DETECTION OF CTX-M-TYPE EXTENDED-SPECTRUM-β-LACTAMASES (ESBLs) IN *Escherichia coli* ISOLATED IN UKMMC.

DR. SITI NORLIA BINTI OTHMAN

DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT FOR MASTERS OF PATHOLOGY (MICROBIOLOGY)

FACULTY OF MEDICINE
UNIVERSITI KEBANGSAAN MALAYSIA
KUALA LUMPUR

2011

MENGESAN ISOLAT *Escherichia coli* YANG MENGHASILKAN ENZIM CTX-M BETA-LACTAMASE SPECTRUM LANJUT DI UKMMC.

DR. SITI NORLIA BINTI OTHMAN

TESIS YANG DIKEMUKAKAN UNTUK MEMENUHI SEBAHAGIAN DARIPADA SYARAT MEMPEROLEHI IJAZAH SARJANA PATOLOGI (MIKROBIOLOGI)

FAKULTI PERUBATAN
UNIVERSITI KEBANGSAAN MALAYSIA
KUALA LUMPUR

2011

DECLARATION

I hereby declare that the work in this thesis is my own except for quotations whose sources are clearly acknowledged. No portion of this work has been submitted for any award of any other degree.

22 December 2010

DR. SITI NORLIA BINTI OTHMAN
P41813

ACKNOWLEDGEMENTS

First and foremost, my thanks to Allah (SWT) for his guidance and help in my journey in completing this thesis.

My deepest thanks to my supervisors, Associate Professor Dr. Salasawati Hussin and Dr. Ramliza Ramli, from Department of Medical Microbiology and Immunology, Universiti Kebangsaan Malaysia Medical Centre, for their invaluable guidance and supervision throughout the course of the study and their constructive criticisms without which this study would not have progressed smoothly.

My sincerest gratitude to all lecturers from Department of Medical Microbiology and Immunology, Universiti Kebangsaan Malaysia Medical Centre.

I am also thankful to all staff of the Medical Microbiology Laboratory, Universiti Kebangsaan Malaysia Medical Centre, especially staff from Infection Control Unit and Molecular Biology Unit. My special thanks to Miss Puteri Fairuz Izyan, Miss Nor A'ain, Puan Aini Hayati, Miss Diana and Miss Nor Hamiza who were there for guidance and lending their helping hands during my project. Without their helps, this study would not be smooth-sailing.

And also to all individuals who have helped me in a way or another.

My deepest appreciation to my dear husband, Dr. Nik Azuan for his faith and understanding. To my children, Fatini, Haziq and Faris, and my other family members, who had been there during my thick and thin times and had tolerated my commitments.

ABSTRACT

Extended-spectrum-beta-lactamase (ESBL) is an enzyme produced by Enterobacteriaceae to confer resistance to extended-spectrum cephalosporins. These enzymes were mostly derived from the classical penicillinases (TEM-1, TEM-2 and SHV-1 β-lactamases). ESBL-producing organisms have spread worldwide and pose a management dilemma when very limited antibiotics are left for treatment of infections caused by these organisms. Infection control also faces a challenge when these plasmid-mediated enzymes are easily transmissible to other organisms. Hence, detection of these organisms is crucial to ensure appropriate antibiotics can be delivered and effective infection control measures can be employed to limit its spread. However, in the last decade, another type of ESBL has been discovered and is not related to TEM- or SHV-type ESBLs. This enzyme has a preference to hydrolyze cefotaxime (CTX) rather than ceftazidime (CAZ), hence the name CTX-M-type ESBLs. Many laboratories have used CAZ as the indicator for ESBL production and with the emergence of CTX-M-type β-lactamase, many CTX-M-type ESBL-producing organisms may be missed if CAZ alone is used for indicator for ESBL production. A cross-sectional descriptive study was carried out in UKMMC from January to June 2009, to determine the prevalence of ESBL-producing ESBLs and to detect the presence of CTX-M-type ESBLs among these isolates by molecular method. A total of 111 *E.coli* isolates were screened positive for ESBL production, however only 96 were confirmed as ESBL-producers by phenotypic confirmatory test using combination disk diffusion method. Out of these, 76 isolates were subjected for detection of $bla_{\text{CTX-M}}$ genes using multiplex PCR. From these, 70 (92.1%) were CTX-M-type ESBLs with majority from CTX-M-1 group (77.1%), followed by CTX-M-9 group (21.4%) and one (1.4%) from CTX-M-2 group. No CTX-M-8 or -25/26 groups isolated. These findings were similar to other studies in Malaysia and Asia, which demonstrated CTX-M-type ESBLs were the predominant ESBL types and CTX-M-1 and -9 groups were the majority among the clinical isolates.

