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Detection of CTX-M-type ESBLs from *Escherichia coli* Clinical Isolates from a Tertiary Hospital, Malaysia

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Abstract:

The present study aims to detect CTX-M-type ESBL from Escherichia coli clinical isolates and to analyze their antibotic susceptibility patterns. One hundred of E. coli isolates were collected from different clinical samples from a tertiary hospital. ESBL positivity was determined by the disk diffusion method. PCR used for amplification of CTX-M-type ESBL produced by E. coli. Out of 100 E. coli isolates, twenty-four isolates (24%) were ESBL-producers. E. coli isolated from pus was the most frequent clinical specimen that produced ESBL (41.66%) followed by urine (34.21%), respiratory (22.23%), and blood (19.05%). After PCR amplification of these 24 isolates, 10 (41.66%) isolates were found to possess CTX-M genes. The CTX-M type ESBL producing E. coli against antibiotics belonging to different families showed the highest resistance rates to Ampicillin (100%), Cefotaxime (97%), Cefuroxime (95%), and Ciprofoxacin (86%). Carbapenem groups of antibiotics, Meropenem (89%) and Imipenem (85%) have the highest susceptibility rate among all antibiotics used in this study. The outcome of the antimicrobial susceptibility testing of significant CTX-M- type ESBL producing E. coli could be useful to avoid failure or prolong treatments.

Key words: CTX-M gene, ESBL, Escherichia coli, PCR.

Introduction:

A heterogeneous enzyme, extended-spectrum β-lactamase (ESBL) produced by *Enterobacteriaceae* showed resistance to a numerous group of antibiotics especially Cephalosporins, Penicillins, Monobactams and Carbapenems. Among the clinical isolates of *Enterobacteriaceae*, the formation of β-lactamases resistance mechanism is very frequent. Infections caused by ESBL producing organisms became a threat to infection control management and spread worldwide.

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ESBL-producing *E. coli* is now a serious concern in infection control therapies and higher prevalence rates in Asia-Pacific countries (1) and many parts of the world (2). The prevalence of extended-spectrum β-lactamases (ESBL), Metallo β-lactamase (MBL) and AmpC producing organisms prolong treatment and effective control (3). ESBL or MBL associated organisms has an effect of higher mortality and morbidity (4). In *Enterobacteriaceae*, resistance mechanisms of ESBLs producing CTX-M, TEM, and SHV types genes exhibit a major problematic alarming concern in various antibiotics (5). The formation of ESBLs producing gene (TEM, SHV, and CTX-M) have been reported in community and nosocomial settings across the world (6).

The CTX-M-type ESBL gene normally hydrolyze third generation antibiotic Cefotaxime (CTX) compared to Ceftazidime (CAZ). Moreover, the CTX-M type ESBL enzymes are resistant to Cefotaxime but persist sensitive to Ceftazidime (6). Therefore, these genes are named CTX-M-type ESBLs which are highly efficient in genetic elements due to the epidemic of plasmids (7). These enzymes are either plasmid-mediated or chromosomally mediated but commonly on a

plasmid in *Enterobacteriaceae* (2). Plasmid mediated CTX-M gene can transfer resistance genes to unrelated antimicrobials and within the bacterial strains (2, 8). CTX-M type ESBL producing *E. coli* developed co-resistance to various classes of antibiotics (9). Ceftazidime (CAZ) normally used to detect ESBL producing organisms, but in many cases, CAZ alone may not be able to detect CTX-M-type ESBL producing organisms (10). Till date, a various number of different variants of blaCTX-M-type enzymes has been identified throughout the world (10-12).

This present study aims to look at the current scenario of CTX-M-type ESBL producing *E. coli* isolates of different infectious specimens from a tertiary hospital, Malaysia using phenotypic methods and molecular based techniques.

Materials and Methods:

Bacterial Isolates

One hundred *E. coli* isolates were collected at Hospital Selayang, Malaysia from June 2017 to June 2018. All the samples were plated on Muller-Hinton agar plate (Oxoid, Basingstoke, United Kingdom) and incubated at 37°C for 24 hours to isolates *E. coli*. All the *E. coli* isolates were selected and reconfirmed by the standard biochemical assay described (13, 14). Among these *E.* coli isolates, 38 isolates were from urine samples, 21 isolates from blood, 13 isolates from stools cultures, 12 isolates from pus, 9 isolates from respiratory secretions blood cultures, and 7 isolates from sputum samples and.

