequency in each pattern of 37 ESBL producing *E. coli* strains from clinical specimens

Type	AM	AMC	CZ	FOX	CPD	CAZ	CRO	CTX	ATM	IMP	GN	NOR	SXT	No. of strains	Strain Code Number
1	S	R	R	S	R	S	R	R	R	S	R	R	S	1	EU20
2	R	S	R	S	R	S	R	R	R	S	S	S	S	1	EU59
3	R	S	R	S	R	S	R	R	S	S	R	S	S	1	EU48
4	R	S	R	S	R	S	R	R	S	S	R	S	R	1	EB9
5	R	S	R	S	R	S	R	R	S	R	R	R	R	1	EU22
6	R	R	S	R	R	R	R	S	R	R	R	R	R	1	EU9
7	R	R	R	S	R	S	R	R	S	S	R	R	R	2	EP18, EU21
8	R	R	R	S	R	S	R	R	R	S	R	R	R	5	EB12, EB25, EP12, EU33, EU51
9	R	R	R	S	R	S	R	R	S	S	R	S	R	2	EB3, EP13
10	R	R	R	S	R	R	S	S	R	S	R	S	S	1	EP9
11	R	R	R	S	R	R	R	R	R	S	R	S	S	3	EB27, EP7, EU5
12	R	R	R	S	R	R	R	R	R	S	R	R	R	4	EB23, EP17, EU19, EU34
13	R	R	R	S	R	R	R	R	R	S	R	R	S	2	EU29, EP2
14	R	R	R	S	R	R	R	R	R	S	R	S	R	1	EU42
15	R	R	R	R	R	S	R	S	S	S	S	S	R	1	EB6

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Table 22 (cont.)

Type	AM	AMC	CZ	FOX	CPD	CAZ	CRO	CTX	ATM	IMP	GN	NOR	SXT	No. of strains	Strain Code Number.
16	R	R	R	R	R	S	R	R	R	S	R	R	R	1	EU56
17	R	R	R	R	R	S	R	R	S	S	R	R	R	1	EU35
18	R	R	R	R	R	R	R	R	R	S	S	R	S	1	EU26
19	R	R	R	R	R	R	R	R	R	S	S	R	R	1	EU8
20	R	R	R	R	R	R	R	R	R	S	R	R	R	5	EU1, EU10, EU12, EU16, EU38
21	R	R	R	R	R	R	R	R	R	S	R	S	R	1	EU41

AM = ampicillin , AMC = amoxycillin/clavulanic acid , CZ = cefazolin , FOX = cefoxitin , CPD = cefpodoxime , CAZ = ceftazidime , CRO= ceftriaxone , CTX = cefotaxime , ATM = aztreonam , IMP = imipenem , GN = gentamicin , NOR = norfloxacin , SXT = trimethoprim/sulfamethoxazole

S = susceptible , R = resistant (Intermediate counts as the resistance)

EB = *E. coli* isolates from blood , EP = *E. coli* isolates from pus, EU = *E. coli* isolates from urine

No. of strains = Number of strains

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Table 23: The antimicrobial susceptibility patterns (antibiogram) and frequency in each pattern of 37 ESBL producing *E. coli* strains from clinical specimens

Specimen (No. of ESBL producing *E. coli*)	Number of susceptible ESBL producing *E.coli* strains (percent)												
	AM	AMC	CZ	FXO	CPD	CAZ	CRO	CTX	ATM	IMP	GN	NOR	SXT
Urine (23)	1 (4.4) *	3 (13.1)	1 (4.4)	12 (52.2)	0	9 (39.1)	0	1 (4.4)	2 (8.7)	22 (95.7)	4 (17.4)	5 (21.8)	6 (26.1)
Blood (7)	0	1 (14.3)	0	6 (85.7)	0	5 (71.4)	0	1 (14.3)	3 (42.9)	7 (100.0)	1 (14.3)	4 (57.1)	1 (14.3)
Pus (7)	0	0	0	7 (100.0)	0	3 (42.9)	2 (28.6)	1 (14.3)	2 (28.6)	7 (100.0)	0	3 (42.9)	3 (42.9)
Total (37)	1 (2.7)	4 (10.8)	1 (2.7)	25 (67.6)	0	17 (46.0)	2 (5.4)	3 (8.1)	7 (18.9)	36 (97.3)	5 (13.5)	12 (32.4)	10 (27.0)

AM = ampicillin , AMC = amoxycillin/clavulanic acid , CZ = cefazolin , FOX = cefoxitin , CPD = cefpodoxime , CAZ = ceftazidime , CRO = ceftriaxone , CTX = cefotaxime, ATM = aztreonam , IMP = imipenem , GN = gentamicin , NOR = norfloxacin , SXT = trimethoprim/sulfamethoxazole

*Percent of susceptible ESBL producing *E. coli* strain

No. of ESBL producing *E. coli* = Number of ESBL producing *E. coli*

Table 24: Prevalence of Integrase (*IntI*1) gene in 120 *E. coli* strains

Specimen (No. of *E. coli* strains)	Number of *IntI* 1 PCR positive in *E.coli* strains
Blood (30)	29* (96.7)**
Pus (30)	30 (100.0)
Urine (60)	60 (100.0)
Total (120)	119 (99.2)

*IntI*1 = integrase gene of class 1 integron

No. of *E. coli* strains = Number of *E. coli* strains

*Number of *IntI*1 PCR positive in *E. coli* strains

**Percent of *IntI*1 PCR positive in *E. coli* strains

Table 25: ESBL genes in ESBL producing *E. coli* strains

<table>
<tr><th colspan="2" rowspan="2">ESBL types*</th><th colspan="4">Number of ESBL producing E.coli strains (%)</th><th rowspan="2">Strain Code Number</th></tr>
<tr><th>Urine (23)</th><th>Blood (7)</th><th>Pus (7)</th><th>Total (37)</th></tr>
<tr><td rowspan="10">Harbouring</td><td rowspan="3">TEM</td><td rowspan="3">18 (78.3)</td><td rowspan="3">5 (71.4)</td><td rowspan="3">6 (85.7)</td><td rowspan="3">29 (78.4)</td><td>EU : 8,9,10,16,19,20,21,22,26,33,34,35,38,41,42,48,51,56</td></tr>
<tr><td>EB : 3,12,23,25,27</td></tr>
<tr><td>EP : 7,9,12,13,17,18</td></tr>
<tr><td rowspan="2">SHV</td><td rowspan="2">2 (8.7)</td><td rowspan="2">1 (14.3)</td><td rowspan="2">0</td><td rowspan="2">3 (8.1)</td><td>EU : 10,42</td></tr>
<tr><td>EB : 27</td></tr>
<tr><td rowspan="2">VEB</td><td rowspan="2">2 (8.7)</td><td rowspan="2">0</td><td rowspan="2">1 (14.3)</td><td rowspan="2">3 (8.1)</td><td>EU : 1,5</td></tr>
<tr><td>EP : 9</td></tr>
<tr><td rowspan="3">CTX-M</td><td rowspan="3">18 (78.3)</td><td rowspan="3">5 (71.4)</td><td rowspan="3">6 (85.7)</td><td rowspan="3">29 (78.4)</td><td>EU : 1,8,16,19,20,21,22,26,29,33,34,35,38,42,48,51,56,59</td></tr>
<tr><td>EB : 3,12,23,25,27</td></tr>
<tr><td>EP : 2,7,12,13,17,18</td></tr>
<tr><td rowspan="5">Co harbouring between</td><td>TEM , SHV</td><td>1 (4.4)</td><td>0</td><td>0</td><td>1 (2.7)</td><td>EU : 10</td></tr>
<tr><td>TEM , VEB</td><td>-</td><td>0</td><td>1 (14.3)</td><td>1 (2.7)</td><td>EP : 9</td></tr>
<tr><td rowspan="3">TEM , CTX-M</td><td rowspan="3">14 (60.9)</td><td rowspan="3">4 (57.1)</td><td rowspan="3">5 (71.4)</td><td rowspan="3">23 (62.2)</td><td>EU : 8,16,19,20,21,22,26,33,34,35,38,48,51,56</td></tr>
<tr><td>EB : 3,12,23,25</td></tr>
<tr><td>EP : 7,12,13,17,18</td></tr>
</table>

Table 25 (cont.)

ESBL types		Number of ESBL producing *E.coli* strains (%)				Strain Code Number
		Urine (23)	Blood (7)	Pus (7)	Total (37)	
Co harbouring between	VEB , CTX-M	1 (4.4)	0	0	1 (2.7)	EU : 1
	TEM , SHV , CTX-M	1 (4.4)	1 (14.3)	0	2 (5.4)	EU : 42 EB : 27
	Total	17 (73.9)	5 (71.4)	6 (85.7)	28 (75.7)	EU : 1,8,10,16,19,20,21,22,26,33,34,35,38,42,48,51,56 EB : 3,12,23,25,27 EP : 7,9,12,13,17,18
Non-TEM , -SHV , -VEB , -CTX-M		1 (4.4)	2 (28.6)	0	3 (8.1)	EU : 12 EB : 6,9

*, the PCR product amplified using universal primers

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Table 26: Various ESBL types in ESBL producing *E. coli* strains and in suspicious ESBL *E. coli* strains

ESBL* types	No. of ESBL producing *E.coli* strains (37)	Number of *E. coli* strains (9) that were negative for confirmtory test
TEM*	29	9
SHV	3	0
VEB	3	0
CTX-M	29	0

No. of ESBL producing *E. coli* strains = Number of ESBL producing *E. coli* strains

*, the PCR product amplified using universal primers

Table 27: Correlation between Integrase (*intI*1) gene and various ESBL genes in 37 ESBL producing *E. coli* strains

Correlation between Integrase gene and ESBL* types	Number of suspicious ESBL producing *E.coli* strains (%)				Strain Code Number
	Urine (23)	Blood (7)	Pus (7)	Total (37)	
Integrase (*intI*1) gene	23 (100.0)	6 (85.7)	7 (100.0)	36 (97.3)	EU : 1,5,8,9,10,12,16,19,20,21,22,26,29,33,34,35,38,41,42,48,51,56,59
					EB : 6,12,23,25,27
					EP : 2,7,9,12,13,17,18
*intI*1, TEM	2 (8.7)	0	0	2 (5.4)	EU : 9,41
*intI*1, VEB	1 (4.4)	0	0	1 (2.7)	EU : 5
*intI*1, CTX-M	2 (8.7)	0	1 (14.3)	3 (8.1)	EU : 29,59
					EP : 2
*intI*1 , TEM , SHV	1 (4.4)	0	0	1 (2.7)	EU : 10
*intI*1 , TEM , VEB	0	0	1 (14.3)	1 (2.7)	EP : 9
*intI*1, TEM , CTX-M	14 (60.9)	4 (57.1)	5 (71.4)	23 (62.2)	EU : 8,16,19,20,21,22,26,33,34,38,35,48,51,56
					EB : 3,12,23,25
					EP : 7,12,13,17,18
*intI*1, VEB , CTX-M	1 (4.4)	0	0	1 (2.7)	EU : 1

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Table 27 (cont.)

Correlation between Integrase gene and ESBL types	Number of suspicious ESBL producing *E.coli* isolates (%)				Strain Code Number
	Urine (23)	Blood (7)	Pus (7)	Total (37)	
*intI*1, TEM , SHV , CTX-M	1 (4.5)	1 (14.3)	0	2 (5.4)	EU : 42
					EB : 27
Non - *intI*1, Non - ESBL types	0	1 (14.3)	0	1 (2.7)	EB : 9

*intI*1 = integrase gene of class 1 integron

*, the PCR product amplified using universal primers

EB = *E. coli* isolates from blood, EP = *E. coli* isolates from pus, EU = *E. coli* isolates from urine

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Table 28: Patterns of the integrase (*intI*1) gene and ESBL genes patterns among the 37 ESBL producing *E. coli* strains

Patterns	*IntI* 1	ESBL genees*				Number of strains	Strains Code Number
		bla_{TEM}	bla_{SHV}	bla_{VEB}	bla_{CTX-M}		
1	+	+	+	-	+	2	EB27,EU42
2	+	+	+	-	-	1	EU10
3	+	+	-	+	-	1	EP9
4	+	+	-	-	+	23	EB : 3,12,23,25
							EP : 7,12,13,17,18
							EU : 8,16,19,20,21,22,26,33,34,35,38,48,51,56
5	+	+	-	-	-	2	EU9,EU41
6	+	-	-	+	+	1	EU1
7	+	-	-	+	-	1	EU5
8	+	-	-	-	+	3	EP2,EU29,EU59
9	+	-	-	-	-	2	EB6,EU12
10	-	-	-	-	-	1	EB9

*intI*1 = integrase gene of class 1 integron

*, The PCR product amplified using universal primers

EB = *E. coli* isolates from blood, EP = *E. coli* isolates from pus, EU = *E. coli* isolates from urine

+ = positive gene, - = negative gene

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Table 29: The correlation between antibiogram and the Integrase (*intI*1) gene and ESBL genes patterns among the 37 ESBL producing *E. coli* strains

Antibiograms	The *intI*1 and ESBL genes patterns									
(37 ESBL producing strains)	1	2	3	4	5	6	7	8	9	10
1				■						
2								■		
3				■						
4										■
5				■						
6					■					
7				■						
8				■						
9				■						
10			■							
11	■			■			■			
12				■						
13								■		
14	■									
15									■	
16				■						
17				■						
18				■						
19				■						
20		■		■		■			■	
21					■					

*intI*1 = integrase gene of class 1 integron

ESBL genes including bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{VEB} genes

Antibiograms of 37 ESBL producers were in Table 22

Table 30: Integrase (*intI*1) gene of 120 *E. coli* strains by PCR and southern blot hybridization

Specimens (No. of *E. coli* strains)	PCR No. of *E. coli* strains (%)	Southern blot hybridization No. of *E. coli* strains (%)
Blood	29* (99.7)**	29* (99.7)**
Pus	30 (100.0)	30 (100.0)
Urine	60 (100.0)	60 (100.0)
Total	119 (99.2)	119 (99.2)

No. of *E. coli* strains = Number of *E. coli* strains

*Number of integrase (*intI*1) gene positive in *E. coli* strains

**Percent of integrase (*intI*1) gene positive in *E. coli* strains

Table 31: Pulsotypes of ESBL producing *E. coli* strains from various clinical specimens

Pulsotypes	Strains Code Number	Number of strains
1	EB3	1
2	EB6	1
3	EB9	1
4	EB12	1
5	EB23, EB25, EU16	3
6	EB27	1
7	EP2	1
8	EP7	1
9	EP9	1
10	EP12	1
11	EP13	1
12	EP17	1
13	EU1	1
14	EU5, EU12	2
15	EU8	1
16	EU10	1
17	EU19	1
18	EU20	1
19	EU21	1
20	EU22	1
21	EU26	1
22	EU29	1
23	EU33	1
24	EU34	1
25	EU35	1

Table 31 (cont.)