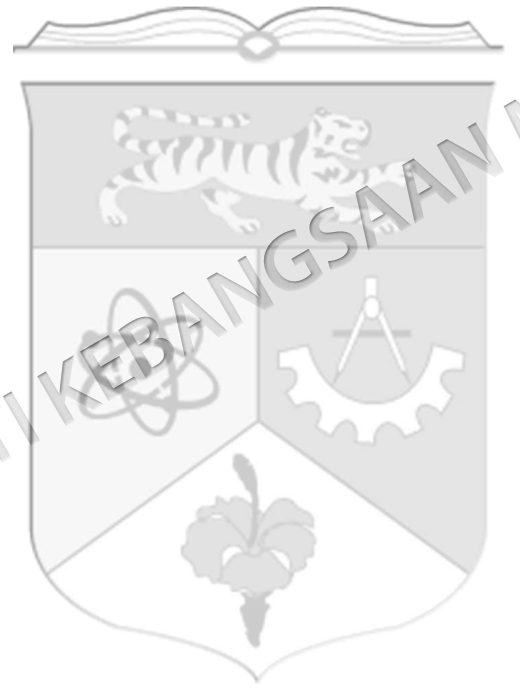
ABSTRAK

Extended-spectrum-beta-lactamase (ESBL) adalah enzım yang dihasilkan oleh Enterobacteriaceae untuk kerintangan terhadap antibiotik *cephalosporin* kelas ketiga (*'extended-spectrum cephalosporin'*). Enzim ini kebanyakannya berasal daripada penicillinase klasik, iaitu TEM-1, TEM-2 dan SHV-1. Organisma yang menghasilkan ESBL telah merebak di seluruh dunia dan menjadi masalah apabila antibiotik yang boleh digunakan untuk merawat jangkitannya adalah sangat terhad. Langkah-langkah kawalan infeksi juga menjadi semakin mencabar apabila enzim ini mudah menular daripada satu organisma kepada organisma yang lain melalui penyebaran plasmid. Namun, dalam sedekad yang lepas, sejenis lagi enzim ESBL yang diberi nama CTX-M telah ditemui dan didapati tidak berkaitan dengan ESBL jenis TEM atau SHV. Enzim ini juga memiliki kecenderungan untuk menghidrolisis antibiotik cefotaxime (CTX) daripada ceftazidime (CAZ). Kebanyakan makmal menggunakan CAZ sebagai penentu penghasilan ESBL di dalam sesuatu organisma. Namun dengan munculnya ESBL jenis CTX-M, ada kemungkinan yang organisma ESBL tidak dapat dikesan sekiranya hanya CAZ digunakan sebagai penentu organisma ESBL. Satu kajian deskriptif telah dijalankan di Pusat Perubatan Universiti Kebangsaan Malaysia (PPUKM) bagi menentukan prevalen isolat *Escherichia coli* yang menghasilkan ESBL dan juga untuk mengesan kewujudan ESBL jenis CTX-M di antara isolat yang dikaji dengan melakukan kaedah molekular. Sebanyak 111 isolat *E.coli* telah disaring positif bagi penghasilan ESBL, tetapi hanya 96 isolat sahaja yang disahkan sebagai organisma ESBL daripada kaedah fenotipik melalui ujian kombinasi disk. Daripada jumlah ini, 76 isolat telah dilakukan ujian PCR multipleks bagi pengesanan gen bla_{CTX-M}. Sebanyak 70 (92.1%) isolat adalah ESBL jenis CTX-M dengan majoritinya adalah daripada kumpulan CTX-M-1 (77.1%), selebihnya pula kumpulan CTX-M-9 (21.4%) dan hanya satu isolat (1.4%) merupakan kumpulan CTX-M-2. Tiada isolat daripada kumpulan CTX-M-8 atau -25/26 yang dijumpai. Penemuan ini adalah sama dengan kebanyakan penemuan kajian lain di Malaysia dan Asia yang menunjukkan ESBL jenis CTX-M merupakan ESBL yang paling kerap ditemui di dalam isolat klinikal dan majoritinya daripada kumpulan CTX-M-1 dan CTX-M-9.

CONTENTS

LIST OF TABLES

LIST OF FIGURES



LIST OF ABBREVIATIONS

A&E	Accident and Emergency Department
AMC	Amoxocilin/clavulanic acid
CLSI	Clinical and Laboratory Standard Institute
CAZ	Ceftazidime
CTX	Cefotaxime
CAZ/CLA	Ceftazidime/Clavulanic acid
CTX/CLA	Cefotaxime/Clavulanic acid
CDC	Centre for Disease Control
DDAT	Double disk approximation test
DNA	Deoxyribonucleic acid
ESBL	Extended-Spectrum-β-Lactamase
ICU	Intensive Care Unit
O&G	Obstetric and Gynaecology
PCR	Polymerase Chain Reaction
UKMMC	Universiti Kebangsaan Malaysia Medical Centre

CHAPTER I

REVIEW OF LITERATURE

1.1 BETA-LACTAMASES ENZYMES

Production of β-lactamases is an important mechanism of resistance to β-lactam antibiotics in gram negative bacteria. These enzymes will hydrolyze the amide bond in the β-lactam ring of β-lactam antibiotics, thus inactivating the β-lactam antibiotics.

These β-lactamases enzymes were classified according to their general spectrum of activity (e.g. penicillinase or cephalosporinase); susceptibility by various inhibitors such as cloxaxillin, *p*-chlormercuribenzoate (pCMB), sulbactam and aztreonam; molecular weight; isoelectric point (pI); and position of genetic determinants (i.e plasmids or chromosome) (Williams, 1990). The first classification was proposed by Richmond in 1970, and later extended by Sykes and Matthew in 1973 and 1976. However, these classifications were deficient when point mutation could greatly alter the substrate specificity and inhibitor susceptibility, thus changing the group of which the enzymes were formerly assigned.

This classification was later reorganized by Bush in 1989 and further revised in 1995. He had classified these enzymes into their functional properties; substrate preference and their susceptibility to inhibition by clavulanic acid. They are grouped into group 1 to 4 and group 2 was further subgrouped into group 2a through group 2d according to their spectrum of activity and inhibition by clavulanic acid. Ambler classification had classified these enzymes based on the nucleotide and amino acid sequences in these enzymes. There are four classes (class A to D) have been recognized, correlating with the functional classification. Class A, C and D act by serine-based mechanisms, whereas class B or metallo-enzymes need zinc for their action. To date, these two classifications are commonly used and referred to (Table 1.1).

