Original article

Detection of CTX-M-type ESBLs *Escherichia coli* at Universiti Kebangsaan Malaysia Medical Centre

Othman SN[1], Hussin S[2], Ramli R[3], Rahman MM[4]

Abstract:

Objective: To determine ESBLs and CTX-M-type ESBL-producing *Escherichia coli* at Universiti Kebangsaan Malaysia Medical Centre (UKMMC) by multiplex PCR. ***Materials and Methods:*** ESBL producing *E. coli* strains were confirmed by disk diffusion method. On the contrary, CTX-M-type ESBL-producing *E.coli* strains were confirmed by multiplex PCR. ***Results:*** Out of 554 collected E.coli isolates from UKMMC, 96 of these were detected as ESBL-producers. In 96 isolates, 76 viable strains were subjected to multiplex PCR for the detection of bla_{CTX} genes. In which 70 (92.1%) were CTX-M-type ESBLs with the majority of CTX-M-1 group (77.1%), followed by CTX-M-9 group (21.4%) and one (1.4%) from CTX-M-2 group. ***Conclusions:*** CTX-M-type ESBLs were the predominant ESBL types isolated at UKMMC. CTX-M-1 and -9 groups were found in majority of the clinical isolates. Although phenotypic characteristics based on disk diffusion test provided similar results, however, molecular detection of genes of organisms is important for further epidemiological investigations.

Keywords: CTX-M; multiplex PCR; Escherichia coli; ESBL

Bangladesh Journal of Medical Science Vol. 15 No. 02 April'16. Page : 257-261

Introduction:

Extended-spectrum-beta-lactamase (ESBL) is an enzyme produced by Enterobacteriaceae to confer resistance to extended-spectrum cephalosporins. ESBL-producing organisms have spread worldwide and pose threat to management of infections caused by these organisms. Infection control also faces a challenge when these plasmid-mediated enzymes are easily transmissible to other organisms. 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 b-lactamase, many CTX-M-type ESBL-producing organisms may be missed if CAZ alone is used for indicator for ESBL production.

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 identity $>94\%$ whereas $\leq 90\%$ identity was observed between the members belonging to distinct groups.[1] Detailed CTX-M-b-lactamases have been classified and reported by various authors.[2]

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% .[3]

In Malaysia, surveillance on the prevalence of ESBL-producing organisms has been going on since the mid 1990s.[4] 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. Another study done at a university hospital in Malaysia

1. Siti Norlia Othman
2. Salasawati Hussin
3. Ramliza Ramli
4. M M Rahman

Department of Medical Microbiology & Immunology, Universiti Kebangsaan Malaysia Medical Centre, 56000 Cheras, Kuala Lumpur, Malaysia.

Corresponds to: Siti Norlia Othman, Department of Medical Microbiology & Immunology, Universiti Kebangsaan Malaysia Medical Centre, 56000 Cheras, Kuala Lumpur, Malaysia.
E-mail: ctnorlia@ppukm.ukm.edu.my/mmr@ppukm.ukm.edu.my

reported bla_{SHV-5} to be dominant in ESBL-producing *E.coli* and was postulated to have been acquired from ESBL-producing *Klebsiella pneumoniae* via plasmid transfer.[5]

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. Therefore the present study was undertaken keeping the above in view.

Materials and Methods:

Patients

This study was carried out for a period of six months at Universiti Kebangsaan Malaysia Medical Centre (UKMMC), one of the tertiary hospitals in the capital city of Malaysia, with 850 beds. Ethical approval was taken from ethical committee of Universiti Kebangsaan Malaysia Medical Centre (UKMMC),

Bacterial isolates

All *Escherichia coli* isolates from all specimens were included in this study. However, specimens from rectal swab and duplicate isolates from the same patient were excluded. All selected strains were collected and maintained on microbeads and stored at -70°C. The kept strains were subcultured on blood agar prior to processing.