Pulsotypes	Strains Code Number	Number of strains
26	EU38	1
27	EU41	1
28	EU42	1
29	EU48	1
30	EU51	1
31	EU56	1
32	EU59	1
untypeable	EP18, EU9	2

EB = *E. coli* isolates from blood, EP = *E. coli* isolates from pus, EU = *E. coli* isolates from urine

Table 32: The correlation between the pulsotypes and the antibiograms of 37 ESBL producing *E. coli* strains

Antibiograms (37 ESBL Producing strains)	Pulsotypes																																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	untypeable
1																																	
2																																	
3																																	
4																																	
5																																	
6																																	
7																																	
8																																	
9																																	
10																																	
11																																	
12																																	
13																																	
14																																	
15																																	
16																																	

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Table 32 (cont.)

Antibiograms (37 ESBL Producing strains)	Pulsotypes																															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	untypeable
17																																	
18																																	
19																																	
20																																	
21																																	

Antibiograms of 37 ESBL producers were in Table 22

Pulsotypes of 37 ESBL producers were in Table 31

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Table 33: The correlation between the pulsotypes and the genes patterns of 37 ESBL producing *E. coli* strains

Genes patterns	Pulsotypes																																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	untypeable
1																																	
2																																	
3																																	
4																																	
5																																	
6																																	
7																																	
8																																	
9																																	
10																																	

Gene patterns including integrase (*intI*1) gene and ESBL genes (*bla*$_{TEM}$, *bla*SHV, *bla*CTX-M, and *bla*VEB genes)

Pulsotypes of 37 ESBL producers were in Table 31

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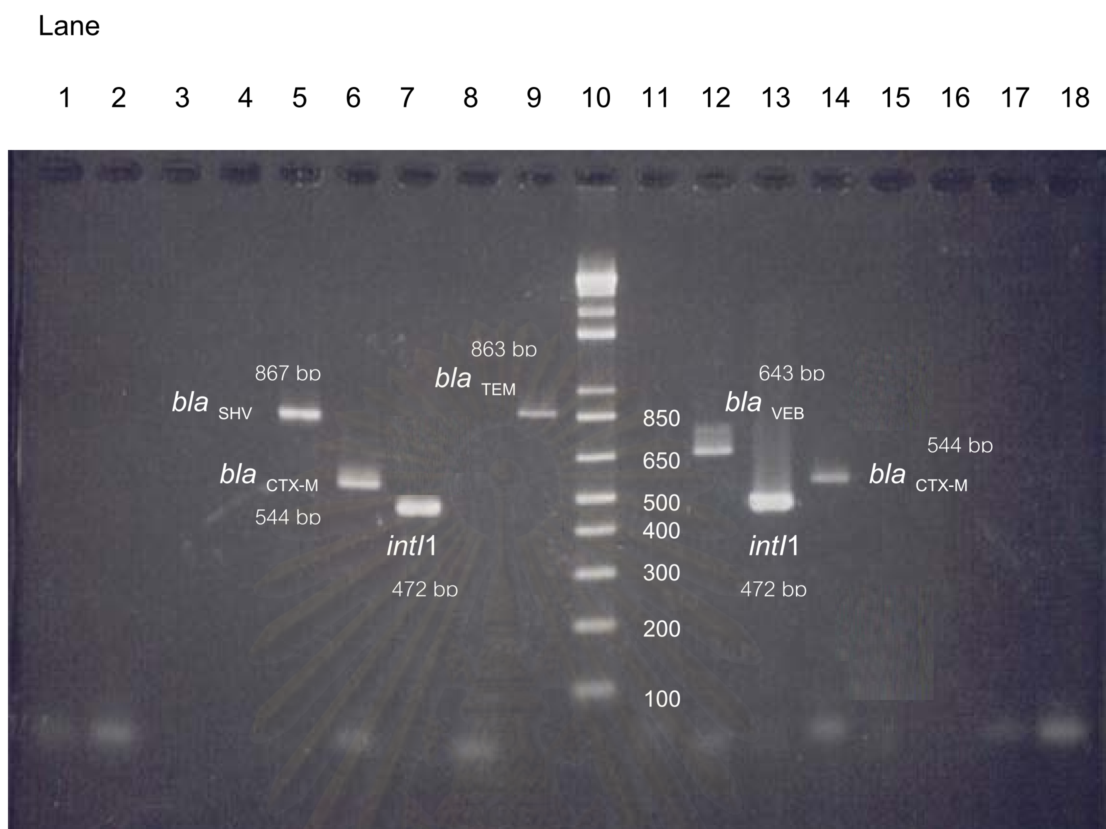

Figure 15: Agarose gel electrophoresis of specific 472, 863, 867, 544, 643-bp amplicon of integrase (*intI*1) gene, bla_{TEM}, bla_{SHV} , bla_{CTX-M} , and bla_{VEB}

Lane 5: 867-bp amplicon of bla_{SHV} (*E. coli* strain EU42)

Lane 6: 544-bp amplicon of bla_{CTX-M} (*E. coli* strain EU42)

Lane 7: 472-bp amplicon of *intI*1 (*E. coli* strain EU42)

Lane 9: 863-bp amplicon of bla_{TEM} (*E. coli* strain EU42)

Lane 10: 100bp DNA ladder

Lane 12: 643-bp amplicon of bla_{VEB} (*E. coli* strain EU1)

Lane 13: 472-bp amplicon of *intI*1 (*E. coli* strain EU1)

Lane 14: 544-bp amplicon of bla_{CTX-M} (*E. coli* strain EU1)

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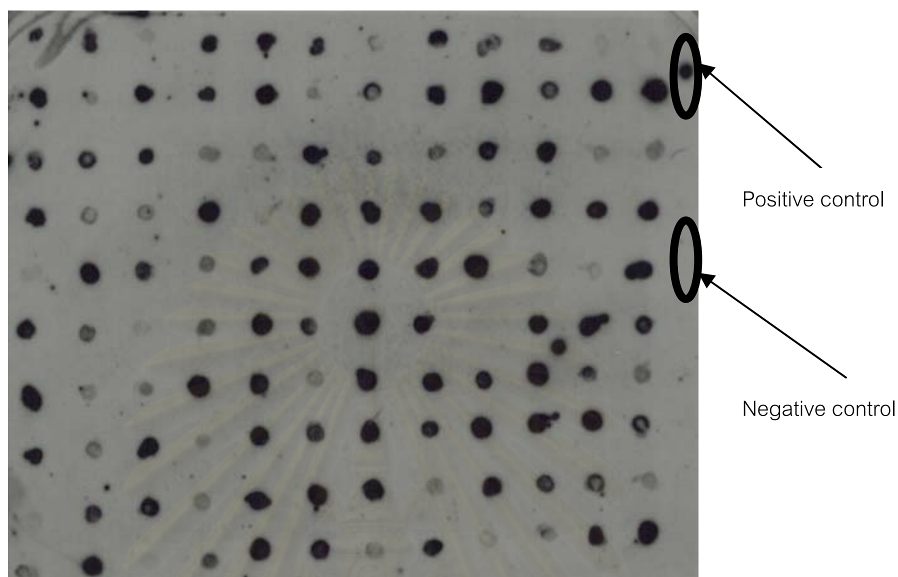

Figure 16: Dot blot hybridization. Genomic DNA of *E. coli* were dot-blotted on positive charged nylon membrane, cell were lysed and subjected to DNA hybridization using integrase gene (*intI* 1) as a probe, which should indicate the presence of an integron elements

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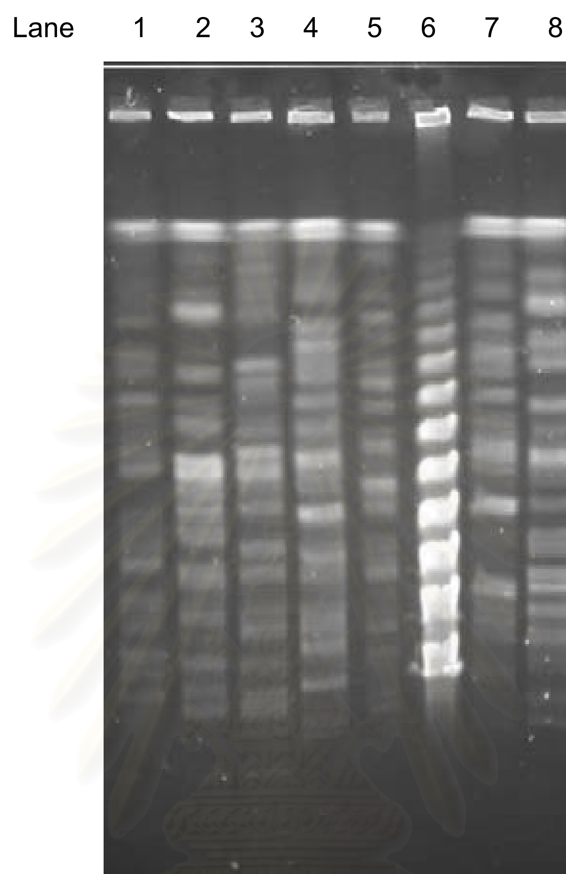

Figure 17: Comparison of PFGE of chromosomal DNA from ESBL producing *E. coli* strains digested with *Xba*I. The pulse time was 5 to 50s at 6 v/cm and run time 22 h. Lane 6 showed the lambda ladder (molecular marker). Lane 1-5 and 7-8 showed the PFGE patterns of isolates from urine; Lane 1: pulsotype 16, Lane 2: pulsotype 17, Lane 3: pulsotype 18, Lane 4: pulsotype 19, Lane 5: pulsotype 20, Lane 7: pulsotype 21, and Lane 8:pulsotyp22

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Lane 1 2 3 4 5 6

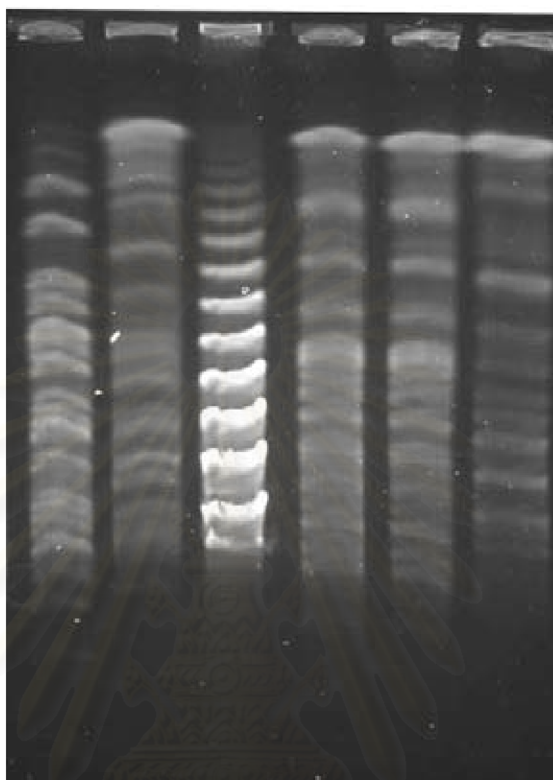

Figure 18: Comparison of PFGE of chromosomal DNA from ESBL producing *E. coli* strains digested with *Xba*I. The pulse time was 5 to 50s at 6 v/cm and run time 22 h. Lane 3 showed the lambda ladder (molecular marker). Lane 1 showed the PFGE patterns of isolates from blood, Lane 2 showed the PFGE patterns of isolates from pus, and Lane 4-6 showed the PFGE patterns of isolates from urine. Lane 1: pulsotype 2, Lane 2: pulsotype 11, Lane 4 and 5: pulsotype 14, Lane 6: pulsotype 1

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CHAPTER VI

DISCUSSION

Beta-lactam antibiotics represent the most common treatment for bacterial infections, whereas beta-lactamases are the leading cause of resistance to beta-lactam antibiotics among gram-negative bacteria worldwide. The persistent exposure of bacterial strains to a multitude of beta-lactams has induced dynamic and continuous production and mutation of beta-lactamases in these bacteria, expanding their activity even against the newly developed beta-lactam antibiotics. These enzymes are known as extended-spectrum beta-lactamases (ESBLs). Infections caused by ESBL producing bacteria are now a problem in many hospitalized patients. ESBL is an enzyme, which hydrolyzes cephalosporins, penicillins and aztreonam, so patients with infections from an ESBL producing organism are at an increased risk of treatment failure.

The prevalence of ESBL production among strains of *Enterobacteriaceae* were most commonly in *K. pneumoniae*, followed by *E. coli*, from country to country. In Asia, the percentage of ESBL-producing *E. coli* strains observed during the years 2002 to 2003 were 9.3% in Korea to 10.3% in Saudi Arabia and up to 18.5% in India (Ryoo et al., 2005; Kader and Kumer, 2005; Supriga et al., 2004). In Thailand, 15% of ESBL producing *E. coli* strains were observed from the multicenter study during the year 2000 to 2003 (Dejsirilert et al., 2004). In the present study, it was shown that 30.8% of the 120 clinical isolates from Siriraj hospital were ESBL producers. The much higher recovery rate than that from the previous data might be due to the fact that all the strains tested were from the large university hospital with excess antibiotic use.

The clinical problem from *E. coli* infection is the failure to response the cephalosporins group particularly the third generation cephalosporins due to the production of the extended spectrum beta-lactamases. The most acceptable methods worldwide are the methods described by the NCCLS including the initial screen test for the detection of the suspicious ESBL producing organisms and the phenotypic confirmatory test by the combination disc method for the confirmed detection of ESBL

producing organisms. According to the NCCLS, the suspicious ESBL producing organisms could be detected when the inhibition zone sizes by either one of the five drugs; cefpodoxime or ceftazidime or ceftriaxone or cefotaxime or aztreonam was in the recommended range. This study showed the interesting result that among all 120 *E. coli* strains that only 46 strains were showed to be the suspicious ESBL producing *E. coli*. After the phenotypic confirmatory test was performed, 80.4% (37 strains) of 46 suspicious ESBL were proved to be ESBL-positive strains by the confirmation test. It has been indicated in this study that the most reliable initial screen test should be done by using the criteria with all five antimicrobial agents. Cefpodoxime seemed to be the most appropriate drug for the initial screen test since all the 37 ESBL producing *E. coli* strains were resistant to it in the phenotypic confirmatory test. In contrary, cefotaxime was the best agent to detect ESBL producing strains because 36 of 37 strains were positive for phenotypic confirmatory test by cefotaxime alone and the combination disc of cefotaxime and clavulanic acid. Consequently, both cefpodoxime and cefotaxime showed high sensitivity of ESBL producer detection in this evaluation study.