Table 1.1: Classification of β-lactamases (Adapted from Jacoby & Munoz-Price 2005)

Bush Functional Group	**Ambler Molecular Class**	**Descriptions**	**Representative enzymes**
1	C	Cephalosporinase; not inhibited by clavulanic acid	Chromosomal (AmpC) ın Enterobacteriaceae, *Acinetobacter baumanii*
2a	A	Penicillinase; inhibited by clavulanic acid	Gram-positive penicillinases
2b	A	Broad-spectrum; inhibited by clavulanic acid	TEM-1, TEM-2, SHV-1
2be	**A**	**Extended broad-spectrum; inhibited by clavulanic acid**	**TEM-3 to TEM-10; SHV-2, SHV-3**
2c	A	Carbenicillinase; inhibited by clavulanic acid	PSE-1, PSE-3, PSE-4, BRO-1, BRO-2
2d	A	Cloxacillinase	OXA-1 to OXA-7; PSE-2

2e	A	Cephalosporinases; inhibited by clavulanic acid	Enzymes from *Proteus vulgaris* and *Citrobacter diversus*
3	B	Metalloenzymes; not inhibited by clavulanic acid	Enzymes (IMP, VIM, GIM, SPM, SIM) from *Pseudomonas maltophilia* and *Acinetobacter spp.*
4	D	Penicillinase; not inhibited by clavulanic acid	Chromosomal enzymes from *Alcaligenes faecalis, Clostridium butyricum, Pseudomonas cepacia*

TEM-1, TEM-2 and SHV-1 β-lactamases (group 2b Bush) by point mutation and have extended their abilities to hydrolyze all penicillins, cephalosporins and monobactam, hence their name 'extended-spectrum β-lactamases'. However, they are inhibited by clavulanic acid and not active against carbapenem and cephamycins. Now, there are over 150 different types of ESBLs have been described (Bradford 2001). They are only differing from the original enzymes by one to few changes in the amino acid sequences. They are found worldwide in many different genera of Enterobacteriaceae and *Pseudomonas aaeruginosa*.

ESBL-producing organism has posed a great clinical impact because the infections caused by these organisms are usually related with high morbidity and mortality as well as healthcare-associated costs. Choice of antimicrobial agents is also limited because these organisms are also resistant to many other groups of antibiotics, including resistance to aminoglycosides and fluoroquinolones. They are also a challenge to infection control measures because they are encoded on plasmids, mobile genetic elements, which are easily transferable to other organisms.

1.3 CTX-M-TYPE ESBLs

In the last decade, a new family of plasmid-mediated ESBLs has been discovered. They are known as CTX-M-type β-lactamases (Bonnet 2004; Bradford 2004). CTX-M-β-lactamases are not related to TEM or SHV ESBLs. They were named CTX-M for their preferences to hydrolyze cefotaxime (CTX) rather than ceftazidime. Organisms producing CTX-M-type β-lactamases typically have higher cefotaxime MICs (>64 μg/mL), while ceftazidime MICs are usually within the apparently susceptible range (2 to 8 μg/mL).

However some CTX-M-producing organisms may actually hydrolyze ceftazidime and confer resistant to this antibiotics (Paterson and Bonomo 2005). When these enzymes were first discovered, they were said not related to the TEM or SHV penicillinases. They were thought to be derived from the chromosomally-encoded K1 enzyme of *Klebsiella oxytoca*. However, this belief was proved to be incorrect, and now believed to be related to β-lactamases of *Kluyvera* spp.

From phylogenetic studies, CTX-M enzymes are divided into five groups based on their amino acids sequence identities, namely groups 1, 2, 8, 9 and 25. The members of each group share >94% identity, whereas ≤90% identity is observed between the members belonging to distinct groups (Bonnet, 2004). To date, more than 40 CTX-M-β-lactamases have been reported (Bonnet 2004; Pitout et al. 2004; Li Xu et al. 2005). The CTX-M-1 group includes nine enzymes, namely CTX-M-1, CTX-M-3, CTX-M-10, CTX-M-12, CTX-M-15, FEC-1, CTX-M-22, CTX-M-23 and CTX-M-28. CTX-M-2-group includes eight enzymes (CTX-M-2, CTX-M-4, CTX-M-4L, CTX-M-5, CTX-M-6, CTX-M-7, CTX-M-20 and Toho-1); CTX-M-8 group comprises of one member only (CTX-M-8); CTX-M-9 group includes nine enzymes namely CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-16, CTX-M-17, CTX-M-19, CTX-M-21, CTX-M-27 and Toho-2) and CTX-M-25 group includes CTX-M-25 and -26 enzymes (Bonnet 2004). However, I believed, these groups are growing with more and more members discovered from time to time.

According to Bonnet (2004), the bla_{CTX-M} genes have been described or presumed to be natural and chromosomally-mediated in the *Kluyvera* spp. (bla_{KLUC-1}, bla_{KLUA} and bla_{KLUG-1}). The DNA sequences surrounding the β-lactamases genes within the organisms and that of bla_{CTX-M} exhibited similarities.

1.4 EPIDEMIOLOGY

1.4.1 Global Epidemiology

ESBLs are the major problem in all hospitals worldwide. The prevalence of ESBLs among clinical isolates varied from country to country. In the United States, occurrence of ESBL production in Enterobacteriaceae ranges from 0 to 25%, with the national average of 3% (CDC National Nosocomial Infections Surveillance, 2006). In Europe, the prevalence also varies greatly from country to country. And in the Asian region, a survey by Antimicrobial Resistance Study Group among Asian countries in 1999, has found the prevalences of between 5% to up to 23%. Singapore/Malaysia were the lowest with 5.6%, whilst Indonesian was the highest with 23%. Japan, Taiwan and Philippines were 8.1%, 16.7% and 13.3% respectively.