Screening tests of ESBL: Escherichia coli isolates were screened as part of our routine antibiotic susceptibility test for ESBL-production by using disk diffusion method with ceftazidime and cefotaxime disks. The inhibition zone diameters were measured and interpreted according to the CLSI guidelines. Confirmation test for ESBL

Suspected ESBL-producing *Escherichia coli* isolates were subjected to confirmatory tests. These tests were performed on all suspected strains.

i) Disk Approximation Test

In disk approximation test, double-disks synergy method was used, using both cefotaxime (CTX) (30 mg) and ceftazidime (CAZ) (30 mg) disks placed 20mm apart (centre to centre) from an amoxicillin/clavulanic acid (AMC) (20/10 mg) disk.[6]Enhancement of inhibition zone indicating synergy between clavulanic acid and the test antibiotic is suggestive of the presence of an ESBL.

ii) Combination Disks Diffusion Test

Combination disks diffusion tests were also carried out for all possible ESBL-producing strains. The disks used were: cefotaxime (30 mg), cefotaxime/ clavulanic acid (CTX/CLA) (30/10 mg) and ceftazidime (30 mg), ceftazidime/ clavulanic acid (CAZ/CLA) (30/10 mg). Regardless of the final zone diameter size, a ≥5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone indicated an ESBL-production.[7]

Quality control strains *E.coli* ATCC^{Ò} 25922 and *Klebsiella pneumoniae* ATCC^{Ò} 700603 were included in the tests.

PCR amplification for detection of bla_{CTX} genes: All confirmed ESBL-producing strains were subjected for CTX-M-type ESBL detection using multiplex PCR using the primers and methods described.[8]

The study was ethically approved by the ethical committee of Universiti Kebangsaan Malaysia.

Results:

Prevalence of ESBL-producing *Escherichia coli*

A total of 554 *E.coli* species were isolated from 545 patients during this six months study period. Out of these, 9 patients had two different strains of *E.coli* which one of each was ESBL-producer. Altogether, 111 isolates (20.0%) were screened positive by routine antibiotic susceptibility test. A total of 96 (86.5%) confirmed as ESBL-producers from combination disks diffusion tests and disk approximations tests. From this, prevalence of ESBL-producing *E.coli* isolated at UKMMC was 17. 3%(Table-I).

Source of isolates: The sources of isolation were classified into 5 representative sources. Specimens of body fluid were grouped together with blood specimens, and tissue specimens were grouped together with swab and pus specimens in respect to the benches they were processed in the laboratories. It was observed that although more than half (53.1%) of the *E.coli* isolates were from urine samples, only 48 (16.3%) were ESBL-producers as compared to swabs/tissue isolates, which 22.2% (n=42) of the isolates were ESBL-producers (Table 4.3.1). The ESBL rates among blood/body

Table-I: ESBL-producing E.coli from different source of isolates

Source of isolates	*E.coli*		Total n=554
	ESBL n=96 (%)	Non-ESBL n=458 (%)	
Urine	48 (16.3)	246 (83.7)	294
Swab/tissue	42 (22.2)	147 (77.8)	189
Blood/body fluid	4 (9.1)	40 (90.9)	44
Respiratory samples	2 (11.1)	16 (88.9)	18
Stool	0 (0.0)	9 (100.0)	9
	Pearson Chi-Square = 7.822 (p = 0.098)		

Table-2: Distribution of CTX-M-type ESBLs detected by PCR

PCR for *bla*CTX-M gene	Frequency (n=76)	Percentage (%)
CTX-M-1 group	54	71.0
CTX-M-2 group	1	1.3
CTX-M-8/25 group	0	0.0
CTX-M-9 group	15	19.7
Non-CTX-M	6	7.9

fluid isolates and respiratory isolates were 9.1% and 11.1%, respectively. They were no stool isolates in ESBL-producer (Table-1) And we found there was no significant difference ($p > 0.05$) between sources of isolation in ESBL-producing *E.coli* isolated. *Antimicrobial resistance rates in ESBL-producing isolates:*ESBL-producing isolates showed significantly higher resistance rate than the non-ESBL-producing isolates in the majority of antimicrobial agents. Such higher resistance rates in ESBL-producing *E.coli* were observed in all antibiotics specifically cephalosporins. Among the ESBL-producing strains, the ceftazidime and cefotaxime resistance rates were 75.0% and 99.0%, respectively. Regarding the non-ESBL-producing strains, the ceftazidime and cefotaxime resistance rates were only 4.8% and 10.3%, respectively. Resistance rates for gentamicin and co-trimoxazole among the ESBL-producers were 40.6% and 64.6% respectively. However, resistance rates for amikacin and piperacillin/tazobactam were slightly lower among the ESBL-producing strains (12.5% and 22.9%), respectively). All ESBL-producing *E.coli* were resistant to ampicillin. No isolates were found to be resistant to imipenem and meropenem.