Antibiotic Susceptibility

Antibiogram analysis were performed among all (100) isolates of *E. coli* by Kirby Bauer disk diffusion method (15) on Mueller-Hinton agar in accordance with CLSI (2017) (16). Ten different types of antibacterial agents namely Ampicillin (10 mcg), Amoxicillin/clavulanic acid (20/10 mcg), Cefotaxime (30 mcg), Ceftazidime (30 mcg), Cefuroxime (30 mcg), Ciprofloxacin (5 mcg), Gentamicin (10 mcg), Imipenem (10 mcg), Meropenem (10 mcg), and Piperacillin/tazaobactum (100/10 mcg) were used in this study. As a reference strains for susceptibility confirmation, ATCC-25922-*E. coli* was used in vitro.

Phenotypic Detection of ESBLs

Double Disk Synergy Test (DDST) was carried out on Muller-Hinton agar plate for the detection of ESBLs gene (4, 17). The synergy between a third-generation cephalosporins group of antibiotics, Cefotaxime 30mg (CTX) and Ceftazidime 30mg (CAZ) disk was placed 20mm center to center apart from Amoxicillin/clavulanic acid (20/10mg) (AMC) disk (17). After the overnight incubation, CLSI-2017 guidelines were strictly followed for measurement, interpretation, and enhancement of inhibition zone indicating synergy (an extended zone of inhibition towards the Amoxicillin-clavulanic acid) confirms the isolates possess ESBL gene (16). ATCC-25922-*E. coli* strains were used as a negative control (13).

CTX-M Gene Detection Using PCR

The boiling method was used to extract the genomic DNA in our study (18). A pure culture of ESBL positive bacterial strains was grown in BHI broth at 37°C for 24 hours. After incubation, 200 µl broth culture was added to 800 µl of distilled water and boiled for 10 minutes at 100°C and then centrifuged at 12,000×g for 2 minutes. The supernatant containing genome was the DNA template for PCR. After amplification, the purified DNA was stored at -20°C for further process. The targeted CTX-M-type gene detection among the 24 ESBL positive isolates was confirmed using PCR as described (19) with minor modification. Briefly, total volume (25 µL/reaction) contained the mixtures of 2.5 µL PCR buffer 10X, 1 µL for each primer (blaCTX-M-F and blaCTX-M-R), $MgCl_2$ (25mM) 1.5 µL, 0.5 µL dNTPs (10mM), *Taq* DNA polymerase 0.2 µL, 8.3 µL of water (nuclease-free) and 10 µL DNA template. Total 25 µL was used in each reaction tube for PCR. The blaCTX primers (Table 1) were used according to conditions (Table 2) for PCR amplification. In agarose gel electrophoresis process, 1.5% agarose gel for PCR product was stained with ethidium bromide (0.5 mg/ml) and was run at 100 volts for 35 minutes and visualised under UV light.

Table 1. Primers used for detection of blaCTX-M.

Genes	Primer sequences	Amplicon size (bp)	References
blaCTX-M-F	ACCGCCGATAAT TCGCAGAT	588	(19)
blaCTX-M-R	GATATCGTTGGT GGTGCCATA		

Table 2. PCR conditions to obtain targeted gene.

Step	Temperature	Time	Cycle
Initial denaturation	94ºC	5 min	1
Denaturation	94ºC	1 min	35
Annealing	59.2ºC	30 sec	
Extension	72ºC	1 min	
Final extension	72ºC	5 min	1
Storage	4ºC		

Statistical Analysis:

In this present study, the Chi square test was used for data analysis. The value of $p< 0.05$ was considered as the significant statistical difference.

Results:

In the present study, disk diffusion synergy test and E-test were carried out to confirm ESBL production. Among the 100 *E. coli* isolates only 24 isolates were found to produce ESBL positive gene (Table 3), and the synergism of ESBL isolates was observed (Fig. 1).

Different types of clinical specimens were the sources of *E. coli* isolates. Among the 12 *E. coli* isolates of pus, 5 (41.66%) isolates were ESBL-producers while 13 (34.21%) out of 38 *E. coli* isolates of urine were ESBL positive. None of the isolates of stool and sputum were positive for ESBL genes. The ESBL production rates among the respiratory and blood isolates were 2 (22.23%) and 4 (19.05%), respectively. These prevalence rates were not significant (p> 0.05) between the clinical specimen and ESBL-producers (Table 3).