The first CTX-M-type ESBL were reported in 1990 (Bauernfeind et al. 1990) in Germany. They reported on a clinical cefotaxime-resistant *E.coli* strain which produced a non-TEM, non-SHV ESBL, designated as CTX-M-1. In 1992, Bernard et al. (1992) had reported the same type of ESBL in clinical *E.coli* strain, in France, named MEN-1. This enzyme was sequenced and presented only 39% identity with the TEM and SHV enzymes (Barthélémy et al. 1992). A few years later, Ishii et al. reported on a MEN-1-related enzyme (83% homologous), designated Toho-1 from a cefotaxime-resistant *E.coli* strain isolated in 1993 in Japan. Further sequencing studies of these enzymes in 1996, revealed that CTX-M-1 is identical to MEN-1 and is variant of Toho-1 (Bauernfiend et al. 1996). Since then, more and more CTX-M-type ESBLs have been reported. They are now forming a rapidly growing family of ESBLs distributed worldwide and in all range of clinical bacteria, particularly among members of Enterobacteriaceae.

CTX-M-type ESBLs have become more prevalent worldwide (Bonnet et al. 2000; Chanawong et al. 2002; Moland et al. 2003; Pagani et al. 2003). They are found mostly in *E.coli* strains and also *Klebsiella pneumoniae*. Other Enterobacteriaceae-producing ESBL were also reported to produce CTX-M-type enzymes. Brigante et al. (2005) have reported that in 2003, CTX-M-type genes accounted for 38.2% of ESBL-positive *E.coli* isolates. In Italian second nationwide survey, CTX-M-type genes were detected in 115 of 583 (19.7%) (Mugnaioli et al. 2006). Worldwide, the most frequent reported CTX-M-β-lactamases are of the CTX-M-1 group (CTX-M-1, M-3, M-10, and M-15 types), CTX-M-2 group (CTX-M-2) and CTX-M-9 group (CTX-M-9, M-13, M-14, M-16, M-17, M-21 and M-27 types).

In Asia, many reports have been seen since the emergence of these enzymes. According to the Study of Monitoring Antimicrobial Resistance Trends (SMART), rates of ESBL-producing *E.coli* in the Asia/Pacific region were between 34.9 and 42.2% (Hawser et al. 2009). Wu et al. (2008) reported that among the 44 of *Proteus mirabilis* strains collected in Taiwan between 1999 and 2005, all were CTX-M-type with 20 of them were of CTX-M-1-like enzymes and the remainings were of CTX-M-9-like enzymes. Kiratisin et al. (2007) reported that blaCTX-M gene was detected in 24 out of 94 (26%) patients, which 18 were *E.coli* strains and and 6 were *K.pneumoniae*.

The predominant ESBLs in the Hunan province of China were CTX-M-14, CTX-M-3 and CTX-M-15 (Liu et al. 2009). In Japan, 65% of 130 CTX-M-producing *E.coli* were of the CTX-M-9 group, 19% were CTX-M-1 group and 16% were CTX-M-2 group (Suzuki et al. 2009). And in Indonesia, from 73 consecutive ESBL-producing *E.coli*, 94.5% were CTX-M-15. This showed that, in our region, three out of five groups of CTX-M-type ESBL were among the majority reported.

1.4.2 Local Epidemiology

In Malaysia, surveillance on the prevalence of ESBL-producing organisms is been going on since the mid 1990s (Ariffin et al. 1999). According to Ministry of Health of Malaysia (MoH), in 2001, the estimated prevalence of ESBLs was 7 to 19% and 27 to 38% for *E.coli* and *Klebsiella* spp. respectively. A data by Cheong et al. (1994) based on her study of resistant patterns of bacteria isolated from six general hospitals in Malaysia from 1991 to 1992, reported that ceftazidime-resistant were 16.6% for *Klebsiella* spp., 5.5% for *E.coli* and 6.8% for *P.aeruginosa*. However, no ESBL detection tests were carried out for these isolates. In 1995, Rahizan et al. has reported that ESBL production among *K.pneumoniae* and *E.coli* were 27% and 19% respectively. The isolates were tested using E-test for their ESBL-productions. Another study done in a university hospital in Malaysia reported $bla_{SHV\text{-}5}$ to be dominant in ESBL-producing *E.coli* and was postulated to have been acquired from ESBL-producing *Klebsiella pneumoniae* via plasmid transfer (Subramaniam et al. 2006).

Locally, CTX-M-type ESBLs are also available and pose the same threat in patient's management and infection control, however the published data on this issue are still lacking. Zamberi et al. (2008) had done a study on 16 *E.coli* strains collected from tertiary hospital in Malaysia in year 2006. They had demonstrated a high prevalence of CTX-M-type ESBLs (81.3%) compared to the TEM-type and SHV-type that were 75% and 12.5% respectively.

1.4.3 CTX-M-Type ESBLs In The Community

An interesting issue of this enzyme is their emergence in the community strains. After many decades, the ESBLs are believed to present only in hospitalized patients or patients who has been exposed to healthcare facilities environments. However today, there are more reports on community-acquired ESBLs especially in urinary tract infections that are believed to be the CTX-M-type ESBLs. This has made the empirical antibiotic therapy for community-acquired infection a more challenging to the clinicians. This fact may also have an implication for control of the spread of CTX-M enzyme.

Kiratisin et al. (2007) had reported that 46 out 94 (43.2%) patients with ESBL infections were community-acquired. All of them were *E.coli* ESBL infections in which 12 (26%) of them were identified as CTX-M-type enzymes. In Spain, 35% of 151 *E.coli* strains resistant to cefotaxime and ceftazidime were associated with community-acquired infections. Of these, 145 isolates were positive for CTX-M-type enzymes (Oteo et al. 2006).