Molecular detection of CTX-M-type ESBL-producing *Escherichia coli*

Out of these 76 tested strains, 70 (92.1%) were positive for $bla_{\text{CTX-M}}$ gene and the other 6 (7.9%) were negative for $bla_{\text{CTX-M}}$ gene. From these, the rate of CTX-M-type ESBL *E.coli* isolated in UKMMC during this study period was 92.1%. Among these tested strains, 54 (71.0%) were positive for $bla_{\text{CTX-M-1 group}}$ gene, 15 (19.7%) were positive for $bla_{\text{CTX-M-9-group}}$ gene and 1 (1.3%) was positive for $bla_{\text{CTX-M-2-group}}$ gene.(Table- II)

Phenotypic testing for the CTX-M-type ESBLs isolates: Among all PCR-tested strains, only 76.0% showed 'suspicious' zone of inhibition to CAZ but almost all (98.7%) showed 'suspicious' zone of inhibition to CTX in routine AST screening test (Table 4.5.1). Meanwhile, in triple approximation disk test, all except four (7.4%) from CTX-M-1 group and two (13.3%) from CTX-M-9 group showed enhancement to both CAZ and CTX substrates in the presence of clavulanic acid in the AMC disk. Among the strains positive for presence of $bla_{\text{CTX-M-1-group}}$ gene, from screening test, seven (13.0%) were tested positive (by the zone diameter difference) by CTX/CLA combination disk only and another 47 (87.0%) were positive for both CAZ/CLA and CTX/CLA combination disks. Whilst among the positive for presence of $bla_{\text{CTX-M-9-group}}$ gene, almost half of them (46.7%) were positive by CTX/CLA combination disks only. For the only strain positive of $bla_{\text{CTX-M-2-group}}$ gene, it showed 'suspicious' zone to both CAZ and CTX disk during screening but was positive for CTX/CLA combination disk only in the confirmatory ESBL test. *Antibiotic susceptibility patterns among the CTX-M-type*

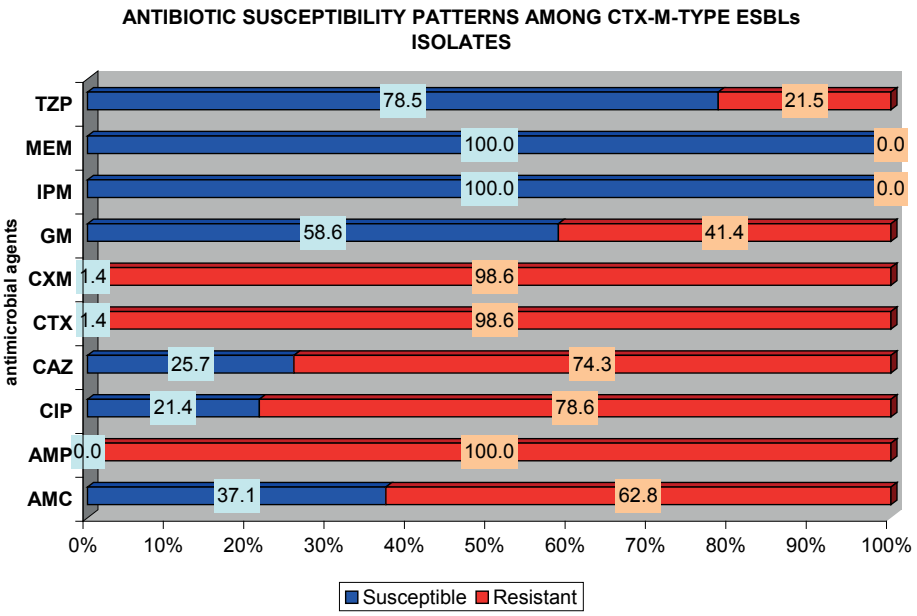

Figure 1: Antibiotic susceptibility and Resistant of CTX-M-type ESBL isolates

Legends: AMC=amoxicillin/clavulanate; AMP=ampicillin; CIP=ciprofloxacin; CAZ=ceftazidime; CTX=cefotaxime; CXM=cefuroxime; GM=gentamicin; IPM=imipenem; MEM=meropenem; TZP=piperacillin/tazobactam

*ESBLs isolates:*We found 70 out of 76 strains (92.1%) were CTX-M-type ESBLs. Among these 70 isolates, the resistance rates to cefotaxime was higher than the rate to ceftazidime (98.6% vs 74.3%). These isolates also showed cross-resistance with ciprofloxacin (78.6%).Gentamicin and piperacillin/ tazobactam resistance rates were slightly lower, 41.4% and 21.5% respectively. No isolates showed resistant to imipenem or carbapenem (Fig.1).