Because of the problem in the antimicrobial therapy in the infections caused by ESBL producing *E.* coli, the informations from the ESBL producing test and antimicrobial susceptibility of the organisms are necessary. The inhibition zone was determined by the Kirby-Bauer susceptibility test with 13 antimicrobial agents. It was shown that none of ESBL producers (37 strains) was susceptible to cefpodoxime, while the organisms were poorly susceptible to ampicillin (3%), amoxicillin/clavulanic acid (11%), cefazolin (3%), gentamicin (14%), trimethoprim/sulfamethoxazole (27%), norfloxacin (32%), and aztreonam (19%). Among the third generation cephalosporins, the present study showed that these strains were more susceptible to ceftazidime (46%) than to cefotaxime (8%) and ceftriaxone (5%). It was shown that the organisms were moderately susceptible to cefoxitin (67%), a cephamycin antibiotic, since the enzymes of this class did not inactivate cephamycin (Jacoby et al., 1988).

Many reports reviewed that ESBL producing bacteria may be multidrug resistant, which includes resistance to non beta-lactam antibiotics such as aminoglycosides and trimethoprim/sulfamethoxazole (Ingviya et al., 2003; Nathisuwan et

al., 2001). This study showed that only 14% and 27% the ESBL producing *E. coli* were susceptible to gentamicin and trimethoprim/sulfamethoxazole, respectively. Babypadmini and Appalaraju reported that 26% and 25% of ESBL producing *E. coli* were susceptible to gentamicin and trimethoprim/sulfamethoxazole, respectively (Babypadmini and Appalaraju, 2004). Even though as high as 97% of ESBL producers were still susceptible to imipenem (carbapenem), but this result indicated the reduction in the susceptibility of *E. coli* isolates as compared to the 100% susceptible of the organism to imipenem for the other studies in Thailand (Ingviya et al., 2003; Dansuputra et al., 2002). Therefore, the rationale use of carbapenem against the ESBL producing *E. coli* should be applied.

This study focused on the distribution of bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{VEB} in all ESBL producing *E. coli* as well as the presence of integrase (*intI*1) gene, a marker of multiple antibiotic resistance class1 integron elements, since they are distributed worldwide and limited data concerning these enzymes has been published in Thailand. However, many strains that were ESBL producing strains produced either CTX-M or VEB ESBLs instead of TEM nor SHV ESBLs.

TEM ESBLs, reported in *Enterobacteriaceae* are most often found in *E. coli* and *K. pneumoniae* (Bradford, 2001). Cao et al. reported that 84.4% of ESBL producing *E. coli* carried bla_{TEM} (Cao et al., 2002). For the present study, most of ESBL producing *E. coli* carried bla_{TEM} (78.4%), which was similar to many other studies. However, the bla_{TEM} universal primers were used in this study so the strains which were bla_{TEM} positive could be either TEM-ESBLs, inhibitor-resistant TEM beta-lactamases (IRT), or broad spectrum beta-lactamases (TEM-1, TEM-2).

SHV ESBLs are often found in clinical isolates of *K. pneumoniae* (Nüesch-Inderbinen et al., 1996; Bush et al., 1995). However, these enzymes have also been found in *E. coli*, *P. aeruginosa* and *Citrobacter diversus* (Naas et al., 1999; Rasheed et al., 1997). For this study, we found only 8.1% of ESBL producing *E. coli* carried bla_{SHV}.

CTX-M ESBLs, like TEM and SHV ESBLs, emerged in the late 1980s, a few years after the introduction of cefotaxime as a treatment for microbial infections (Bonnet,

2004). Although the worldwide expansion of CTX-M producing strains has not been observed until 1995, it is now a major concern in certain areas, such as South America, the Far East, and Eastern Europe (Baraniak et al., 2002). CTX-M ESBLs are capable of hydrolyzing broad-spectrum cephalosporin. They confer a high level of resistance to cefotaxime but have a low level of activity against ceftazidime (Tzouvelekis et al., 2000). They have been identified in various clinical strains, but mostly in *E. coli*, *K. pneumoniae*, and *Salmonella enterica* serovar Typhimurium (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 isolates (Baraniak et al., 2002; Sabate et al., 2000). For this study, it was shown that 78.4% of ESBL producers carried $bla_{\text{CTX-M}}$, and these strains were more resistant to cefotaxime than to ceftazidime.

VEB-1 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. 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 (Girlich et al., 2001). In this study, only 8.1% of ESBL producers carried bla_{VEB}.

Similar study was performed by Dansuputra et al. who repoted the detection of bla_{TEM} (72.2%), $bla_{\text{CTX-M}}$ (52.8%) and bla_{VEB} genes (16.7%) in ESBL producing *E. coli* isolated from various clinical specimens in King Chulalongkorn Memorial Hospital. However, these investigators could not detected bla_{SHV} in any *E. coli* (Dansuputra *et al.*, 2002). In this study, it was found that ESBL producing *E. coli* isolated from Siriraj Hospital carried bla_{TEM} (78.4%), bla_{SHV} (8.1%), $bla_{\text{CTX-M}}$ (78.4%) and bla_{VEB} genes (8.1%).

However, all four ESBL genes (bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, and bla_{VEB} genes) were not detected in 8% of ESBL producers. It is possible that these strains produced the other types of ESBLs including SFO-1, TOHO-1, -2, SME-1, NMC-A, IMI-1, PER-1, -2, and GES-1 (Brown et al., 2000). Besides, the PCR results indicated that 9 strains, which were negative ESBL by the confirmatory test gave the positive result for bla_{TEM}. The nine strains could be interpreted as the restricted-spectrum class A beta-lactamase producer, e.g. $bla_{\text{TEM-1}}$ hyperproducer.

Class1 Integron Elements play an important role in antibiotic resistance of clinical *E. coli* due to their abilities to capture, integrate, and express gene cassettes encoding antibiotic resistance. The prevalence of class1 integrons in *E. coli* are clearly increasing, and common worldwide. In Spain, Elisabete et al. reported that 67.0% of strains carried integrase gene (*intI*1) (Machado et al., 2005). In France, Skurnik et al. reported that 11.0% of strains carried an *intI*1 (Skurnik et al., 2003). In Korea, Kang et al. reported that 30.8% of strains carried an *intI*1 (Kang et al., 2004). The gene cassettes of frequently found integron were encoding the resistance to trimethoprim (*dfr*), sulfonamide (*sul*), and aminoglycoside (*aadA*) (Kang et al., 2005; Machado et al., 2005; Yu et al., 2003). There have been a few reports on the prevalence of class1 integron elements in clinical isolates from *E. coli* in Thailand. The present study showed that 99.7% of *E. coli* (119 of 120) carried an *intI*1. Among these, 68.9% (82 of 119) were resistant to trimethoprim/sulfamethoxazole and 44.5% (53 of 119) were resistant to gentamicin (aminoglycoside). Surprisingly, high incidence of *intI*1 among *E. coli* isolated from clinical specimens would indicate a spread of eminent resistant bacteria in the hospital. Girlich et al. reported that class 1 integron from *E. coli* carried multiple resistance genes including bla_{VEB-1}, bla_{OXA-10} and rifampicin (*arr*-2) resistant gene (Girlich et al., 2001). Besides, gene cassettes encoded the trimethoprim (*dfr*) resistant gene to co-occur with aminoglycoside (*aad*) resistant gene, quaternary ammonium compounds (*qacEΔ1*), sulphonamides (*sul1*), and $bla_{CTX-M-9}$ in *E. coli* (Sabate et al., 2002). In the present study, the most predominate co-resistant gene from 23 ESBL producers were bla_{TEM} and bla_{CTX-M} which have never been reported in gene cassettes of integron. Moreover, the transfer of the entire integron, via a plasmid or transposon, is more frequent than single gene mobilization or integration within the integron (Martinez-Freijo et al., 1999). Interestingly, the future study could be emphasized on the research into characterization of drug resistant gene cassettes associated with class 1 integron in these clinical strains.

The molecular typing of all 37 ESBL producing *E. coli* was also performed using Pulsed-Field Gel Electrophoresis (PFGE) technique. The purpose to do this part of the study was to determine the variation of pulsotypes among ESBL producing *E. coli*

strains isolated from various specimens from different patient wards at Siriraj Hospital during the 3 months of the study period.

It was found that most of the strains showed unrelated patterns, because there were as many as 32 pulsotypes of ESBL producing *E. coli* among all 37 strains. It seemed that there was neither particular endemic nor epidemic strain among the ESBL producing *E. coli* isolated from different patient units. Many reports reviewed that PFGE analysis of ESBL producing *E. coli* strains showed extensive diversity in clonality. Diversity of clones have been reported in Taiwan, Korea, Netherland, U.S.A, and also France (Kim et al., 2002; Morris et al., 2003; Saurina et al., 2000; Siu et al.,1999; Vercauteren et al., 1997). PFGE data from the present study as well as and many other studies demonstrated that most of the strains had the spread of antibiotic resistance via horizontal gene transfer. This process thought to be a significant cause of increase drug resistance because the bacteria can quickly transfer the resistance gene to many genus and species. However, a few PFGE data showed that ESBL producing *E. coli* isolated from each another patient was same pulsotype due to vertical gene transfer occur when an organism receives genetic material from its ancestor.

In conclusion, the rapid increase in antibiotic resistance of pathogenic bacteria has been considered to be one of the major problems in human medicine today. The study of ESBLs genes and integrons could provide information about which antibiotics should be more carefully used to prevent further accumulation of resistance. The clinical laboratory detection of ESBL producing bacteria is simple to performed, easy to interprete and economic. However, the molecular methods for study the antibiotic resistant genes as well as integrase gene showed that the transmission of antibiotic resistant genes was via the horizontal transfer. This could lead to rapid emergence of antibiotic resistance among clinical strains of bacteria. Essential infection control practices should include hand washing by hospital personnel, increased barrier precautions, isolation of patients infected by ESBL producers, especially concerning the empirical use of broad spectrum antimicrobial agents such as third and fourth generation cephalosporins and imipenem.

The present study provides the new information to the successful utilization of antimicrobials in patient treatment, as well as the attempt to solve the problems of antimicrobial agent resistant bacteria transmission.

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CHAPTER VII

CONCLUSION

The study indicated the epidemiology of extened-spectrum beta-lactamases (ESBLs) producing *E. coli* strains and prevalence of an integrase (*intI*1) gene, a marker of multiple antibiotic resistance class 1 integron elements among *E. coli* isolated from clinical specimens in Thailand.

One hundred and twenty *E. coli* strains, which were isolated from clinical specimens of the patients at Siriraj hospital during June to August 2004, were initially screened for ESBL producer by mean of disc diffusion test then confirmed by combination disc test a as recommended to NCCLS. Overall, 30.8% (37/120) of the strains produced ESBLs.

The antimicrobial susceptibility test of 37 *E. coli* ESBL producing strains showed that 18.9% (7 of 37) susceptible to aztreonam and a few of strains susceptible to third cephalosporin. All strains were resistant to cefpodoxime, but 5.4% (2 of 37) were susceptible to ceftriaxone, and 8.1% (3 of 37) were susceptible to cefotaxime. As high as 46.0% (17 of 37) were still susceptible to ceftazidime. The susceptibility test with the other antimicrobial agents showed that 2.7%, 10.8%, 2.7%, 67.6%, 97.3%, 13.5%, 32.4%, and 27.0% of ESBL producers were susceptible to amplicillin, amoxicillin/clavulanic acid, cefazolin, cefoxitin, imipenem, gentamicin, norfloxacin, and trimethoprim/sulfamethoxazole, respectively.

Thirty-seven ESBL producing *E. coli* strains were investigated for the ESBL-type by PCR method using bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{VEB} primers. TEM positive were found in 78.4% of 37 strains, while 8.1% were SHV positive, 78.4% were CTX-M positive, and 8.1% were VEB positive. Most of the strains carried at least two different *bla* genes, including 62.2% coharboured bla_{TEM} and $bla_{CTX\text{-}M}$, which were the most common coharbouring genes detected in ESBL producer. There was high incidence of integrase (*intI*1) gene positive results among ESBL producing strains and *E. coli* isolated from clinical specimens.

PFGE data can imply that both vertical and horizontal gene transfer were the mode of antibiotic resistance transmission, although it seemed that the latter were more predominate.

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APPENDICES

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX A

Media, Chemical agents, Materials, Instruments and Identification procedures

Media

1. Eosin methylene blue agar (Difco, USA)

Peptone	10.0	g
Lactose	5.0	g
Sucrose	5.0	g
Dipotassium Phosphate	2.0	g
Agar	13.5	g
Eosin Y	0.4	g
Methylene Blue	0.065	g

pH 7.2 ± 0.2 at 25°C

2. Mac Conkey agar (BBL, USA)

Pancreatic digest of gelatin	17.0	g
Pancreatic digest of casein	1.5	g
Peptic digest of animal tissue	1.5	g
Lactose	10.0	g
Bile salts	1.5	g
Sodium chloride	5.0	g
Neutral red	0.03	g
Crystal violet	0.001	g
Agar	13.5	g

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3. Mueller Hinton agar medium (Becton Dickinson, USA)

Beef , Infusion form	300.0	g
Bacto casamino acids , Technical	17.5	g
Starch	1.5	g
Bacto agar	17.0	g
Distilled water	1000.0	ml

pH: 7.3 ± 0.1 at 25°C

4. Tryptic soy agar (Mearck, Germany)

Peptone from casein	15.0	g
Peptone from soymeal	5.0	g
Sodium chloride	5.0	g
Agar	15.0	g
Distilled water	1000.0	ml

pH: 7.3 ± 0.2 at 25°C

5. Tryptic soy broth (Mearck, Germany)

Peptone from casein	17.0	g
Peptone from soymeal	3.0	g
D(+)Glucose	2.5	g
Sodium chloride	5.0	g
di-Potassium Hydrogen Phosphate	15.0	g
Distilled water	1000.0	ml

pH: 7.3 ± 0.2 at 25°C

Media preparation :

All of ingradients were dissolved in distilled water, heat to boiling and then steriled by autoclaving at 120°C, 15 pounds/inch^2 pressure, for 15 minutes. The sterile medium was cooled to 45°C to 50°C, and dispensed into sterile plates. For sterile Tryptic soy broth, 5 ml were dispensed into each tube before autoclaving.