These data have shown the predilection of CTX-M-type enzymes in the community-acquired ESBL strains. Some researchers believed this is because of the close relation of the CTX-M-type enzymes to those β-lactamases found in environmental organism, *Kluyvera spp.* Another potential reservoir for community-acquired CTX-M enzymes is our gut, where these multidrug resistant colonists are believed to come from our food supply, with the finding of diverse ESBL-producing bacteria including CTX-M-15 in poultry and other farm animals (Duan et al. 2006). Many studies also have shown that ESBL-producing *E.coli* are among human guts colonizer. In 141 healthy individuals in Thailand, 51.8% had ESBL-producing *E.coli* in their stool samples, with all except one

were belonged to CTX-M type (Sasaki et al. 2010). These findings have proven that ESBLs have evolved and causing more challenges to the clinicians for patients management, to the laboratories for the detection of these enzymes as well as for the infection control measures in limiting the spread of these enzymes.

1.5 DETECTION OF ESBLs AND CTX-M-TYPE ESBLs

1.5.1 Phenotypic Detection

Like all ESBLs, CTX-M enzymes hydrolyze broad-spectrum cephalosporins and aztreonam but not cephamycins or carbapenems. But in contrast to TEM- and SHV-type ESBLs, CTX-M-type enzymes are much more active against cefotaxime than against ceftazidime. Most laboratories were using ceftazidime-resistant as the indicator of ESBL-producing strains because ceftazidime is the best substrate for TEM- and SHV-type ESBLs. However, with the knowledge that CTX-M-type ESBLs hydrolyze cefotaxime (CTX) more than ceftazidime, use of ceftazidime alone is no longer recommended (Livermore, 2005). Using ceftazidime alone for marker, could fail to detect CTX-M-producing strains that show susceptibility to ceftazidime

With the increasing rates of ESBLs, their early and correct detection has become more important, not only for patients' optimum management, but as well as for infection control measures to limit its spread especially in the healthcare institutions.

The CLSI has proposed disk diffusion and broth microdilution methods for screening of ESBL production by *E.coli, Proteus mirabilis, K.pneumoniae and K.oxytoca*. The substrates used are ceftriaxone, cefotaxime, cefpodoxime, ceftazidime or aztreonam. CLSI also recommends use of more than one antimicrobial agent for screening to improve sensitivity of detection. For disk diffusion method, there are specific zone diameter which indicate a high level of suspicious for ESBL production (Table 1.5.1).For broth microdilution method, the growth at or above the screening concentrations (Table 1.3.1) may indicate ESBL production. Phenotypic confirmatory tests should be used to ascertain the diagnosis of ESBL production in all 'suspicious' strains.

Table 1.5.1.1: MIC and Inhibition Zone Criteria for the Detection of ESBLs in *K.pneumoniae* and *E.coli* (Adapted from CLSI, 2009).

Antimicrobial Disks Concentration	**Diameter for susceptible**	**Zone diameter for possible ESBL-producing strains**	**MIC for susceptible strains**	**MIC for possible ESBL-producing strains**
Cefpodoxime 10	≥ 21 mm	≤ 17 mm	≤ 8 mg/L	≥ 8 mg/L
Ceftazidime 30 µ	≥ 18 mm	≤ 22 mm	≤ 8 mg/L	≥ 2 mg/L
Aztreonam (30 µ)	≥ 22 mm	≤ 27 mm	≤ 8 mg/L	≥ 2 mg/L
Cefotaxime (30 µ	≥ 23 mm	≤ 27 mm	≤ 8 mg/L	≥ 2 mg/L
Ceftriaxone (30 µ)	≥ 21 mm	≤ 25 mm	≤ 8 mg/L	≥ 2 mg/L

However, the criteria for screening for ESBL production in Enterobacteriaceae other than those mentioned earlier have not been established by CLSI and have remain problematic and controversial issue. This is because the clavulanic acid effect with these ESBL-producing organisms is not always present. The clavulanic acid inhibition may be masked by other types of β-lactamases, such as *AmpC* enzymes that produced by *Enterobacter spp.*

Phenotypic confirmatory tests are achieved by usıng disk diffusion method (combination disks tests), broth microdilution method or commercially available Etest strip (AB Biodisk, Sweden). For confirmatory tests using disk diffusion method, both cefotaxime (30μg) and ceftazidime (30μg), alone and in combination with clavulanic acid (30/10μg) are used. A ≥ 5 mm increase in zone diameter for either antimicrobial agents tested in combination with clavulanic acid versus its zone when tested alone indicates ESBL-production (CLSI 2009).

For broth microdilution method, ceftazidime (0.25 to 128 μg/mL), ceftazidime plus clavulanic acid (0.25/4 to 128/4 μg/mL), cefotaxime (0.25 to 64 μg/mL) and cefotaxime plus clavulanic acid (0.25/4 to 64/4 μg/mL) are used. Phenotypic confirmation is considered as a ≥ 3-twofold-serial-dilution decrease in MIC of either cephalosporin in the presence of clavulanic acid compared to its MIC when tested alone (CLSI 2009).

The Etest strip is a plastic drug-impregnated strip on one side which generates a stable concentration gradient for ceftazidime and the other side generates the concentration gradient of ceftazidime/clavulanic acid. The manufacturer recommends a ≥8-fold reduction in cephalosporin MICs in the presence of clavulanic acid for

confirmation of ESBL production. Similar strip containing cefotaxime and cefotaxime/clavulanic acid are now available. This has improved the ability to detect ESBL types which preferentially hydrolyze cefotaxime such as CTX-M-type enzymes.