Discussion:

In the study period, the prevalence of ESBL-producing *E.coli* isolated in the centre was 17.4%. This finding was similar to the estimated prevalence by Ministry of Health of Malaysia[9] [12] (2001), ranged between 17% and 27%. However, from the National Survey conducted amongst some of the Asian countries – Korea, Japan, Singapore and Malaysia reported the presence of ESBL in 5 to 8% of *E.coli* isolates. Thailand, Taiwan, Philippines and Indonesia reported slightly higher rates of 12 to 20% .[10]

Among the CTX-M-producing *E.coli*, the highest resistance rates were to cefuroxime and cefotaxime (98.6% respectively) and ceftazidime (74.3%). This supports the findings that CTX-M-type ESBLs hydrolyze CTX more than they do on CAZ. For detection of ESBL-producing organisms, use of CAZ alone is no longer adequate as indicator of ESBL productions. In our study, only 76% of CTX-M-type ESBLs showed resistance or 'suspicious' zone of inhibition on our routine screening test. The result showed that if we only used CAZ disk alone for the screening test, we might have missed another 24.0% of CTX-M-type ESBL strains.

In the present study all isolates including the non-CTX-M-type ESBLs showed enhancement to at least one of the substrates, and 70 strains (92.1%) showed enhancement to both substrates. However, the degree of enhancement were varied, thus any enhancement was regarded as positive for ESBL-producers. The interpretation of the enhancement zones is quite subjective and the sensitivity may be reduced when ESBL activity is very low leading to a wide zone of inhibition around the cephalosporin disks.[11] From this study, we also found some strains with straightening patterns instead of the usual enhancement patterns. These strains were determined for their ESBL-producers by the zone difference in the combination disks tests, and all turned out to be negative for ESBL-producers and were not tested further. These strains may harbour both AmpC-type b-lactamases and ESBLs.[12] The co-existence of both enzyme types in the same strain results in false negative tests for the detection of ESBLs. This is because AmpC b-lactamase resists inhibition by CLA. Hence obscure the synergistic effect of CLA and cephalosporins against ESBL. Unfortunately, we did not test the presence of ESBL enzymes by molecular methods for these strains. The advantage of this double approximation disk test is inexpensive and technically simple method for the detection of clavulanic acid synergy.[13] But the distance of disk placement for optimal sensitivity has not been standardized.

For confirmatory test, we used combination disk test using both ceftazidime/clavulanic acid (CAZ/CLA) and cefotaxime/clavulanic acid (CTX/CLA) against ceftazidime (CAZ) and cefotaxime (CTX) alone. Prior to this study, all suspected ESBL-producing Enterobacteriaceae were subjected for confirmation test using ceftazidime-ceftazidime/clavulanic acid Etest strip alone. With the advent of presence of CTX-M-type ESBL, and the recommendations by CLSI[7] to use both ceftazidime and cefotaxime, alone and in combination with clavulanic acid. And due to the financial constraint, this combination disks diffusion test were used in our study. The combination disks is cheaper than the Etest strip and the test is technically easy to perform and results is easy to interpret too, even by an inexperience staff because it only requires difference in the diameter of zone of inhibition. However, correct storage of these clavulanic-containing disks and appropriate control tests are very critical to the potency of the disks and the sensitivity of this method. [14]

Currently, molecular method can be used for detection of ESBL enzymes in clinical isolates. For ESBL-producers caused by bla_{TEM} or bla_{SHV} genes, oligonucleotide sequencing following PCR amplification is a gold standard for identification of specific point mutation of bla_{TEM} and bla_{SHV} genes.[15]In our study, we tested 76 ESBL-producing *E.coli* isolates for presence of bla_{CTX-M} genes. Out of these, 70 (92.1%) were positive for bla_{CTX-M} genes. Of these, 54 (71.0%) were from CTX-M-1 group, 15 (19.7%) were of CTX-M-9 group, one (7.9%) was CTX-M-2 group and no isolates were from CTX-M-8 or -25/26 groups. These findings demonstrated that CTX-M-type ESBLs were prevalence among *E.coli* isolates in our centre, and these were in parallel to another Malaysia study[16] and other worldwide trends where the CTX-M-type ESBLs have been dominant in most studies. However, we did not look for presence of bla_{TEM} and

bla$_{SHV}$ genes to demonstrate the actual prevalences of all types of ESBL enzymes in our clinical isolates. From the study it is recommended that a good and reliable laboratory phenotypic technique is essential to ensure all types of ESBLs, especially the CTX-M-type ESBLs. PCR amplification and sequencing of *bla*$_{CTX-M}$ genes could be efficiently used to characterize the organisms producing CTX-M-b-lactamases and other types of beta-lactamases.