Chemical agents

Low melting point agarose (Promega, USA)

Ultrapure agarose (Gibco BRL, Spain)

Brij-58 (Sigma, USA)

Sodium deoxycholate (Sigma, USA)

Sodium lauroyl sarcosine (Sigma, USA)

Proteinase K (Ameresco, USA)

Tris (hydroxymethyl) (Ameresco, USA)

Sodium chloride (Merck, USA)

EDTA (Ameresco, USA)

Boric acid (Bio-Rad, USA)

Materials

15-ml snap-top tubes (Fisher, USA)

5.-ml snap-top tubes (Fisher, USA)

15-ml round bottom tube, screw cap (Pyrex, USA)

Insert mold (Bio-Rad, USA)

Instruments

Incubator 37°C (Memmert, Germany)

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Shaking waterbath (United Instrument, USA)

Turbidity meter

Vortex Mixer (Scientifix, USA)

Digital sliding vernier caliper

Roller (Life Scuience, USA)

Refrigerator (-20°C) (Listed Household Freezer, USA)

Refrigerator centrifuge (4°C) (Sigma, USA)

Automatic pipette, p20/p200/p1000 (Gilson Medical, France)

pH meter (Beckman, USA)

Millipore filter

Pulsed-Field Gel Box (Bio-Rad, USA)

Pump, Gel Molds (Bio-Rad, USA)

Cooling system (Bio-Rad, USA)

Power supply, Pulse wave switcher (Bio-Rad, USA)

Thermo Hybaid PCR (PCR Sprint, USA)

Nylon transfer membrane (Schleicher and Schuell, USA)

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Enzyme and Molecular Marker

Deoxynucleotide triphosphates (Finnzymes, Finland)

Taq DNA polymerase (Finnzymes, Finland)

1 Kb Plus DNA ladder (Invitrogen, USA)

RNase (Amresco, USA)

Lysozyme (Amresco, USA)

Proteinase K (Amresco, USA)

XbaI (Invitrogen, USA)

λ ladder marker (Bio-Rad, USA)

Identification procedures

Gram staining procedure

Gram crystal violet solution

Gram iodine solution

Gram safranin solution

95% ethanol

Staining procedure: The organisms were smeared on a clean slide and allowed to dry. The slide was heated with a flame to fix smear. Gram crystal violet was dropped on the smear. After minute, the slide was then washed with water and drained. Next, gram iodine solution was dropped on the smear, and washed with the water after 1 minute. The smear was decolorized with 95% ethanol and then washed with water. Gram safranin solution was next dropped on the smear in order to use as counterstanin for 30 seconds. The smear was allowed to dry and then examined by microscope under 100x objective lens over the entire smear.

Biochemical tests

1. Citrate agar, Simmons

Purpose: Simmons citrate agar is used to distinguish gram-negative bacteria based on their ability to utilize as a sole source of carbon.

Principle and interpretation: Several theories have been proposed to explain the mechanism of citrate agar. Only one is presented here. Organisms that metabolize citrate as a sole source of carbon cleave citrate to oxaloacetate and acetate via the citritase enzyme. Another enzyme, oxaloacetate decarboxylase, then converts oxaloacetate to pyruvate and CO_2. CO_2 combines with sodium and water to form Na_2CO_3, an alkaline compound. As a result, the pH of the medium rises and the indicator (bromthymol blue) changes from green to Prussian blue. Presence of the blue color constitutes a positive finding for citrate utilization.

Ingredients and Preparation: Mix the following ingredients, heat to boiling, dispense into test tubes, and sterilize at 121°C for 15 minutes. Cool each tube of medium in a slanted position.

Sodium citrate	2	g
NaCl	5	g
$MgSO_4$	0.2	g
Ammonium dihydrogen phosphate	1	g
Dipotassium phosphate	1	g
Bromthymol blue	80	g
Agar	15	g
Distilled water	1	L

Final pH 6.9

Procedure: Lightly inoculate the test organism to the surface of citrate medium, incubate at 35°C for 24 to 48 hours, and observe for a Prussian blue color change.

2. Indole test

Purpose: Indole broth is used for distinguishing between bacteria based on ability to produce indole form tryptophan.

Principle and interpretation: Indole broth contains tryptophan-rich peptone and NaCl. The tryptophan present in peptone is oxidized by certain bacteria to indole, skatole, and indoleacetic acid. The intracellular enzymes that are responsible for metabolizing tryptophan to these compounds are collectively termed tryptophanase. Indole is detected in broth cultures of bacteria with an alcoholic *p*-dimethylaminobenzaldehyde reagent. Indole reacts with the aldehyde to give a red product in the alcoholic layer of the broth-reagent mixture.

Two reagents were used to detect indole: Kovac's and Ehrlich. Ehrlich reagent is believed to be more sensitive than Kovac's and is recommended for detection of indole production by anaerobic bacteria and nonfermentative gram negative organisms. Kovac's reagent was used initially to classify members of the family *Enterobacteriaceae* and should be used with these organisms.

Ingredients and preparation: Mix the ingredients, heat to boiling, dispense into tubes, and sterilize at 121°C for 15 minutes.

Indole broth:

Pancreatic digest of casein, USP	20	g
NaCl	5	g
Distilled water	1	L

Final pH 7.2

Reagents:

Kovac's indole reagent. Dissolve the aldehyde in the alcohol and slowly add acid to the mixture.

Alcohol, amyl or isoamyl	150	ml
p- dimethylaminobenzaldehyde	10	g
Hydrocholic acid, concentrated	50	ml

Procedure: Inoculate the test organism into indole broth, incubate at 35°C for 18 to 24 hours, and test as follows.

Indole test: Add five drops of Kovac's reagent directly to the broth culture, shake gently, and observe for development of a red color in the upper alcohol layer.

3. Malonate broth

Purpose: Malonate broth is used for differentiation of members of the family *Enterobacteriaceae*, especially *Salmonella* spp.

Principle and interpretation: Malonate brothtests for utilization of sodium malonate as a sole source of carbon. The medium contains buffer, pH indicator, sodium malonate, required salts, and a small amount of yeast extract and glusoce. The pH indicator, bromthymol blue, is a deep Prussian blue at its alkaline end point (pH 7.6), yellow at its acidic end point (pH 6.0), and green when uninoculated (pH 6.7). Bacteria that are capable of using malonate as a source of energy and carbon produce alkaline by products that change the color of the medium to blue. Bacteria that are

unable to use malonate as a carbon source usually do not grow and the pH of the medium does not change; the indicator remains green. Some malonate-negative strains may produce a yellow color owing to fermentation of glucose.

Ingredients and preparation: Mix the ingredients, heat to boiling, dispense into tubes, and sterilize at 121°C for 15 minutes.

Yeast extract	1	g
Ammonium sulfate	2	g
Dipotassium phosphate	0.6	g
Monopotassium phosphate	0.4	g
NaCl	2	g
Sodium malonate	3	g
D-Glucose	0.25	g
Bromthymol blue	0.025	g
Distilled water	1	L

Final pH 6.7

Procedure: Inoculate the test organism into malonate broth and incubate at 35°C for 18 to 24 hours.

4. Methyl red-Voges-Proskauer broth

Purpose: Methyl red-Voges-Proskauer (MR-VP) broth is useful for distinguishing between members of the family *Enterobacteriaceae* based on their ability to produce acetylmethylcarbinol (acetoin) and strong acids from fermentation of glucose. The broth, which contains protein, glucose, and phosphate buffer, is used for the MR test and the VP test.

Principle and interpretation: Members of the family *Enterobacteriaceae* may be divided metabolically into two groups: the mixed acid producers and the butylene glycol producers. The mixed acid producers such as *E. coli* produce large amounts of organic acids including lactic, acetic, formic, and succinic. Butylene glycol producers such as *Klebsiella* and *Enterobacter* spp.

produce smaller amounts of organic acids and large amounts of neutral products, especially 2,3-butanediol.

The MR test is used to distinguish the mixed acid producers. In this test a methyl red indicator is added to the MR-VP test broth after incubation. At a pH of 4.4 the indicator remains red, and at the pH of 6.0 it became yellow. The MR-positive organisms are those that produce large amounts of acid and a red color, whereas the MR-negative organisms produce a yellow color.

The VP test detects the presence of acetoin, or acetylmethylcarbinol, an intermediate in the production of butylenes glycol. In this test two reagents, α-naphthol and 40% KOH, are added to the test broth after appropriate incubation. The broth-reagent mixture is then mixed thoroughly to expose the medium atmospheric oxygen. If acetoin is present, it is oxidized in the presence of air and KOH to diacetyl. Diacetyl then reacts with the guanidine components of peptone, in the presence of α-naphthol, to form a red color (α-naphthol serves as a catalyst and acts as a color intensifier). Development of a red color is a positive VP test result.

Ingredients and preparation: Mix the ingredients, heat to boiling, dispense into tubes (1 ml per tube), and sterilize at 121°C for 15 minutes.

Pancreatic digest of casein and peptic Digest of animal tissue, USP	7	g
D-Glucose	5	g
Dipotassium phosphate	5	g
Distilled water	1	L

Final pH 6.9

Reagents:

Methyl red reagent: Dissolve the methyl red in alcohol and add the distilled water. Store at room or refrigerator temperature.

Methyl red	50	mg
Ethyl alcohol, 95%	150	ml
Distilled water	100	ml

Voges-Proskauer reagents:

VP-1:

α-naphthol	5	g
Ethyl alcohol, absolute	100	ml

VP-2:

Potassium hydroxide	40	g
Distilled water, q.s. to	100	ml

Procedure: Inoculate the test organism to two tubes of MR-VP broth, each containing 1 ml, and incubate for 1 to 3 days at 35°C.

Methyl red test: Add five drops of methyl red reagent to one broth culture and observe for development of a red color. This is a positive MR test, which is indicative of mixed acid fermentation.

Voges-Proskauer test: Add 0.6 ml of VP-1 reagent to another broth culture, shake the tube, and add 0.2 ml of VP-2 reagent. The reagents must be added in the preceding sequence. Shake the tube gently. Allow the tube to stand for at least 15 minutes and observe for formation of a red color. This is a positive VP test and indicates butylene glycol fermentation. Hold tubes in which results are negative for an additional 45 minutes, since maximum color development occurs within 1 hour after the reagent is added. Ignore a copper color of the medium, which occurs after 1 hour's incubation. This color is due to reaction between α-naphthol and KOH.

5. Triple sugar iron agar

Purpose: Triple sugar iron (TSI) agar is a screening medium used to identify gram-negative bacilli based on ability to ferment the carbohydrates glucose, sucrose, and lactose to produce H_2S gas.

Principle and interpretation: TSI agar contain protein, NaCl, lactose, sucrose, dextrose, a sulfur source, an H_2S indicator, a pH indicator, and agar. The medium includes ten times as much lactose and sucrose as glucose. Bacteria that ferment glucose produce a variety of acids, turning the color of the medium from red to yellow. Large amounts of acid are produced in the butt of the tube (fermentation) than in the slant of the tube (respiration). Organisms growing on TSI also from alkaline products from the oxidative decarboxylation of peptone. These alkaline products neutralize

the small amounts of acids present in the slant but are unable to neutralize the large amounts of acid present in the butt. Thus, the appearance of an alkaline (red) slant and an acid (yellow) butt after 24 hours incubation indicates that the organism is a glucose fermenter but is unable to ferment lactose and sucrose.

Bacteria that ferment lactose or sucrose (or broth), in addition to glucose, reduce such large amounts of acid that the oxidative deamination of protein that may occur in the slant does not yield enough alkaline products to cause a reversion of pH in that region. Thus, these bacteria produce an acid slant and acid butt. It is impossible to determine from the TSI reaction whether both lactose and sucrose are being fermented or only one of these carbohydrates is being fermented; individual carbohydrate fermentation tests are required to make this assessment.

Gas production (CO_2 and hydrogen) is detected by the presence of cracks or bubbles in the medium. These are formed when the accumulated gas escapes.

H_2S gas is produced as a results of the reduction of thiosulfate. H_2S is a colorless gas and can be detected only in the presence of an indicator, in this case ferric ammonium sulfate. H_2S combines with the ferric ions of ferric ammonium sulfate to produce the insoluble black precipitate ferrous sulfide. Reduction of thiosulfate proceeds only in an acid environment, and blackening usually occurs in the butt of the tube. Although the black precipitate may frequently obscure the color of the butt, it can be assumed that the organism is a glucose fermenter because of the requirement for an acid environment. The reactions can be summarized as follow:

Alkaline slant/acid butt: glucose only fermented

Acid slant/acid butt: glucose and sucrose fermented or glucose and lactose fermented or glucose, lactose, and sucrose fermented

Bubbles or cracks present: gas produced

Black precipitate present: H_2S gas produced

Ingredients and preparation: Mix the ingredients, heat to boiling, dispense into tubes, and sterilize at 121°C for 15 minutes, and allow tubes of medium to cool in a slanted position.

Pancreatic digest of casein, USP	10	g
Peptic digest of animal tissue, USP	10	g
NaCl	1	g

Lactose	10	g
Sucrose	10	g
D-Glucose	1	g
Ferric ammonium sulfate	0.2	g
Sodium thiosulfate	0.2	g
Phenol red	25	mg
Agar	13	g
Distilled water	1	L

Final pH 7.3-7.4

Procedure: Inoculate test cultures to TSI agar by first touching a sterile bacteriologic needle to a colony and then stabbing the needle into the deep agar region of the medium. Hence withdrawing the needle, move it from side to side over the surface of the medium. Incubate cultures at 35°C for 18 to 24 hours. Examine cultures for color of the slant, butt, gas cracks, and blackening caused by H_2S.

6. Urea agar

Purpose: Urea agar are used for distinguish between species of aerobic bacteria based on ability to hydrolyze urea.

Principle and interpretation: A variety of media are used to test for ability to hydrolyze urea. The hydrolysis of urea by urease to ammonia is accompanied by a rise in pH of the medium and a concomitant change in the color of the indicator from yellow to red.