Another confirmatory test for ESBL production is the double disk approximation test (DDAT) described by Jarlier et al. in 1998. This test is done by placing cephalosporin disks (e.g. ceftazidime and/or cefotaxime) close (20 to 30 mm centre to centre) to an amoxicillin/clavulanic acid disk on a plate inoculated with the test organism. Enhancement of zone of inhibition of the cephalosporins caused by the synergy of the clavulanic acid in amoxicillin/clavulanic acid disk exhibits positive results of ESBL-production (Jarlier et al.) For this test, precise placement of the disks, correct storage of the clavulanic acid-containing disks and performance of appropriate control tests are critical to the sensitivity of this method (Moland and Thompson 1994). However, in a study comparing Etest and DDAT by Florijn et al. (2002) had concluded that DDAT is a cheap and reliable method for detection of *E.coli*, *Klebsiella* spp., and *Proteus mirabilis* isolates suspected of carrying ESBL. This test performs better in a routine setting than the Etest, which often yields a result that cannot be interpreted. This was because of the reading of one of the antibiotic was out of the range on the strip and no ratio could be determined.

In a study by Wiegand et al. (2007), Etest strips and combination disks have showed sensitivity of 94 and 93%, and specificity of 85 and 81%, respectively. The double-disk test with ceftazidime, cefotaxime, cefpodoxime, and cefpirome showed the highest specificity and positive predictive value among all test methods (i.e. 97 and 98%, respectively).

However, all of the tests mentioned above, did not define how to differentiate the CTX-M-type ESBLs from other types of enzymes. With the understanding that these enzymes may produce less activity against ceftazidime, they are easily missed if the laboratories are using ceftazidime alone for screening.

1.5.2 Genotypic Detection

Phenotypic tests do not differentiate different enzymes in ESBL-producing organisms. To differentiate different enzymes of ESBLs, molecular methods have been developed. Genotypic or molecular method helps to detect TEM, SHV or CTX-M types of ESBLs. However this test usually restricted to reference laboratories and to molecular surveillance studies (Pitout and Laupland, 2008). PCR amplification is a commonly used molecular method for detection of ESBL genes, and sometimes followed by sequencing. Sequencing is essential to discriminate between the non-ESBL parent enzymes (e.g. TEM-1, TEM-2 or SHV-1) and different variants of TEM or SHV ESBLs (e.g. TEM-3, SHV-2 etc) (Bradford 2001).

For CTX-M-type enzymes, four sets of primers are required to amplify group-specific CTX-M β-lactamases genes. Several recent studies have described various approaches for the detection of different $bla_{\text{CTX-M}}$ genes. This includes a study by Pitout et al. (2004), they have developed four sets of primers which able to amplify single DNA fragment for each groups of $bla_{\text{CTX-M}}$ genes. Li Xu et al. (2005) have developed a rapid and accurate multiplex PCR assay for simultaneous amplification of all $bla_{\text{CTX-M}}$ genes and differentiation of the five groups. Other methods described were real time PCR and pyrosequencing (Birkett et al. 2007; Naas et al. 2007).

1.6 TREATMENT AND MANAGEMENT

With the ability of ESBL-producing organisms to hydrolyze many β-lactams antibiotics, the choice of antibiotic for treatment of its infection is greatly reduced. And this situation has been made worse by the fact that the ESBLs genes are encoded on plasmids, a mobile genetic element. This plasmid also carries genes encoding resistance to other class of antibiotics such as aminoglycosides and trimetophrim/sulfamethoxazole. Plasmids are easily transmissible to other bacterial strains and makes control of these multidrug resistant organisms difficult. Though some ESBL strains may appear susceptible to cephalosporins *in vitro*, they should not be reported as 'susceptible' as treatment of ESBL infection with any cephalosporins may lead to treatment failure. The failure rates are high when MICs of the cephalosporins are elevated but still within the susceptible range (Paterson et al. 2001).

β-lactam/β-lactamase inhibitor combinations may appear active against ESBL-producing strains. However, there are many organisms produce multiple β-lactamase enzymes, which may reduce the effectiveness of β-lactam/β-lactamase inhibitor combinations (Chanawong et al. 2002). Furthermore, published clinical experience with this combination is very limited, thus this combination cannot be considered as first-line therapy for serious infections caused by ESBL-producing strains (Paterson and Bonomo 2005).

Other non-β-lactam antibiotics such as quinolones may be regarded as the treatment of choice for infections caused by these organisms, provided they appear susceptible in vitro. However, with the increasing reports of quinolones-resistant among ESBL-producing organisms, this may limit its use especially as empiric therapy. Two

studies have suggested that synergy may occur when ciprofloxacin is added to β-lactam antibiotics in vitro. However no published data on the clinical use of this combination (Paterson and Bonomo 2005).

Carbapenems, including imipenem, meropenem and ertapenem have remained the antibiotic of choice for serious infections caused by ESBL-producing organisms. Carbapenems have a consistent susceptibility against ESBL-producing organisms. Furthermore, their activity appears to be least affected by increasing the size of the inoculum compared with other ß-lactam agents tested. Large-scale surveillance studies demonstrate that >98% of ESBL-producing *E.coli, Klebsiella* spp. and *P.mirabilis* isolates were susceptible to carbapenems (Hirakata et al. 2005; Sader et al. 2007; Goossens et al. 2005). Carbapenems should be considered as the first-line of therapy for infections caused by these organisms.