Table 3. ESBL producing clinical isolates.

Sources / specimens	*E. coli*		Total (n=100)
	ESBL (n=24)	Non ESBL (n= 76)	
Urine	13 (34.21%)	25 (65.79%)	38
Blood	4 (19.05%)	17 (80.95%)	21
Respiratory	2 (22.23%)	7 (77.77%)	9
Stool	0	13 (100.00%)	13
Pus	5 (41.66%)	7 (58.34%)	12
Sputum	0	7 (100.00%)	7
Pearson Chi-Square = 10.839 (p = 0.054)			

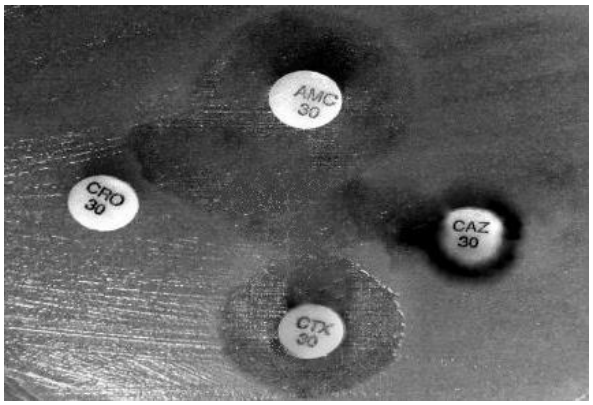

Figure 1. Double disk synergy test with ESBL producing E. coli.

Different types of antibacterial agents showed higher resistance rate by *E. coli* isolates (CTX-M-type ESBL) while less resistance in non-ESBL isolates. From results, the resistance rates of cephalosporins were higher than any other groups of antibiotics used. Among the *E. coli* strains (ESBL producers), highest resistance was shown towards Ampicillin with the rate of (100%) and subsequently Cefotaxime (97%), Cefuroxime (95%), Ciprofloxacin (86%), and Ceftazidime (72%) (Fig. 2). In non-ESBL producing strains, resistance rate was for Ampicillin 78%, Cefotaxime 67%, Cefuroxime 85%, Ciprofloxacin 66%, and Ceftazidime 52%. Surprisingly, Meropenem (11%), and Imipenem (15%) showed lower resistance among ESBL producing isolates.

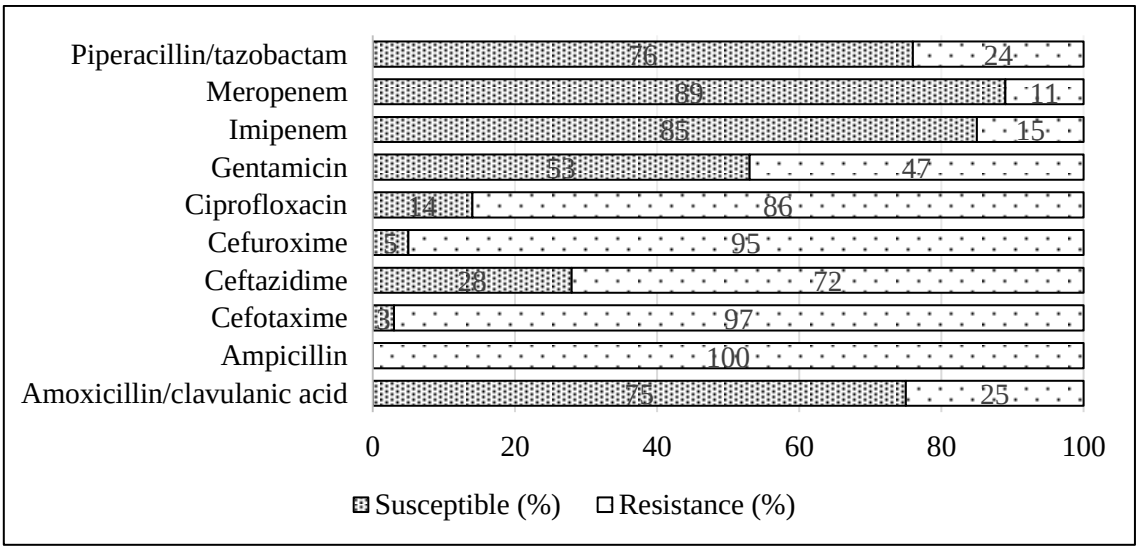

Figure 2. Susceptibility of different types of antibiotic among CTX-M-type ESBLs positive E. coli isolates.