Acknowledgement:
The authors thank the patients for their consent to participate in the study and wish to acknowledge for every support by Department of Medical Microbiology & Immunology, Universiti Kebangsaan Malaysia Medical Centre to conduct the study.
Conflict of interest: None declared.

References:

1. Ariffin H, Navaratnam P, Mohamed M, Arasu A, Abdullah WA, Lee CL, Peng LH. Ceftazidime-resistant *Klebsiella pneumoniae* bloodstream infection in children with febrile neutropenia. *International Journal of Infectious Diseases*. 2000; **4:**21–25.
2. Bradford PA. Extended-spectrum b-lactamases in the 21st Century: Characterization, Epidemiology, and Detection of This Important Resistance Threat. Clinical Microbiology Reviews. 2001; 14(4):933-951
3. Kiratisin P, Apisarnthanarak A, Saifon P, Laesripa C, Kitphati R, Mundy LM. The Emergence of a Novel ceftazidime-resistant CTX-M extended-spectrum b-lactamase, CTX-M-55, in both community-onset and hospital-acquired infections in Thailand. *Diagnostic Microbiology and Infectious Disease*. 2007; **58:**349-355.
4. Lim K, Yasin R, Yeo C, Puthucheary S. Thong KL. Characterization of Multidrug Resistant ESBL-producing *Escherichia coli* Isolates from Hospitals in Malaysia. Journal of Biomedicine and Biotechnology. 2009;**165;**637.
5. Mugnaioli C, Luzzaro F, De Luca F, Brigante G, Perilli M, Amicosante G, Stefani S, Toniolo A, Rossolini GM. CTX-M-type extended-spectrum beta-lactamases in Italy: molecular epidemiology of an emerging countrywide problem. *Antimicrobial Agents & Chemotherapy*. 2006; **50**:2700-2706.
6. Chanawong A, M'Zali FH, Heritage J, Xiong JH. Hawkey PM. 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. 2002; **46:**630–637.
7. CLSI.2012. Antimicrobial Susceptibility Testing Standards. http://clsi.org/blog/2012/01/13/clsi-publishes-
8. Bonnet, R. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. *Antimicrobial Agents and Chemotherapy*. 2004;**48**:1-14.
9. Ministry of Health of Malaysia. Consensus Guidelines for the Management of Infections by ESBL-producing Bacteria. http://www.acadmed.org.my/index.cfm?&menuid=28. Accessed December, 2013
10. Pitout JD, Hossain A, Hanson ND. Phenotypic and molecular detection of CTX-M-b-lactamases produced by Escherichia coli and *Klebsiella spp*. *Journal of Clinical Microbiology*. 2004;**42**:5715-5721.
11. Pitout JD, & Laupland KV. Extended-spectrum β-lactamase-producing Enterobacteriaceae: an emerging public-health concern. *Lancet Infectious Disease*. 2008; **8**:159-166.
12. Subramaniam G, Palasubramaniam S. Navaratnam P. SHV-5 Extended spectrum b-lactamases in clinical isolates of *Escherichia coli* in Malaysia, *Indian Journal of Medical Microbiology*. 2006; **24(**3):205-207.
13. Tofteland S, Haldorsen B, Dahl KH, et al. Effects of phenotype and genotype on methods for detection of Extended-Spectrum-b-Lactamase-producing clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* in Norway. *Journal of Clinical Microbiology*. 2007; **45(**1):199-205.
14. Tzouvelekis LS, Vatopoulos AC, Katsanis G. Tzelepi E. 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*. 1999;**37:**2388.
15. Wu JJ, Chen HM, Ko WC, Wu HM, Tsai SH, Yan JJ. Prevalence of extended-spectrum b-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. 2008; **60**:169-175
16. Sekawi Z, Yusof R, Shamsudin MN. Extended-spectrum-Beta-Lactamase-producing E.coli from a tertiary hospitals in Malaysia: Emergence of CTX-M-type b-Lactamases Variation. *Research Journal of Microbiology*. 2008;**3**(6):489-493