A review by Wong-Beringer (2001) on antibiotic therapy for ESBL-producing Enterobacteriaceae, has found that cumulative experience with imipenem consistently shows its superior value in treating serious infections due to ESBL-producing Enterobacteriaceae. And based on *in vitro* susceptibility data, meropenem is also a reasonable choice. Although carbapenems have been suggested for empirical therapy, its use must be based on the local epidemiological data as well as patients' risks. With the increasing reports of emergence of community-acquired ESBL infections, particularly the CTX-M-type enzymes, the empirical antibiotic therapy must be chose carefully.

For control of these organisms, infection control measures must be implemented in the hospitals. There are evidences that ESBL-producing Enterobacteriaceae are spread

from patient to patient. Infection control measures or guidelines must be employed to limit the spread of these multidrugs resistant organisms. The measures include hand hygienes, contact precautions with proper personal protective equipment and isolation or cohorting of patients where possible. Screening of high risk patients will allow identification of colonized patients so further measures can be employed to prevent infection. Discharge and transfer of patients that are colonized or infected by ESBL-producing organisms (or any multidrug resistant organisms for that matter) must be made clear to the receiving ends. This is to prevent the transmission of such organisms to other patients or staff. Continuous surveillance may also be employed to detect the emergence of such organisms or the in the event of outbreaks.

Other measure that may be taken probably is the restriction of third-generation cephalosporins and other unrelated antibiotics that may lead to selective pressure for the emergence of ESBL-producing strains. Control of multidrug resistant organisms requires full commitment from not only clinicians and other healthcare workers but also from patients, visitors and their family members.

REFERENCES

Ariffin, H., Navaratnam, P., Mohamed, M., Arasu, A., Abdullah, W.A., Lee, C.L. and Peng, L.H. 2000. Ceftazidime-resistant *Klebsiella pneumoniae* bloodstream infection in children with febrile neutropenia. *International Journal of Infectious Diseases*. **4**:21–25.

Asensio, A., Oliver, A., Gonzalez-Diego, P., Baquero, F., Perez-Diaz, J.C., Ros, P., Cobo, J., Palacios, M., Lasheras, D. and Canton, R. 2000. Outbreak of a multiresistant *Klebsiella pneumoniae* strain in an intensive care unit: antibiotic use as risk factor for colonization and infection. *Clinical Infectious Diseases*. **30:**55–60.

Barthélémy, M., Peduzzi, J., Bernard, H., Tancrede, C., Labia, R. 1992. Close amino acid sequence relationship between the new plasmid-mediated extended-spectrum β-lactamase MEN-1 and chromosomally-encoded enzymes of *Klebsiella oxytoca*. *Biochimica et Biophysica Acta*. **1122**:15-22

Bauernfeind, A., Grimm, H., Schweightart, S. 1990. A new plasmidic cefotaximase in a clinical isolate of *Escherichia coli*. *Infection.* **18**:294-298.

Bauernfeind, A., Stemplinger, I., Jungwirth, R., Ernst, S., Casellas, J.M. 1996. Sequence of beta-lactamases genes encoding CTX-M-1 (MEN-1) and CTX-M-2 and relationship of their amino acid sequences with those of other beta-lactamases. *Antimicrobial Agents and Chemotherapy*. **40**:509-513.

Bell, J.M., Turnidge, J.D., Gales, A.C., Pfaller, M.A., Jones, R.N. 2002. Prevalence of extended-spectrum beta-lactamase (ESBL)-producing clinical isolates in the Asia-Pacific region and South Africa: regional results from SENTRY Antimicrobial Surveillance Programme (1998-99). *Diagnostic Microbiology and Infectious Diseases.* **42**:193-198.

Bernard, H., Tancrede, C., Livrelli, C., Morand, A., Barthélémy, M., Labia, R. 1992. A novel plasmid-mediated extended-spectrum β-lactamase not derived from TEM- or SHV-type enzymes. *Journal of Antimicrobial Chemotherapy*. **29**:590-592.

Birkett, C.I., Ludlam, H.A., Woodford, N. et al. 2007. Real-time TaqMan PCR for rapid detection and typing of genes encoding CTX-M extended-spectrum-β-lactamases. *Journal of Medical Microbiology*. **56**:52-55.

Bradford, P.A. 2001. Extended-spectrum β-lactamases in the 21st Century: Characterization, Epidemiology, and Detection of This Important Resistance Threat. *Clinical Microbiology Reviews*. **14**(4):933-951.

Chanawong, A., M'Zali, F.H., Heritage, J., Xiong, J.H. and Hawkey, P.M. 2002. Three cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among

Enterobacteriaceae in the People's Republic of China. *Antimicrobial Agents and Chemotherapy*. **46**:630–637.

Duan, R.S., Sit, T.H., Wong, S.S., Wong, R.C., Chow, K.H., Mak, G.C., Yam, W.C., Ng, L.T., Yuen, K.Y., Ho, P.L. 2006. *Escherichia coli* producing CTX-M beta-lactamases in food animals in Hong Kong. *Microbial Drug Resistance.* **12**:145-148.

Florijn, A., Nijssen, S., Schmitz, F.J., Verhoef, J., Fluit, A.C. 2002. Comparison of Etest and Double Disk Diffusion Test for Detection of Extended Spectrum Beta-Lactamases. *European Journal of Clinical Microbiology and Infectious Disease.* **21**:241–243

Hawser, S.P., Bouchillon, S.K., Hoban, D.J. et al. 2009. Emergence of high levels of extended-spectrum-beta-lactamase-producing Gram-negative bacilli in the Asia-Pacific region: data from the Study for Monitoring Antimicrobial Resistance Trends (SMART) program, 2007. *Antimicrobial Agents and Chemotherapy*. **53**:3280–3284.