However, resistance rates are higher in most of the *E. coli* isolates which produce the ESBL gene than non-producing ESBL isolates. Out of these 24 ESBL positive strains, 10 (41.66%) isolates showed positive for the blaCTX-M gene, and the rest 14 (58.34%) isolates did not produce any blaCTX-M gene (Fig.3).

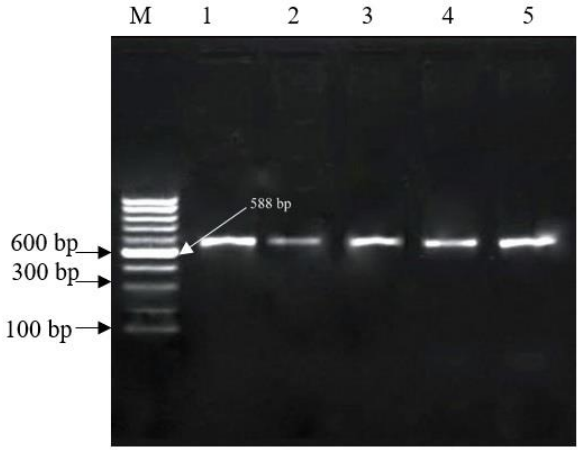

Figure 3. CTX-M-type ESBL positive E. coli isolates after PCR study, M: DNA ladder, Lanes: from 1-5 represent amplified product (588 bp) of CTX-M positive isolates.

Discussion:

The higher prevalence rates of ESBL producing *E. coli* due to various resistance mechanisms causes hospital-acquired infections. We have observed the ESBL phenotype, CTX-M-type gene and their antibiotics susceptibility patterns among the 100 *E. coli* isolates from a tertiary hospital, Malaysia.

Various ESBLs genes production cause outbreaks throughout the world. It is essential to do epidemiological identification and molecular classification of ESBL genes (20). In the present study, 24 isolates were ESBL producer among 100 clinical isolates of *E. coli*. The projected prevalence rate of *E. coli* isolates (ESBL producer) ranged between 7% and 19% by the Ministry of Health, Malaysia (2001) (21) which is in agreement with our study. Moreover, a recent study has reported that 18.8% of isolates were ESBL producers from Hospital Tengku Ampuan Afzan (HTAA) in 2016 (22). According to the recent studies, the high prevalence rates of 56.92% (23), 62.9% (24), 62.8% (25), 46.3% (26), 72.3 % (27), 57.7% (28), 91.7% (29), and 90.91% (30) ESBL producing isolates was observed across the world. Similarly, studies have reported the closer prevalence rates of 24% (31), 25.83% (32), and 26.87 % (33) ESBL producing *E. coli* compared to our study, respectively. However, the estimated presence of ESBLs producing *E. coli* should be between 5 to 8% in Asian countries (10).

Within the past decades, E. coli genotype CTX-M has extensively found and spread across the world (34). These enzymes have become a severe public health concern causing outbreaks throughout the worldwide. Studies have revealed that the predominant gene among the β-lactamase is CTX-M gene. Currently, the molecular method (PCR amplification) is the standard method for the detection of ESBL producing (blaTEM, blaSHV, and blaCTX-M) gene (10). Our study found that, among the 24 ESBL producing *E. coli* isolates, 10 (41.66%) were blaCTX-M genes producer (Figure 3) while 90% isolates were blaCTX-M gene producer in 2016 (22). However, we did not test for other ESBL producing gene (blaTEM and blaSHV) among our clinical isolates. Similarly, various prevalence rates of 92.1% (10), 20% (35), and 11.8% (36) CTX-M gene were observed in Malaysia. Moreover, different prevalence rates of 82.6% (37), 28% (38), 56% (39), 95.2% (40), 90.6% (26) and 82.5% (23) CTX-M have been dominant across the world.