Ingredients and preparation: Mix urea basal ingredients, sterilize by filtration, and add sterile agar solution (50°C). Mix and dispense into tubes, and allow tubes of medium to cool in a slanted position.

Urea base:

Pancreatic digest of gelatin, USP	1	g
NaCl	5	g

Monopotassium phosphate	2	g
D-Glucose	1	g
Urea	20	g
Phenol red	12	mg
Distilled water	100	ml

Final pH 6.8

Agar solution:

Agar	15	g
Distilled water	900	ml

Urea agar:

Urea base	100	ml
Agar solution	900	ml

Procedure: Inoculate the organism to the urea agar, incubate for 24 to 48 hours at 35°C, and observe for a red color change in the medium.

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APPENDIX B

Reagents

1. Reagents for clinical laboratories

1.1 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 OD. Value is 0.08 to 0.10

1.2 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/inches^2 pressure, for 15 minutes. Store at room temperature.

2. Reagents for agarose gel electrophoresis

2.1 10X Tris-borate buffer (TBE)

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

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

2.2 10 mg/ml Ethidium bromide

Ethidium bromide	1	g
Distilled water	100	ml

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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

2.3 1% Agarose gel

Agarose ultrapure	0.4	g
1XTBE buffer	40	ml

3. Reagents for Hybridization

3.1 Hybridization buffer

For overnight hybridizations use the following:

5x SSC	12.5	ml
0.1% SDS	0.5	ml
5% (w/v)	2.5	g
1 in 20 dilution liquid block	2.5	ml
Distilled water	34.5	ml

3.2 TE buffer

10mM Tris-HCl

1mM EDTA

pH 8.0

3.3 SDS stock

10% or 20% (w/v) SDS

3.4 Denaturation solution

0.4 M NaOH

3.5 Neutralization solution

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1 M NaCl

0.5 M Tris-HCl

pH adjusted to pH 7.5

3.6 20xSSC

0.3 M Na_3 citrate

3 M NaCl

4. Reagents for Pulsed-field gel elctrophoresis

4.1 PIV buffer

10mM Tris (pH 7.6)	0.0726	g
1M NaCl	29.2200	g
Ultrapure water	500.0000	g

All ingradients were dissolved in 500 ml of ultrapure water. The buffer adjusted the pH to 7.6, and then sterile at 121°C, 15 pounds/inches^2 pressure. The PIV buffer was stored at 4°C

4.2 Lysis solution

20 µg of RNase per ml

1 mg of lysozyme per ml

Lysis buffer

The lysis solution was prepared by mixing the 80 µl of RNase stock solution, 800 µl of lysozyme stock solution, and 40 ml of lysis buffer together.

4.2.1 Lysis buffer

6mM Tris (pH7.6)	0.3630	g
1M NaCl	29.4200	g
100mM EDTA (pH 7.6)	18.6120	g

0.5% Brij-58	2.5000	g
0.2% Sodium deoxycholate	1.0000	g
0.5% Sodium lauroyl sarcosine	2.5000	g
Ultrapure water	500.0000	ml

All ingredients were dissolved in 500 ml of ultrapure water, and then adjusted the pH to 7.6. The buffer was steriled by Millipore filter, and then stored at 4°C

4.2.2 RNase stock solution

The 10 mg of RNase was dissolved in 1 ml of sterile water, and then heated in boiling water for 20 to 30 min to destroy DNase. The 100 µl of RNase was dispensed in each microcentrifuge tube, and freezed at -20°C. (These aliquots could be refrozen up to two times)

4.2.3 Lysozyme stock solution

The 0.5 g of lysozyme was dissolved in 10 ml of sterile water (final concentration, 50 mg/ml or 10 mg/ml). The 200 µl of lysozyme was dispensed in each microcentrifuge tube, and freezed at -20°C. (These aliquots could be refrozen once)

4.3 ESP solution

100 µg of Proteinase K per ml of ES buffer

ES buffer

Ten milliliters of 20x Proteinase K was added to 190 ml of ES buffer, and then mixed thoroughly. The solution was stored at 4°C.

4.3.1 ES buffer

0.5 M EDTA (pH 8.0)	93.06	g
10% Sodium lauryl sarcosine	50.00	g
Ultrapure water	500.00	ml

All ingradients were dissolved in 500 ml of ultrapure water, and then adjusted the pH to 8.0. The buffer was sterile by millipore filter and stored at 4°C.

4.3.2 **20x Proteinase K stock solution**

One hundred grams of proteinase K was dissolved in 50 ml of ES buffer and then incubated at 50°C for 1 hour. The solution was stored at 4°C

4.4 1xTE buffer

10 mM Tris (pH 7.6)	0.6057	g
0.1 M EDTA (pH 7.6)	0.0186	g
Ultrapure water	500.0000	ml

All ingradients were dissolved in 500 ml of ultrapure water, and then adjusted the pH to 7.6. The buffer was steriled at 121°C, 15 pounds/inches^2 pressure and stored at 4°C.

4.5 10x TBE buffer

0.1 M Tris (pH 8.5)	108.0000	g
0.1 M Boric acid	55.0000	g
4 mM EDTA	9.3000	g
Sterile ultrapure water	1000.0000	ml

All ingradients were dissolved in 500 ml of sterile ultrapure water and then adjusted the pH to 8.2-8.4, 500 ml of sterile ultrapure water was added and then mixed thoroughly. The solution was stored at 4°C.

4.6 Ethidium bromide solution

The 300 μl of eithidium bromide (1 μg/μl) was dissolved in 300 ml of ultrapure water.

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APPENDIX C

THE RESULTS OF ALL TESTS IN THIS STUDY

Table A-1: Inhibition zone sizes of the initial screen test of *E. coli* isolated from blood

Strain Code No.	Inhibition zone sizes (mm.)				
	CPD≤ 22	CAZ ≤ 22	CRO ≤ 25	CTX ≤ 27	ATM ≤ 27
EB001	29.52	30.16	33.48	33.37	35.34
EB002	29.74	27.13	34.40	31.06	36.06
EB003	NZ	23.69	12.02	13.26	24.56
EB004	28.19	29.07	31.85	32.94	34.36
EB005	27.44	26.92	30.99	32.27	32.65
EB006	NZ	19.99	14.59	23.55	24.39
EB007	33.79	30.08	31.61	32.18	33.79
EB008	37.51	27.08	34.84	31.07	37.51
EB009	NZ	27.75	13.50	14.85	28.64
EB010	27.64	32.03	32.24	34.06	36.29
EB011	29.49	31.65	33.21	35.93	36.40
EB012	NZ	26.26	NZ	10.92	NZ
EB013	26.33	29.25	29.74	30.87	32.25
EB014	28.87	31.40	32.53	35.31	33.66
EB015	34.33	28.56	32.40	33.00	34.33
EB016	34.64	27.58	32.30	32.28	34.64
EB017	27.82	29.20	30.31	34.17	32.54
EB018	27.97	30.18	32.98	34.99	35.63
EB019	33.82	30.19	27.02	35.07	33.82
EB020	34.49	31.03	27.62	29.84	34.49
EB021	32.87	27.45	26.35	32.94	32.87

Table A-1: (cont.)

Strain Code No.	Inhibition zone sizes (mm.)				
	CPD ≤22	CAZ ≤ 22	CRO ≤ 25	CTX ≤27	ATM ≤ 27
EB022	35.12	33.42	30.95	38.07	35.12
EB023	NZ	14.46	8.10	8.11	10.60
EB024	32.22	29.65	23.99	31.50	32.22
EB025	NZ	18.43	NZ	NZ	10.37
EB026	33.50	30.56	28.58	35.23	33.50
EB027	NZ	8.61	NZ	NZ	NZ
EB028	36.53	30.92	30.3	31.51	36.53
EB029	29.43	29.10	24.80	32.35	29.43
EB030	30.40	28.75	26.34	31.49	30.40

*CPD = cefpodoxime, CAZ = ceftazidime, CRO = ceftriaxone, CTX = cefotaxime, ATM =aztreonam

**NZ = no zone

EB = *E.coli* isolates from blood

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Table A- 2: Inhibition zone sizes of the initial screen test of *E. coli* isolated from pus

Strain Code No.	Inhibition zone sizes (mm.)				
	CPD ≤ 22	CAZ ≤ 22	CRO ≤ 25	CTX ≤ 27	ATM ≤ 27
EP001	25.75	28.44	29.64	30.28	31.77
EP002	NZ	14.07	7.78	NZ	12.38
EP003	23.88	32.09	30.06	28.96	32.62
EP004	25.45	31.16	29.73	30.79	31.62
EP005	NZ	17.47	16.31	18.71	20.99
EP006	23.86	30.32	28.21	30.71	29.71
EP007	NZ	17.77	NZ	NZ	9.03
EP008	24.59	31.24	30.30	33.36	30.30
EP009	13.22	15.83	21.41	25.01	19.91
EP010	27.73	32.35	31.67	32.14	33.57
EP011	NZ	14.21	14.16	13.52	21.34
EP012	NZ	24.42	11.67	9.96	19.57
EP013	NZ	27.02	14.31	9.42	25.57
EP014	NZ	10.20	17.84	11.50	23.07
EP015	26.45	30.10	30.12	31.67	31.41
EP016	27.15	30.29	31.77	32.56	33.07
EP017	NZ	10.52	NZ	NZ	8.85
EP018	NZ	24.54	11.73	8.49	23.45
EP019	27.63	29.12	31.78	31.69	33.93
EP020	25.12	30.08	29.25	31.53	29.92
EP021	26.07	29.17	30.17	31.70	30.44
EP022	NZ	10.03	12.00	10.66	18.81
EP023	27.05	29.16	30.59	32.31	32.81

Table A-2: (cont.)

Strain Code No.	Inhibition zone sizes (mm.)				
	CPD ≤ 22	CAZ ≤ 22	CRO ≤ 25	CTX ≤ 27	ATM ≤ 27
EP024	23.51	26.67	28.44	31.94	29.39
EP025	NZ	15.55	19.66	21.61	20.30
EP026	24.37	28.03	28.14	31.31	29.70
EP027	24.71	31.62	27.07	35.16	30.12
EP028	25.24	29.14	28.57	32.88	29.32
EP029	23.40	28.57	26.24	31.87	29.05
EP030	24.46	28.87	30.85	31.51	32.20

*CPD = cefpodoxime, CAZ = ceftazidime, CRO = ceftriaxone, CTX = cefotaxime, ATM = aztreonam

**NZ = no zone

EP = *E. coli* isolates from pus

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Table A- 3: Inhibition zone sizes of the initial screen test of *E. coli* isolated from urine

Strain Code No.	Inhibition zone sizes (mm.)				
	CPD ≤ 22	CAZ ≤ 22	CRO ≤ 25	CTX ≤ 27	ATM ≤ 27
EU001	NZ	9.87	8.94	9.25	12.70
EU002	32.52	30.46	42.75	40.35	43.80
EU003	7.20	21.54	21.73	26.34	20.18
EU004	30.21	30.84	35.22	34.51	35.26
EU005	9.58	11.63	18.56	17.78	12.70
EU006	NZ	12.09	16.21	17.80	21.04
EU007	29.96	28.54	34.80	36.23	37.16
EU008	NZ	13.65	NZ	NZ	7.70
EU009	13.55	16.82	8.83	32.80	11.68
EU010	NZ	10.69	7.24	NZ	NZ
EU011	30.23	30.35	33.68	33.08	35.72
EU012	NZ	16.16	7.90	11.92	14.88
EU013	25.75	25.64	33.23	31.93	34.41
EU014	31.26	30.41	34.26	32.89	36.67
EU015	29.33	31.17	34.27	36.16	38.87
EU016	NZ	17.43	7.84	NZ	10.26
EU017	29.34	26.79	33.81	34.40	34.91
EU018	27.72	32.03	31.95	32.87	34.64
EU019	NZ	10.66	NZ	NZ	NZ
EU020	NZ	24.75	12.73	13.83	20.85
EU021	NZ	27.20	11.35	14.19	22.89
EU022	NZ	22.18	9.60	10.99	16.28
EU023	30.22	30.17	34.04	33.91	36.55
EU024	28.83	27.24	33.62	31.84	35.25

Table A-3: (cont.)

Strain Code No.	Inhibition zone sizes (mm.)				
	CPD ≤ 22	CAZ ≤ 22	CRO ≤ 25	CTX ≤ 27	ATM ≤ 27
EU025	18.96	25.24	26.97	28.45	28.49
EU026	NZ	14.36	7.24	NZ	14.24
EU027	29.54	28.09	33.31	33.77	35.55
EU028	29.75	28.99	32.32	35.27	35.65
EU029	NZ	10.92	NZ	NZ	7.47
EU030	29.68	32.08	30.88	36.51	35.09
EU031	29.75	33.48	34.60	36.43	36.19
EU032	28.80	31.75	33.31	38.23	34.60
EU033	NZ	24.47	8.77	9.79	14.40
EU034	NZ	14.33	7.25	NZ	10.04
EU035	NZ	22.02	8.94	11.00	14.97
EU036	26.98	29.56	30.65	33.71	33.67
EU037	27.44	29.61	32.40	30.26	33.85
EU038	NZ	17.74	8.43	NZ	12.47
EU039	33.44	32.80	40.70	36.23	42.41
EU040	26.08	31.43	30.72	34.12	32.95
EU041	NZ	16.15	9.49	8.35	12.54
EU042	NZ	16.98	9.37	NZ	11.15
EU043	NZ	19.59	18.11	10.49	15.38
EU044	25.51	29.87	29.08	30.18	30.17
EU045	27.84	29.31	33.20	35.39	33.85
EU046	25.57	31.05	32.40	31.40	34.79
EU047	29.33	31.11	32.14	34.99	34.90
EU048	8.31	32.70	15.42	15.38	25.84
EU049	28.31	29.88	34.12	35.03	35.37

Table A- 3: (cont.)