Jacoby, G.A. and Munoz-Price, L.S. 2005. The new β-lactamases. *New England Journal of Medicine.* **352**:380-391.

Kim, J., Lim, Y.M., Jeong, Y.S., Seol, S.Y. 2005. Occurrence of CTX-M-3, CTX-M-15 and CTX-M-9 extended-spectrum-beta-lactamases in Enterobacteriaceae clinical isolates in Korea. *Antimicrobial Agents and Chemotherapy*. **49**:1572-1575.

Kiratisin, P., Apisarnthanarak, A., Saifon, P., Laesripa, C., Kitphati, R., Mundy, L.M. 2007. The Emergence of a Novel ceftazidime-resistant CTX-M extended-spectrum β-lactamase, CTX-M-55, in both community-onset and hospital-acquired infections in Thailand. *Diagnostic Microbiology and Infectious Disease.* **58**:349-355.

Liu, W., Chen, L., Li, H. et al. 2009. Novel CTX-M b-lactamase genotype distribution and spread into multiple species of Enterobacteriaceae in Changsha, Southern *China. Journal of Antimicrobial Chemotherapy*. **63**:895–900.

Moland, E.S. and Thompson, K.S. 1994. Extended-spectrum β-lactamases of Enterobacteriaceae. *Journal of Antimicrobial Chemotherapy* **33**:666–668.

Munday, C.J., Xiong, J., Li, C., Shen, D., Hawkey, P.M. 2004. Dissemination of CTX-M type β-lactamases in Enterobacteriaceae isolates in the People's Republic of China. *International Journal of Antimicrobial Agents*. **23**:175-180.

Naas, T., Oxacelay, C., Nordman, P. 2007. Identification of CTX-M-type extended-spectrum-β-lactamase genes using real-time PCR and pyrosequencing. *Antimicrobial Agents and Chemotherapy*. **51**:223-230.

Paterson, D.L., Ko, W.C., Von Gottberg, A., Casellas, J/M., Mulazimoglu, L., Klugman, K.P., Bonomo, R.A., Rice, L.B., McCormack, J.G. and Yu, V.L. 2001. Outcome of cephalosporin treatment for serious infections due to apparently susceptible organisms producing extended-spectrum beta-lactamases: implications for the clinical microbiology laboratory. *Journal of Clinical Microbiology*. **39**:2206–2212.

Paterson, D.L., Bonomo, R.A. 2005. Extended-spectrum β-lactamases: a Clinical Update. *Clinical Microbiology Reviews*. **18**(4):657-686.

Perez, F., Endimiani, A., Hujer, K.M., Bonomo, R.A.2007. The Continuing Challenges of ESBLs. *Current Opinion in Pharmacology*. **7**:459-469.

Pitout, J.D., Hossain, A., Hanson, N.D. 2004. Phenotypic and molecular detection of CTX-M-β-lactamases produced by Escherichia coli and *Klebsiella spp. Journal of Clinical Microbiology*. **42**:5715-5721.

Sasaki, T., Hirai, I., Niki, M. et al. 2010. High prevalence of CTX-M beta-lactamase producing Enterobacteriaceae in stool specimens obtained from healthy individuals in Thailand. *Journal of Antimicrobial Chemotherapy.* **65**:666–668.

Subramaniam, G., Palasubramaniam, S. and Navaratnam, P. 2006. SHV-5 Extended spectrum β-lactamases in clinical isolates of *Escherichia coli* in Malaysia, *Indian Journal of Medical Microbiology*. 24(3):205-207.

Suzuki, S., Shibata, N., Yamane, K. et al. 2009. Change in the prevalence of extended spectrum-b-lactamase-producing Escherichia coli in Japan by clonal spread. *Journal of Antimicrobial Chemotherapy.* **63**:72–79.

Tofteland, S., Haldorsen, B., Dahl, K.H., et al. 2007. Effects of phenotype and genotype on methods for detection of Extended-Spectrum-β-Lactamase-producing clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* in Norway. *Journal of Clinical Microbiology*. **45**(1):199-205.

Tzouvelekis, L.S., Vatopoulos, A.C., Katsanis, G. and Tzelepi, E. 1999. Rare case of failure by an automated system to detect extended-spectrum beta-lactamase in a cephalosporin-resistant *Klebsiella pneumoniae* isolate. *Journal of Clinical Microbiology*. **37:**2388.

Wiegand, I., Geiss, H.K., Mack, D., Sturenburg, E., Seifert, H. 2007. Detection of extended-spectrum beta-lactamases among Enterobacteriaceae by use of semiautomated microbiology systems and manual detection procedures. *Journal of Clinical Microbiology* **45**:1167-1174

Williams, R.J. 1990. β-lactamase resistance. *Current Opinion in Infectious Diseases.* **3**:751-756.

Wong-Beringer, A. 2001. Therapeutic Challenges Associated with Extended-Spectrum, Beta-Lactamase-Producing *Escherichia coli* and *Klebsiella pneumoniae.* Pharmacotherapy Publications. 21(5). http://www.medscape.com/viewarticle/409716 (accessed 28th September 2010)

Woodford, N., Fagan, E.J., Ellington, M.J. 2006. Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum-β-lactamases. *Journal of Antimicrobial and Chemotherapy*. **57**:154-155.

Wu, J.J., Chen, H.M., Ko, W.C., Wu, H.M., Tsai, S.H., Yan, J.J. 2008. Prevalence of extended-spectrum β-lactamases in *Proteus mirabilis* in a Taiwanese university hospital, 1999 to 2005: identification of a novel CTX-M enzyme (CTX-M-66). *Diagnostic Microbiology and Infectious Disease*. **60**:169-175.