The highest resistance rates were to Cefotaxime (97%) and Cefuroxime (95%) respectively while Ceftazidime (72%) among the CTX-M-types ESBL isolates. These findings support that CAZ alone is not appropriate to confirm ESBL productions. Moreover, cephalosporin groups of antibiotics showed a higher resistance rate in Gram negative bacteria (10, 41). This present study revealed that the carbapenems groups of antibiotic such as Meropenem (89%) and Imipenem (85%) are more susceptible to ESBL-producing CTX-M-type *E. coli* compared to cephalosporins antibiotics (Cefotaxime 3%, Cefuroxime 5%, and Ceftazidime 28%). These findings could be important to treat Gram negative infections with carbapenems.

The most prevalent CTX-M type ESBLS has become an alarming phenomenon due to their unpredictable epidemiological changes in antibiotics resistance, allotypic diversity, rapid and global spread in *Enterobacteriaceae* especially encountered in *E. coli*. Some of the possible factors such as geographical locations, proficiency level of technical staffs, different types of antibiotics usages, varied guidelines and techniques might be involved in resistance mechanisms across the world. In this regards, strict surveillance on antibacterial therapeutic agents, emphasise the efficacy of molecules, essential laboratory detection and overcome the limitation of alternative therapeutic management could be possible features to solve or decreases the rapid dissemination of CTX-M type ESBLs.

Conclusion:

The higher prevalence rate of ESBL producing (CTX-M) E. coli strains has extended into a serious level in Malaysia as well as worldwide. The molecular classification of CTX-M-type ESBL producing E. coli isolates may harbour multiple ESBL genes too. Our findings recommend, an early and regular screening process on clinically important isolates demands an extra concern.

Reliable monitoring is essential to stop spreading ESBLs genes in the local community as well as worldwide. Our findings on antibiotic susceptibility patterns could be useful for quality assurance, and implementation of infectious diseases control managements. However, molecular characterization of genes requires additional epidemiological investigations.

Conflicts of Interest: None.

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لكشف عن ESBLs من النوع CTX-M من الإشريكية القولونية السريرية المعزولة من المستشفى الثالث، ماليزيا

فضل م.ك.ك **فرزانة .ي** **نجنين أ** **راشد م.ا** **نازمول .م.ح.م**

الخلاصة:

تهدف هذه الدراسة إلى الكشف عن نوع CTX-M ESBL من الإشريكية القولونية العزلات السريرية ولتحليل أنماط حساسيتها للمضادات الحيوية. تم جمع مئة من عزلات الإشريكية القولونية من عينات سريرية مختلفة من المستشفى الثالث. تم تحديد إيجابية ESBLبواسطة طريقة نشر القرص. PCR تستخدم لتضخيم ESBL CTX- نوع من إنتاج الإشريكية القولونية من بين 100 عزلة من الإشريكية القولونية ، كانت أربع وعشرون عزلة (24 ٪) من منتجي ESBL. كانت الإشريكية القولونية المعزولة من القيح أكثر العينات السريرية التي أنتجت (41.66 ESBL٪) متبوعة بالادرار بنسبة (34.21٪)، والجهاز التنفسي (22.23٪)، والدم (19.05٪). بعد تضخيم PCR لهذه الاربع والعشرون عزلة، تم العثور على أن 10 (41.66 ٪) منها تمتلك جينات CTX-M. أظهرت CTX-M من نوع ESBL الذي ينتج الإشريكية القولونية مضادة للمضادات الحيوية التي تنتمي إلى عائلات مختلفة أعلى معدلات مقاومة للأمبيسيلين (100 ٪)، السيفوتاكسيم (97 ٪) ، السيفوروكسيم (95 ٪)، والسيبروفوكساسين (86 ٪). مجموعات الكاربابينيم من المضادات الحيوية، الميروبينيم (89 ٪) وإيميبينيم (85 ٪) لديها أعلى معدل حساسية بين جميع المضادات الحيوية المستخدمة في هذه الدراسة. يمكن أن تكون نتائج اختبار الحساسية المضادة للميكروبات لـ E. ESBL من نوع CTX-M- إنتاج الإشريكية القولونية مفيدة لتفادي الفشل أو إطالة مدة العلاج.

الكلمات المفتاحية : CTX-M جينات، ESBL ، الإشريكية القولونية ، PCR.