Strain Code No.	Inhibition zone sizes (mm.)				
	CPD ≤ 22	CAZ ≤ 22	CRO ≤ 25	CTX ≤ 27	ATM ≤ 27
EU050	24.83	23.72	31.57	33.47	31.64
EU051	NZ	21.90	7.71	NZ	12.15
EU052	28.97	31.41	34.26	35.43	34.85
EU053	29.79	30.80	33.90	35.15	35.77
EU054	27.71	28.29	31.87	29.71	34.38
EU055	27.63	29.96	31.16	33.22	33.23
EU056	NZ	20.91	7.38	NZ	12.53
EU057	27.04	32.25	29.57	34.90	30.69
EU058	30.97	30.59	35.11	36.78	38.78
EU059	NZ	28.69	12.12	14.21	18.02
EU060	29.6	28.61	35.88	31.84	37.86

*CPD = cefpodoxime, CAZ = ceftazidime, CRO = ceftriaxone, CTX = cefotaxime, ATM = aztreonam

**NZ = no zone

EU = *E. coli* isolates from urine

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Table B-1: Inhibition zone sizes of suspicious ESBL producing *E. coli* isolated from blood by the phenotypic confirmatory test (combination disc method)

Strains Code Number	Inhibition zone sizes (mm.)								
	CPD	CPD/CA	CPD/CA - CPD ≥ 5	CAZ	CAZ/CA	CAZ/CA - CAZ ≥ 5	CTX	CTX/CA	CTX/CA - CTX ≥ 5
EB003	NZ	19.91	+	26.04	29.10	-	13.08	26.10	+
EB006	NZ	10.56	+	21.84	22.71	-	17.84	27.14	+
EB009	NZ	24.07	+	30.04	33.91	-	16.98	30.37	+
EB012	NZ	15.92	+	8.59	25.23	+	NZ	22.72	+
EB023	NZ	21.09	+	15.85	29.39	+	NZ	27.37	+
EB025	NZ	21.16	+	16.24	28.36	+	NZ	26.43	+
EB027	NZ	20.29	+	9.33	26.12	+	NZ	25.70	+

*CPD = cefpodoxime, CPD/CA = cefpodoxime/clavulanic acid

CAZ = ceftazidime, CAZ/Ca = ceftazidime/clavulanic acid

CTX = cefotaxime, CTX/CA = cefotaxime/clavulanic acid

**NZ = no zone

EB = *E. coli* isolates from blood

+ = positive ESBL producer by combination disc test

- = negative ESBL producer by combination disc test

Table B-2: Inhibition zone sizes of suspicious ESBL producing *E. coli* isolated from pus by the phenotypic confirmatory test (combination disc method)

Strains Code Number	Inhibition zone sizes (mm.)								
	CPD	CPD/CA	CPD/CA - CPD ≥ 5	CAZ	CAZ/CA	CAZ/CA - CAZ ≥ 5	CTX	CTX/CA	CTX/CA - CTX ≥ 5
EP002	NZ	22.10	+	16.35	32.67	+	NZ	30.37	+
EP005	NZ	NZ	-	13.97	15.01	-	16.94	17.33	-
EP007	NZ	19.97	+	16.02	28.85	+	7.90	25.94	+
EP009	13.22	25.60	+	13.74	27.53	+	22.95	30.67	+
EP011	NZ	NZ	-	15.31	16.38	-	15.92	16.95	-
EP012	NZ	17.60	+	23.58	27.19	-	10.86	24.24	+
EP013	NZ	22.93	+	25.33	28.94	-	16.41	28.59	+
EP014	NZ	NZ	-	11.25	15.32	-	14.56	16.18	-
EP017	NZ	18.21	+	16.67	29.98	+	NZ	24.95	+
EP018	NZ	19.03	+	23.59	26.80	-	13.40	24.75	+
EP022	NZ	NZ	-	10.28	13.33	-	12.22	12.54	-
EP025	NZ	NZ	-	15.85	17.51	-	20.84	21.57	-

*CPD = cefpodoxime, CPD/CA = cefpodoxime/clavulanic acid

CAZ = ceftazidime, CAZ/Ca = ceftazidime/clavulanic acid

CTX = cefotaxime, CTX/CA = cefotaxime/clavulanic acid

**NZ = no zone

EP = *E. coli* isolates from pus

+ = positive ESBL producer by combination disc test

- = negative ESBL producer by combination disc test

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Table B-3: Inhibition zone sizes of suspicious ESBL producing *E. coli* isolated from urine by the phenotypic confirmatory test (combination disc method)

Strain Code Number	Inhibition zone sizes (mm.)								
	CPD	CPD/CA	CPD/CA - CPD ≥ 5	CAZ	CAZ/CA	CAZ/CA - CAZ ≥ 5	CTX	CTX/CA	CTX/CA - CTX ≥ 5
EU001	NZ	15.22	+	8.11	24.18	-	8.72	23.13	+
EU003	7.20	8.57	-	18.55	19.98	-	23.92	24.43	-
EU005	9.58	24.43	+	7.80	26.12	+	17.92	29.98	+
EU006	NZ	NZ	-	13.88	15.34	-	14.83	16.52	-
EU008	NZ	15.56	+	11.98	26.40	+	NZ	23.52	+
EU009	13.55	19.30	+	16.82	27.94	+	27.05	31.68	-
EU010	NZ	14.57	+	7.55	24.52	+	NZ	24.52	+
EU012	NZ	NZ	-	15.13	25.59	+	NZ	25.52	+
EU016	NZ	18.12	+	13.74	15.40	-	9.96	17.87	+
EU019	NZ	15.69	+	8.18	24.00	+	NZ	22.92	+
EU020	NZ	17.24	+	24.85	28.30	-	11.51	24.35	+
EU021	NZ	17.15	+	27.38	29.39	-	13.01	26.17	+
EU022	NZ	18.57	+	24.42	27.57	-	12.08	27.01	+
EU025	18.96	21.14	-	26.05	26.82	-	27.86	28.29	-
EU026	NZ	NZ	-	14.41	15.63	-	8.61	14.55	+
EU029	NZ	16.33	+	9.45	28.00	+	NZ	23.88	+
EU033	NZ	14.84	+	22.94	27.11	+	8.77	24.49	+
EU034	NZ	15.79	+	12.06	23.61	+	7.15	22.72	+
EU035	NZ	14.92	+	19.42	23.51	-	11.37	22.30	+
EU038	NZ	18.55	+	13.82	24.71	+	7.17	25.32	+
EU041	NZ	NZ	-	13.42	15.84	-	8.03	18.65	+
EU042	NZ	19.46	+	12.41	26.45	+	9.34	26.83	+

Table B-3: (cont.)

Strain Code Number	Inhibition zone sizes (mm.)								
	CPD	CPD/CA	CPD/CA - CPD ≥ 5	CAZ	CAZ/CA	CAZ/CA - CAZ ≥ 5	CTX	CTX/CA	CTX/CA - CTX ≥ 5
EU043	NZ	NZ	-	17.21	17.43	-	15.49	16.01	-
EU048	8.31	20.52	+	25.35	26.78	-	15.58	27.39	+
EU051	NZ	14.29	+	19.48	24.29	-	NZ	20.35	+
EU056	NZ	13.20	+	18.52	22.99	-	NZ	19.56	+
EU059	NZ	20.99	+	24.26	28.16	-	12.85	26.07	+

*CPD = cefpodoxime, CPD/CA = cefpodoxime/clavulanic acid

CAZ = ceftazidime, CAZ/Ca = ceftazidime/clavulanic acid

CTX = cefotaxime, CTX/CA = cefotaxime/clavulanic acid

**NZ = no zone

EU = *E. coli* isolates from urine

+ = positive ESBL producer by combination disc test

- = negative ESBL producer by combination disc test

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Table C-1: Inhibition zone sizes and antimicrobial susceptibility pattern of 30 *E. coli* isolated from blood

Strain Code No.	Inhibition zone sizes of antimicrobial agents (mm.) / Antimicrobial susceptiility pattern																									
	AM		AMC		CZ		FOX		CPD		CAZ		CRO		CTX		ATM		IMP		GN		NOR		SXT	
EB001	NZ	R	23.32	S	22.58	S	23.77	S	29.52	S	30.16	S	33.48	S	33.37	S	35.34	S	29.53	S	22.42	S	34.84	S	NZ	R
EB002	NZ	R	24.80	S	22.60	S	26.54	S	29.74	S	27.13	S	34.40	S	31.06	S	36.06	S	31.11	S	22.17	S	36.99	S	24.88	S
EB003	NZ	R	15.64	I	NZ	R	21.30	S	NZ	R	23.69	S	12.02	R	13.26	R	24.56	S	27.40	S	8.02	R	22.32	S	NZ	R
EB004	18.15	S	21.59	S	22.08	S	25.39	S	28.19	S	29.07	S	31.85	S	32.94	S	34.36	S	31.66	S	20.09	S	34.33	S	26.51	S
EB005	NZ	R	23.32	S	24.37	S	24.49	S	27.44	S	26.92	S	30.99	S	32.27	S	32.65	S	28.51	S	18.97	S	28.94	S	NZ	R
EB006	NZ	R	11.41	R	11.26	R	10.21	R	NZ	R	19.99	S	14.59	I	23.55	S	24.39	S	28.61	S	20.65	S	22.07	S	NZ	R
EB007	NZ	R	17.47	I	21.91	S	22.78	S	33.79	S	30.08	S	31.61	S	32.18	S	33.79	S	28.61	S	NZ	R	26.98	S	NZ	R
EB008	NZ	R	20.65	S	20.55	S	27.46	S	37.51	S	27.08	S	34.84	S	31.07	S	37.51	S	30.05	S	8.68	R	34.53	S	NZ	R
EB009	NZ	R	19.83	S	NZ	R	27.88	S	NZ	R	27.75	S	13.50	R	14.85	R	28.64	S	29.34	S	14.35	I	24.38	S	NZ	R
EB010	NZ	R	16.77	I	8.52	R	23.83	S	27.64	S	25.25	S	32.24	S	25.05	S	36.29	S	28.55	S	20.44	S	26.97	S	NZ	R
EB011	NZ	R	19.29	S	NZ	R	20.17	S	29.49	S	16.04	I	33.21	S	17.52	I	36.40	S	29.22	S	24.42	S	NZ	R	NZ	R
EB012	NZ	R	17.38	I	NZ	R	21.14	S	NZ	R	26.26	S	NZ	R	10.92	R	NZ	R	27.67	S	NZ	R	NZ	R	NZ	R

Table C-1: (cont.)

Strain Code No.	Inhibition zone sizes of antimicrobial agents (mm.) / Antimicrobial susceptiility pattern																									
	AM		AMC		CZ		FOX		CPD		CAZ		CRO		CTX		ATM		IMP		GN		NOR		SXT	
EB013	NZ	R	18.22	S	NZ	R	22.43	S	26.33	S	25.67	S	29.74	S	10.70	R	32.25	S	24.76	S	NZ	R	22.24	S	NZ	R
EB014	18.33	S	17.97	I	NZ	R	23.07	S	28.87	S	17.55	I	32.53	S	19.98	I	33.66	S	24.73	S	NZ	R	23.10	S	22.35	S
EB015	NZ	R	18.19	S	17.85	I	22.14	S	34.33	S	28.56	S	32.40	S	33.00	S	34.33	S	28.05	S	19.11	S	22.12	S	NZ	R
EB016	NZ	R	20.17	S	19.94	S	23.48	S	34.64	S	27.58	S	32.30	S	32.28	S	34.64	S	28.54	S	20.02	S	28.19	S	NZ	R
EB017	NZ	R	20.74	S	NZ	R	23.26	S	27.82	S	10.17	R	30.31	S	NZ	R	32.54	S	28.44	S	21.15	S	21.28	S	NZ	R
EB018	NZ	R	15.84	I	NZ	R	25.70	S	27.97	S	24.81	S	32.98	S	12.22	R	35.63	S	28.74	S	21.55	S	32.33	S	NZ	R
EB019	NZ	R	16.14	I	18.00	S	18.03	S	33.82	S	30.19	S	27.02	S	35.07	S	33.82	S	26.38	S	20.46	S	33.66	S	28.82	S
EB020	NZ	R	20.95	S	20.38	S	21.20	S	34.49	S	31.03	S	27.62	S	29.84	S	34.49	S	28.21	S	NZ	R	32.52	S	NZ	R
EB021	18.15	S	18.34	S	18.67	S	17.47	I	32.87	S	27.45	S	26.35	S	32.94	S	32.87	S	29.52	S	17.45	S	32.45	S	25.64	S
EB022	NZ	R	24.91	S	26.14	S	28.39	S	35.12	S	33.42	S	30.95	S	38.07	S	35.12	S	32.58	S	21.11	S	36.77	S	NZ	R
EB023	NZ	R	15.87	I	NZ	R	19.97	S	NZ	R	14.46	R	8.10	R	8.11	R	10.60	R	26.56	S	NZ	R	NZ	R	NZ	R
EB024	NZ	R	19.60	S	20.55	S	21.90	S	32.22	S	29.65	S	23.99	S	31.50	S	32.22	S	29.33	S	18.69	S	NZ	R	20.45	S
EB025	NZ	R	17.41	I	NZ	R	22.80	S	NZ	R	18.43	S	NZ	R	NZ	R	10.37	R	30.56	S	NZ	R	NZ	R	NZ	R

Table C-1: (cont.)

Strain Code No.	Inhibition zone sizes of antimicrobial agents (mm.) / Antimicrobial susceptiility pattern																									
	AM		AMC		CZ		FOX		CPD		CAZ		CRO		CTX		ATM		IMP		GN		NOR		SXT	
EB026	NZ	R	18.55	S	20.23	S	26.90	S	33.50	S	30.56	S	28.58	S	35.23	S	33.50	S	27.85	S	20.16	S	30.79	S	21.53	S
EB027	NZ	R	11.43	R	NZ	R	24.00	S	NZ	R	8.61	R	NZ	R	NZ	R	NZ	R	28.17	S	NZ	R	18.02	S	16.36	S
EB028	NZ	S	23.04	S	23.91	S	27.68	S	36.53	S	30.92	S	30.30	S	31.51	S	36.53	S	27.80	S	19.76	S	33.94	S	27.17	S
EB029	NZ	R	21.67	S	22.10	S	26.85	S	29.43	S	29.10	S	24.80	S	32.35	S	29.43	S	28.10	S	8.38	R	25.20	S	NZ	R
EB030	NZ	R	20.85	S	21.62	S	26.45	S	30.40	S	28.75	S	26.34	S	31.49	S	30.40	S	29.74	S	7.94	R	26.51	S	NZ	R

EB = *E. coli* isolates from blood

Strain Code No. = Strain Code Number

* AM = ampicillin, AMC = amoxicillin/clavulanic acid, CZ = cefazolin, FOX = cefoxitin, CPD = cefpodoxime, CAZ = ceftazidime, CRO = ceftriaxone, CTX = cefotaxime, ATM = aztreonam, IMP = imipenem, GN = gentamicin, NOR= norfloxacin, SXT = trimethoprim/sulfamethoxazol

**NZ = no zone

***S = susceptible, I = intermediate, R = resistant

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Table C-2: Inhibition zone sizes and antimicrobial susceptibility pattern of 30 *E. coli* isolated from pus

Strain Code No.	Inhibition zone sizes of antimicrobial agents (mm.) / Antimicrobial susceptiility pattern																									
	AM		AMC		CZ		FOX		CPD		CAZ		CRO		CTX		ATM		IMP		GN		NOR		SXT	
EP001	NZ	R	18.86	S	19.61	S	21.08	S	25.75	S	28.44	S	29.64	S	30.28	S	31.77	S	26.48	S	20.70	S	36.17	S	NZ	R
EP002	NZ	R	15.55	I	NZ	R	25.24	S	NZ	R	14.07	R	7.78	R	NZ	R	12.38	R	29.13	S	9.25	R	15.95	I	16.89	S
EP003	NZ	R	11.21	R	20.99	S	25.37	S	23.88	S	32.09	S	30.06	S	28.96	S	32.62	S	29.99	S	19.92	S	37.80	S	19.15	S
EP004	18.15	S	19.92	S	23.22	S	25.70	S	25.45	S	31.16	S	29.73	S	30.79	S	31.62	S	30.58	S	23.02	S	24.18	S	NZ	R
EP005	NZ	R	NZ	R	NZ	R	9.11	R	NZ	R	17.47	I	16.31	I	18.71	I	20.99	I	23.49	S	23.85	S	NZ	R	NZ	R
EP006	NZ	R	17.41	I	19.24	S	25.02	S	23.86	S	30.32	S	28.21	S	30.71	S	29.71	S	28.67	S	12.75	R	19.28	S	NZ	R
EP007	NZ	R	13.64	R	NZ	R	22.24	S	NZ	R	17.77	I	NZ	R	NZ	R	9.03	R	30.39	S	NZ	R	29.01	S	20.76	S
EP008	NZ	R	18.13	S	20.11	S	26.96	S	24.59	S	31.24	S	30.30	S	33.36	S	30.30	S	29.96	S	22.25	S	11.74	R	NZ	R
EP009	NZ	R	17.78	I	15.00	I	26.80	S	13.22	R	15.83	I	21.41	S	25.01	S	19.91	I	31.64	S	14.49	I	37.56	S	23.23	S
EP010	NZ	R	24.27	S	23.43	S	26.26	S	27.73	S	32.35	S	31.67	S	32.14	S	33.57	S	30.10	S	21.45	S	37.29	S	26.87	S
EP011	NZ	R	8.82	R	NZ	R	NZ	R	NZ	R	14.21	R	14.16	I	13.52	R	21.34	I	26.55	S	20.76	S	8.81	R	NZ	R
EP012	NZ	R	15.97	I	NZ	R	17.82	I	NZ	R	24.42	S	11.67	R	9.96	R	19.57	I	27.60	S	NZ	R	NZ	R	NZ	R

Table C-2: (cont.)

Strain Code No.	Inhibition zone sizes of antimicrobial agents (mm.) / Antimicrobial susceptiility pattern																									
	AM		AMC		CZ		FOX		CPD		CAZ		CRO		CTX		ATM		IMP		GN		NOR		SXT	
EP013	NZ	R	17.40	I	NZ	R	27.01	S	NZ	R	27.02	S	14.31	I	9.42	R	25.57	S	26.88	S	NZ	R	25.87	S	NZ	R
EP014	18.33	S	8.19	R	NZ	R	NZ	R	NZ	R	10.20	R	17.84	I	11.50	R	23.07	S	24.82	S	18.96	S	25.23	S	25.00	S
EP015	NZ	R	17.20	I	17.14	I	22.66	S	26.45	S	30.10	S	30.12	S	31.67	S	31.41	S	29.34	S	NZ	R	NZ	R	NZ	R
EP016	NZ	R	20.56	S	21.14	S	23.43	S	27.15	S	30.29	S	31.77	S	32.56	S	33.07	S	30.30	S	21.95	S	26.42	S	NZ	R
EP017	NZ	R	12.57	R	NZ	R	18.62	S	NZ	R	10.52	R	NZ	R	NZ	R	8.85	R	26.97	S	NZ	R	NZ	R	NZ	R
EP018	NZ	R	15.10	I	NZ	R	23.75	S	NZ	R	24.54	S	11.73	R	8.49	R	23.45	S	26.62	S	8.57	R	NZ	R	NZ	R
EP019	NZ	R	20.16	S	20.78	S	25.76	S	27.63	S	29.12	S	31.78	S	31.69	S	33.93	S	28.18	S	21.74	S	33.26	S	26.87	S
EP020	NZ	R	19.66	S	20.07	S	26.12	S	25.12	S	30.08	S	29.25	S	31.53	S	29.92	S	28.96	S	21.38	S	31.70	S	NZ	R
EP021	18.15	S	23.54	S	22.52	S	25.00	S	26.07	S	29.17	S	30.17	S	31.70	S	30.44	S	28.38	S	20.42	S	33.31	S	25.19	S
EP022	NZ	R	8.66	R	NZ	R	NZ	R	NZ	R	10.03	R	12.00	R	10.66	R	18.81	I	25.75	S	NZ	R	NZ	R	NZ	R
EP023	NZ	R	19.09	S	19.25	S	27.72	S	27.05	S	29.16	S	30.59	S	32.31	S	32.81	S	26.73	S	9.59	R	33.97	S	NZ	R
EP024	NZ	R	16.71	I	20.70	S	22.86	S	23.51	S	26.67	S	28.44	S	31.94	S	29.39	S	28.71	S	21.29	S	29.38	S	NZ	R
EP025	NZ	R	8.54	R	NZ	R	11.75	R	NZ	R	15.55	I	19.66	I	21.61	I	20.30	I	26.05	S	21.41	S	23.29	S	23.28	S

Table C-2: (cont.)

Strain Code No.	Inhibition zone sizes of antimicrobial agents (mm.) / Antimicrobial susceptiility pattern																									
	AM		AMC		CZ		FOX		CPD		CAZ		CRO		CTX		ATM		IMP		GN		NOR		SXT	
EP026	NZ	R	14.49	I	13.92	R	21.81	S	24.37	S	28.03	S	28.14	S	31.31	S	29.70	S	27.04	S	20.80	S	28.09	S	NZ	R
EP027	NZ	R	21.61	S	23.18	S	27.83	S	24.71	S	31.62	S	27.07	S	35.16	S	30.12	S	28.55	S	21.62	S	25.24	S	28.34	S
EP028	NZ	R	21.34	S	22.20	S	23.18	S	25.24	S	29.14	S	28.57	S	32.88	S	29.32	S	27.12	S	18.06	S	25.97	S	26.54	S
EP029	NZ	R	17.94	I	18.71	S	23.22	S	23.40	S	28.57	S	26.24	S	31.87	S	29.05	S	28.21	S	19.97	S	32.13	S	NZ	R
EP030	NZ	R	20.62	S	23.83	S	26.42	S	24.46	S	28.87	S	30.85	S	31.51	S	32.20	S	30.55	S	21.83	S	28.97	S	NZ	R

EP = *E. coli* isolates from pus

Strain Code No. = Strain Code Number

* AM = ampicillin, AMC = amoxicillin/clavulanic acid, CZ = cefazolin, FOX = cefoxitin, CPD = cefpodoxime, CAZ = ceftazidime, CRO = ceftriaxone, CTX = cefotaxime, ATM = aztreonam, IMP = imipenem, GN = gentamicin, NOR= norfloxacin, SXT = trimethoprim/sulfamethoxazol

**NZ = no zone

***S = susceptible, I = intermediate, R = resistant

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Table C-3: Inhibition zone sizes and antimicrobial susceptibility pattern of 120 *E. coli* isolated from urine

Strain Code No.	Inhibition zone sizes of antimicrobial agents (mm.) / Antimicrobial susceptiility pattern																									
	AM		AMC		CZ		FOX		CPD		CAZ		CRO		CTX		ATM		IMP		GN		NOR		SXT	
EU001	NZ	R	14.94	I	NZ	R	16.73	I	NZ	R	9.87	R	8.94	R	9.25	R	12.70	R	27.12	S	NZ	R	NZ	R	NZ	R
EU002	NZ	R	20.09	S	26.30	S	27.22	S	32.52	S	30.46	S	42.75	S	40.35	S	43.80	S	31.54	S	22.90	S	39.14	S	NZ	R
EU003	NZ	R	10.50	R	10.50	R	10.03	R	7.20	R	21.54	S	21.73	S	26.34	S	20.18	I	29.76	S	NZ	R	26.08	S	NZ	R
EU004	18.15	S	20.16	S	23.72	S	26.15	S	30.21	S	30.84	S	35.22	S	34.51	S	35.26	S	30.08	S	21.67	S	NZ	R	NZ	R
EU005	NZ	R	15.29	I	NZ	R	26.02	S	9.58	R	11.63	R	18.56	I	17.78	I	12.70	R	29.30	S	14.33	I	34.63	S	25.91	S
EU006	NZ	R	NZ	R	NZ	R	7.83	R	NZ	R	12.09	R	16.21	I	17.80	I	21.40	I	27.03	S	21.23	S	24.90	S	NZ	R
EU007	NZ	R	19.61	S	22.37	S	26.42	S	29.96	S	28.54	S	34.80	S	36.23	S	37.16	S	28.12	S	NZ	R	NZ	R	NZ	R
EU008	NZ	R	15.03	I	NZ	R	15.16	I	NZ	R	13.65	R	NZ	R	NZ	R	7.70	R	30.45	S	22.89	S	NZ	R	NZ	R
EU009	NZ	R	NZ	R	19.80	S	NZ	R	13.55	R	NZ	R	8.83	R	32.80	S	11.68	R	10.07	R	NZ	R	NZ	R	NZ	R
EU010	NZ	R	12.19	R	NZ	R	16.15	I	NZ	R	10.69	R	7.24	R	NZ	R	NZ	R	28.54	S	NZ	R	NZ	R	NZ	R
EU011	NZ	R	17.94	I	20.86	S	23.13	S	30.23	S	30.35	S	33.68	S	33.08	S	35.72	S	29.22	S	20.81	S	34.74	S	22.70	S
EU012	NZ	R	8.08	R	NZ	R	9.54	R	NZ	R	16.16	I	7.90	R	11.92	R	14.88	R	26.60	S	8.33	R	8.15	R	NZ	R
EU013	NZ	R	15.22	I	19.60	S	17.54	I	25.75	S	25.64	S	33.23	S	31.93	S	34.41	S	27.71	S	18.86	S	NZ	R	NZ	R
EU014	18.33	S	21.15	S	22.68	S	24.22	S	31.26	S	30.41	S	34.26	S	32.89	S	36.67	S	30.53	S	22.93	S	31.06	S	26.65	S

Table C-3: (cont.)

Strain Code No.	Inhibition zone sizes of antimicrobial agents (mm.) / Antimicrobial susceptiility pattern																									
	AM		AMC		CZ		FOX		CPD		CAZ		CRO		CTX		ATM		IMP		GN		NOR		SXT	
EU015	NZ	R	16.59	I	17.28	I	22.87	S	29.33	S	31.17	S	34.27	S	36.16	S	38.87	S	31.16	S	21.72	S	NZ	R	NZ	R
EU016	NZ	R	17.09	I	NZ	R	17.07	I	NZ	R	17.43	I	7.84	R	NZ	R	10.26	R	30.25	S	NZ	R	NZ	R	NZ	R
EU017	NZ	R	17.22	I	19.37	S	24.66	S	29.34	S	26.79	S	33.81	S	34.40	S	34.91	S	28.22	S	20.88	S	18.15	S	NZ	R
EU018	NZ	R	23.39	S	23.26	S	26.52	S	27.72	S	32.03	S	31.95	S	32.87	S	34.64	S	30.70	S	21.57	S	32.96	S	28.98	S
EU019	NZ	R	13.33	R	NZ	R	18.65	S	NZ	R	10.66	R	NZ	R	NZ	R	NZ	R	27.53	S	NZ	R	NZ	R	NZ	R
EU020	18.15	S	17.16	I	NZ	R	23.60	S	NZ	R	24.75	S	12.73	R	13.83	R	20.85	I	28.81	S	NZ	R	NZ	R	23.91	S
EU021	NZ	R	17.50	I	NZ	R	26.15	S	NZ	R	27.20	S	11.35	R	14.19	R	22.89	S	27.71	S	10.81	R	NZ	R	NZ	R
EU022	NZ	R	18.38	S	NZ	R	24.24	S	NZ	R	22.18	S	9.60	R	10.99	R	16.28	I	28.35	S	8.15	R	NZ	R	NZ	R
EU023	NZ	R	19.80	S	20.41	S	26.16	S	30.22	S	30.17	S	34.04	S	33.91	S	36.55	S	28.74	S	NZ	R	NZ	R	NZ	R
EU024	NZ	R	11.13	R	12.35	R	23.50	S	28.83	S	27.24	S	33.62	S	31.84	S	35.25	S	27.50	S	8.12	R	NZ	R	NZ	R
EU025	NZ	R	12.71	R	11.48	R	13.72	R	18.96	I	25.24	S	26.97	S	28.45	S	28.49	S	28.09	S	22.15	S	NZ	R	NZ	R
EU026	NZ	R	8.49	R	NZ	R	8.85	R	NZ	R	14.36	R	7.24	R	NZ	R	14.24	R	25.65	S	20.16	S	NZ	R	20.11	S
EU027	NZ	R	17.71	I	20.62	S	24.61	S	29.54	S	28.09	S	33.31	S	33.77	S	35.55	S	28.85	S	NZ	R	NZ	R	NZ	R
EU028	NZ	R	19.65	S	22.42	S	24.76	S	29.75	S	28.99	S	32.32	S	35.27	S	35.65	S	28.18	S	19.60	S	21.43	S	27.38	S

Table C-3: (cont.)

Strain Code No.	Inhibition zone sizes of antimicrobial agents (mm.) / Antimicrobial susceptiility pattern																									
	AM		AMC		CZ		FOX		CPD		CAZ		CRO		CTX		ATM		IMP		GN		NOR		SXT	
EU029	NZ	R	12.36	R	NZ	R	23.54	S	NZ	R	10.92	R	NZ	R	NZ	R	7.47	R	29.32	S	NZ	R	NZ	R	28.10	S
EU030	18.33	S	20.79	S	23.96	S	27.18	S	29.68	S	32.08	S	30.88	S	36.51	S	35.09	S	29.43	S	NZ	R	38.29	S	28.78	S
EU031	NZ	R	20.58	S	22.58	S	28.23	S	29.75	S	33.48	S	34.60	S	36.43	S	36.19	S	29.84	S	20.67	S	NZ	R	NZ	R
EU032	NZ	R	21.06	S	23.66	S	27.68	S	28.80	S	31.75	S	33.31	S	38.23	S	34.60	S	29.78	S	20.85	S	37.23	S	22.37	S
EU033	NZ	R	16.52	I	NZ	R	23.45	S	NZ	R	24.47	S	8.77	R	9.79	R	14.40	R	30.36	S	8.24	R	NZ	R	NZ	R
EU034	NZ	R	11.32	R	NZ	R	20.15	S	NZ	R	14.33	R	7.25	R	NZ	R	10.04	R	27.18	S	NZ	R	NZ	R	NZ	R
EU035	NZ	R	16.09	I	NZ	R	17.05	I	NZ	R	22.02	S	8.94	R	11.00	R	14.97	R	29.80	S	22.41	S	NZ	R	10.99	R
EU036	NZ	R	21.10	S	22.29	S	27.90	S	26.98	S	29.56	S	30.65	S	33.71	S	33.67	S	28.43	S	22.33	S	29.07	S	22.22	S
EU037	18.15	S	18.27	S	21.48	S	22.04	S	27.44	S	29.61	S	32.40	S	30.26	S	33.85	S	29.71	S	22.05	S	14.74	I	NZ	R
EU038	NZ	R	14.73	R	NZ	R	17.71	I	NZ	R	17.74	I	8.43	R	NZ	R	12.47	R	28.86	S	9.83	R	NZ	R	NZ	R
EU039	NZ	R	19.61	S	23.97	S	28.15	S	33.44	S	32.80	S	40.70	S	36.23	S	42.41	S	29.13	S	21.85	S	37.34	S	16.42	S
EU040	NZ	R	18.09	S	21.99	S	24.31	S	26.08	S	31.43	S	30.72	S	34.12	S	32.95	S	28.09	S	9.41	R	NZ	R	NZ	R
EU041	NZ	R	10.23	R	NZ	R	9.14	R	NZ	R	16.15	I	9.49	R	8.35	R	12.54	R	30.13	S	NZ	R	31.39	S	NZ	R
EU042	NZ	R	15.50	I	NZ	R	27.76	S	NZ	R	16.98	I	9.37	R	NZ	R	11.15	R	30.70	S	NZ	R	30.70	S	NZ	R

Table C-3: (cont.)

Strain Code No.	Inhibition zone sizes of antimicrobial agents (mm.) / Antimicrobial susceptiility pattern																									
	AM		AMC		CZ		FOX		CPD		CAZ		CRO		CTX		ATM		IMP		GN		NOR		SXT	
EU043	NZ	R	9.21	R	NZ	R	12.90	R	NZ	R	19.59	S	18.11	I	10.49	R	15.38	R	27.43	S	9.55	R	NZ	R	NZ	R
EU044	NZ	R	18.78	S	NZ	R	26.35	S	25.51	S	29.87	S	29.08	S	23.76	S	30.17	S	29.10	S	21.74	S	NZ	R	NZ	R
EU045	NZ	R	16.58	I	18.37	S	21.70	S	27.84	S	29.31	S	33.20	S	35.39	S	33.85	S	30.38	S	21.36	S	NZ	R	NZ	R
EU046	NZ	R	13.44	R	15.23	I	24.88	S	25.57	S	31.05	S	32.40	S	31.40	S	34.79	S	31.24	S	NZ	R	NZ	R	NZ	R
EU047	18.33	S	25.15	S	NZ	R	23.66	S	29.33	S	31.11	S	32.14	S	34.99	S	34.90	S	28.69	S	21.60	S	33.73	S	NZ	R
EU048	NZ	R	20.99	S	NZ	R	27.43	S	8.31	R	32.70	S	15.42	I	15.38	I	25.84	S	31.47	S	NZ	R	37.30	S	23.85	S
EU049	NZ	R	19.15	S	20.19	S	23.46	S	28.31	S	29.88	S	34.12	S	35.03	S	35.37	S	30.49	S	20.87	S	NZ	R	28.72	S
EU050	NZ	R	11.60	R	14.13	R	20.57	S	24.83	S	23.72	S	31.57	S	33.47	S	31.64	S	27.50	S	20.99	S	NZ	R	14.96	I
EU051	NZ	R	15.39	I	NZ	R	22.46	S	NZ	R	21.90	S	7.71	R	NZ	R	12.15	R	28.17	S	NZ	R	NZ	R	NZ	R
EU052	NZ	R	21.55	S	24.66	S	22.67	S	28.97	S	31.41	S	34.26	S	35.43	S	34.85	S	27.57	S	NZ	R	28.04	S	30.10	S
EU053	NZ	R	20.95	S	23.35	S	24.82	S	29.79	S	30.80	S	33.90	S	35.15	S	35.77	S	28.81	S	21.93	S	31.05	S	NZ	R
EU054	18.15	S	15.90	I	15.72	I	24.29	S	27.71	S	28.29	S	31.87	S	29.71	S	34.38	S	28.00	S	20.83	S	30.16	S	NZ	R
EU055	NZ	R	17.79	I	18.55	S	21.93	S	27.63	S	29.96	S	31.16	S	33.22	S	33.23	S	29.58	S	15.77	S	10.43	R	NZ	R
EU056	NZ	R	13.12	R	NZ	R	16.45	I	NZ	R	20.91	S	7.38	R	NZ	R	12.53	R	28.64	S	8.27	R	NZ	R	NZ	R

Table C-3: (cont.)

Strain Code No.	Inhibition zone sizes of antimicrobial agents (mm.) / Antimicrobial susceptiility pattern																									
	AM		AMC		CZ		FOX		CPD		CAZ		CRO		CTX		ATM		IMP		GN		NOR		SXT	
EU057	NZ	R	16.24	I	19.94	S	22.74	S	27.04	S	32.25	S	29.57	S	34.90	S	30.69	S	29.80	S	NZ	R	NZ	R	NZ	R
EU058	NZ	R	20.35	S	22.41	S	26.56	S	30.97	S	30.59	S	35.11	S	36.78	S	38.78	S	30.84	S	20.23	S	28.42	S	NZ	R
EU059	NZ	R	23.20	S	NZ	R	26.02	S	NZ	R	28.69	S	12.12	R	14.21	R	18.02	I	30.76	S	22.09	S	30.03	S	30.14	S
EU060	NZ	R	15.65	I	21.42	S	22.45	S	29.60	S	28.61	S	35.88	S	31.84	S	37.86	S	27.81	S	20.51	S	NZ	R	23.45	S

EU = *E. coli* isolates from urine

Strain Code No. = Strain Code Number

* AM = ampicillin, AMC = amoxicillin/clavulanic acid, CZ = cefazolin, FOX = cefoxitin, CPD = cefpodoxime, CAZ = ceftazidime, CRO = ceftriaxone, CTX = cefotaxime, ATM = aztreonam, IMP = imipenem, GN = gentamicin, NOR= norfloxacin, SXT = trimethoprim/sulfamethoxazol

**NZ = no zone

***S = susceptible, I = intermediate, R = resistant

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Table D-1: ESBL genes (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{VEB} genes) and an integrase (*intI*1) gene of ESBL producing *E. coli* and suspicious ESBL producing *E. coli* by PCR

Strain Code Number	Specimen	ESBL producing *E. coli*	ESBL genes				*IntI* 1
			bla_{TEM}	bla_{SHV}	bla_{CTX-M}	bla_{VEB}	
EB003	blood	+	+	-	-	+	+
EB006	blood	+	-	-	-	-	+
EB009	blood	+	-	-	-	-	-
EB012	blood	+	+	-	-	+	+
EB023	blood	+		-	-	+	+
EB025	blood	+	+	-	-	+	+
EB027	blood	+	+	+	-	+	+
EU001	urine	+	-	-	+	+	+
EU003	urine	-	+	-	-	-	+
EU005	urine	+	-	-	+	-	+
EU006	urine	-	+	-	-	-	+
EU008	urine	+	+	-	-	+	+
EU009	urine	+	+	-	-	-	+
EU010	urine	+	+	+	-	-	+
EU012	urine	+	-	-	-	-	+
EU016	urine	+	+	-	-	+	+
EU019	urine	+	+	-	-	+	+
EU020	urine	+	+	-	-	+	+
EU021	urine	+	+	-	-	+	+
EU022	urine	+	+	-	-	+	+
EU025	urine	-	+	-	-	-	+
EU026	urine	+	+	-	-	+	+
EU029	urine	+	-	-	-	+	+
EU033	urine	+	+	-	-	+	+

Table D-1: (cont.)

Strain Code Number	Specimen	ESBL producing *E. coli*	ESBL genes				*IntI* 1
			bla_{TEM}	bla_{SHV}	$bla_{CTX\text{-}M}$	bla_{VEB}	
EU034	urine	+	+	-	-	+	+
EU035	urine	+	+	-	-	+	+
EU038	urine	-	+	-	-	+	+
EU041	urine	+	+	-	-	-	+
EU042	urine	+	+	+	-	+	+
EU043	urine	-	+	-	-	-	+
EU048	urine	+	+	-	-	+	+
EU051	urine	+	+	-	-	+	+
EU056	urine	+	+	-	-	+	+
EU059	urine	-	-	-	-	+	+
EP002	pus	+	-	-	-	+	+
EP005	pus	-	+	-	-	-	+
EP007	pus	+	+	-	-	+	+
EP009	pus	+	+	-	+	-	+
EP011	pus	-	+	-	-	-	+
EP012	pus	+	+	-	-	+	+
EP013	pus	+	+	-	-	+	+
EP014	pus	-	-	-	-	-	+
EP017	pus	+	+	-	-	+	+
EP018	pus	+	+	-	-	+	+
EP022	pus	-	+	-	-	-	+
EP025	pus	-	-	-	-	-	+

EB = *E. coli* isolates from blood, EP = *E. coli* isolates from pus, EU = *E. coli* isolates from urine

*intI*1 = integrase gene of class 1 integron

+ = positive gene, - = negative gene

Table E-1: Integrase (*intI*1) gene of *E. coli* isolated from blood by PCR and Dot blot hybridization

Strain Code No.	Integrase (*intI*1) gene		Strain Code No.	Integrase (*intI*1) gene	
	PCR	Dot blot hybridization		PCR	Dot blot hybridization
EB001	+	+	EB016	+	+
EB002	+	+	EB017	+	+
EB003	+	+	EB018	+	+
EB004	+	+	EB019	+	+
EB005	+	+	EB020	+	+
EB006	+	+	EB021	+	+
EB007	+	+	EB022	+	+
EB008	+	+	EB023	+	+
EB009	-	-	EB024	+	+
EB010	+	+	EB025	+	+
EB011	+	+	EB026	+	+
EB012	+	+	EB027	+	+
EB013	+	+	EB028	+	+
EB014	+	+	EB029	+	+
EB015	+	+	EB030	+	+

EB = *E. coli* isolates from blood

+ = positive integrase gene, - = negative integrase gene

Strain Code No. = Strain Code Number

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Table E-2: Integrase (*intI*1) gene of *E. coli* isolated from pus by PCR and Dot blot hybridization

Strain Code No.	Integrase (*intI*1) gene		Strain Code No.	Integrase (*intI*1) gene	
	PCR	Dot blot hybridization		PCR	Dot blot hybridization
EP001	+	+	EP016	+	+
EP002	+	+	EP017	+	+
EP003	+	+	EP018	+	+
EP004	+	+	EP019	+	+
EP005	+	+	EP020	+	+
EP006	+	+	EP021	+	+
EP007	+	+	EP022	+	+
EP008	+	+	EP023	+	+
EP009	+	+	EP024	+	+
EP010	+	+	EP025	+	+
EP011	+	+	EP026	+	+
EP012	+	+	EP027	+	+
EP013	+	+	EP028	+	+
EP014	+	+	EP029	+	+
EP015	+	+	EP030	+	+

EP = *E. coli* isolates from pus

+ = positive integrase gene, - = negative integrase gene

Strain Code No. = Strain Code Number

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Table E-3: Integrase (*intI* 1) gene of *E. coli* isolated from urine by PCR and Dot blot hybridization

Strain	Integrase (*intI*1) gene		Strain	Integrase (*intI*1) gene	
Code No.	PCR	Dot blot hybridization	Code No.	PCR	Dot blot hybridization
EU001	+	+	EU028	+	+
EU002	+	+	EU029	+	+
EU003	+	+	EU030	+	+
EU004	+	+	EU031	+	+
EU005	+	+	EU032	+	+
EU006	+	+	EU033	+	+
EU007	+	+	EU034	+	+
EU008	+	+	EU035	+	+
EU009	+	+	EU036	+	+
EU010	+	+	EU037	+	+
EU011	+	+	EU038	+	+
EU012	+	+	EU039	+	+
EU013	+	+	EU040	+	+
EU014	+	+	EU041	+	+
EU015	+	+	EU042	+	+
EU016	+	+	EU043	+	+
EU017	+	+	EU044	+	+
EU018	+	+	EU045	+	+
EU019	+	+	EU046	+	+
EU020	-	+	EU047	+	+
EU021	+	+	EU048	+	+
EU022	-	+	EU049	+	+
EU023	+	+	EU050	+	+
EU024	+	+	EU051	+	+
EU025	+	+	EU052	+	+
EU026	+	+	EU053	+	+
EU027	+	+	EU054	+	+

Table E-3 (cont.)

Strain	Integrase (*intI*1) gene		Strain	Integrase (*intI*1) gene	
Code No.	PCR	Dot blot hybridization	Code No.	PCR	Dot blot hybridization
EU055	+	+	EU058	+	+
EU056	+	+	EU039	+	+
EU057	+	+	EU060	+	+

EU = *E. coli* isolates from urine

+ = positive integrase gene, - = negative integrase gene

Strain Code No. = Strain Code Number

BIOGRAPHY

Miss Yupin Taipobsakul was born in April 2, 1979 in Bangkok, Thailand. She graduated with the Bachelor degree of Pharmaceutical Sciences from the Faculty of Pharmaceutical Sciences, Chulalongkorn University in 2001. She started to work as a pharmacist in hospital during 2001-2003. She has enrolled for the master's degree in Microbiology at the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University in 2003.

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