Molecular Characterization of CTX-M15 Beta Lactamase Producing Escherichia coli Isolates in Intensive Care Units

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ABSTRACT

The rapid emergence of multiresistant microbial pathogens is a serious threat to human health. Extended spectrum beta lactamase (ESBL)-producing Escherichia coli is a superbug causing worldwide outbreaks, CTX-M has become the most commonly detected ESBL genotype; these enzymes have become predominant worldwide, the most common type of CTX-M reported in various geographic regions is CTX-M15. A total of 80 Escherichia coli (E. coli) isolates were recovered from different clinical samples of intensive care units (ICUs) patients. These isolates were identified by Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), in vitro antimicrobial susceptibility was investigated by VITEK 2 instrument, ESBL production was assessed phenotypically and ESBL genes (TEM, SHV and CTX-M15) confirmed genotypically by PCR and further confirmation for CTX-M15 by DNA sequencing. More than 50% of ESBL E. coli isolates were multidrug resistant; while carbapenams remains the most active compound among the studied isolates. Among ESBL E. coli, CTX-M-15 was 90% which was confirmed by sequencing. To our knowledge, this is the first report to study CTX-M-15 in E. coli isolates in Sharkia governorate. This study showed a high rate of ESBL E. coli with the widespread dissemination of CTX-M-15 which emphasize the need for employing an excellent management program in antibiotic therapy.

Keywords
CTX-M15, ICUs, E. coli, PCR, ESBL, Sequencing

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Introduction

ESBLs are beta-lactamase group of enzymes that break down antibiotics belonging to the penicillin and extended-spectrum/third generation cephalosporins (e.g., ceftriaxone, cefotaxime and/or ceftazidime) and render them ineffective. ESBL has generally been defined as transmissible beta lactamases that can be inhibited by clavulanic acid, tazobactam or sulbactam (Bouxom et al., 2018).

ESBLs are of great concern because they are often plasmid-associated that carry antimicrobial resistance genes for other antibiotics which results in treatment problems, higher morbidity, mortality, and increased health care costs (Zhang et al., 2019).

More than 125 CTX-M enzymes are currently known. Despite their name, a few are more active on ceftazidime than cefotaxime. They have mainly been found in strains of
Salmonella enterica serovar Typhimurium and E. coli, but have also been described in other species of enterobacteriaceae, currently CTX-M-15 is the most widespread type in E. coli (Bonnedahl et al., 2014).

While E. coli was the first species in which the CTX-M type ESBLs was reported as early as 1990. Now, the CTX-M phenotype has also been reported in E. coli strains isolated from healthy humans, livestock, companion animals, food products, and sewage indicating a large scale of the reservoirs harboring and disseminating these ESBLs (Melo et al., 2018).

Infections with ESBL E coli have been linked with poorer clinical outcomes and prolonged hospitalization on average compared to infections. This is likely exacerbated in resource-limited settings, where access to newer antibiotics is often limited by cost (Maina et al., 2017).

The aim of this study was to characterize CTX-M15 beta lactamase producing E. coli isolates as a cause of infections in Zagazig university hospitals ICUs and to study its incidence, antimicrobial resistant profile and to investigate the presence of ESBL genes as a cause of antimicrobial resistance among the studied isolates.

Materials and Methods

This study is descriptive cross-sectional study, a total of 80 E. coli isolates were recovered from patients admitted to the ICUs of Zagazig University Hospitals over 6 months period from June 2016 to December 2017. Approval for this study was obtained from Research Administration and Research Ethics Committee of Faculty of Medicine, Zagazig University; the samples were sputum, urine, blood, pus, and body fluid. The collected samples were transported to the microbiology laboratory and inoculated on suitable media that incubated at 37°C for 24-48 hours.

Identification

All the isolates were subjected to Gram stain then identified by (MALDI-TOF MS) using the VITEK MS system (Biomerieux) small amount of freshly grown E. coli isolates were picked and smeared on the wells then overlaid with 1µL of cyano hydroxy cinnamic acid (CHCA) matrix solution and allowed to dry before sent to acquisition station of MALDI-TOF MS. E. coli isolates were further investigated by:

Antibiotic susceptibility testing

Antibiotic susceptibility testing was carried out using Vitek 2 System (card no 71) for Gram negative bacilli (Biomerieux) in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2018), the following antibiotics were included: Ampicillin, Amoxyclov, Trimethoprim/sulfamethoxazole (SXT), Amikacin, Imipenem, Meropenem, Tobramycin, Azetronam, Cefipeme Ceftazidime, Cefotaxime, Ceftriaxone Piperacillin/Tazobactam, Gentamicin, Ciprofloxacine, Norfloxacin, Tigecycline Nitrofurantoin, Ampicillin/Subbactam

ESBL production detection

ESBL production was detected using Phenotypic screening test by Kirby –Bauer disk diffusion method using Mueller-Hinton agar, according to manufacture structure and to the CLSI criteria (CLSI, 2018), ESBL phenotypes confirmed by the double-disk synergy (DDS) test that carried out with amoxicillin-clavulanat (20 + 10 µg), cefotaxime (30 µg), ceftazidime (30 µg) Oxoid, ESBL E test strip (BioMérieux SA, France) CT/CTL and TZ/TZL and Vitek2 system.
PCR

All ESBL E. coli isolates were analyzed by PCR to detect TEM, SHV and CTX-M15 genes as follow:

DNA was extracted from isolated E. coli colonies by using QIAamp®DNA Mini kit QIAGEN), for DNA amplification PCR was performed using thermal cycler (Gene Amp PCR system 2400, Roche and ready to use (Taq PCR Master Mix Kit QIAGEN, GERMANY) were used as described by the manufacturer. PCR amplification conditions for CTXM-15 were as follows: with cycling condition of initial denaturation step at 94 ºc for 2 min followed by 35 cycles of Denaturation at 95 ºc for 20 sec. Annealing at 55 ºc for 90 sec. Extension at 72 ºc for 90 sec and final extension at 72 ºc for 10 min. The amplified PCR products were visualized on 2 % agarose gel stained with ethidium bromide and examined under ultraviolet light; the molecular size marker is Sizer TM-50 Plus DNA Marker Solution iNtRON, A single DNA band at 840 bp bp was recorded as positive for the TEM gene and at 776 bp bp was recorded as positive for the SHV gene, for CTX-M15 bands were expected to be at 995 bp Primers used in this study are shown on table 1

Sequencing of CTX-M15

Amplified DNAs of CTX-M15 genes were sequenced by an automated sequencing analyzer (3500xL Genetic Analyzer Applied Biosystem, USA). Sequence data were analyzed using the sequence software alignment and were compared to the identified CTX-M15 gene in the GenBank nucleotide database available on the Internet at the National center of biotechnology information website (http://www.ncbi.nlm.nih.gov) (NCBI), and nucleotide BLAST (Basic Local Alignment Search Tool) web site for sequencing analysis services and for detection of CTXM15 gene.

Statistical analysis

Data were analyzed using SPSS 20. Chi Square was used to compare categorical variables. P value of 0.05 was considered statistically significant

Results and Discussion

There were 70 out of 80 E. coli isolates ESBL positive by screening test; the highest percentage of ESBL producing E. coli was found in internal medicine ICU (38/70) 98%, followed by anesthetic ICU (16/70) 83%. The most frequent ESBL ICU positive E. coli isolated from urine with high percentage 35/70 (95%) followed by blood 14/70(81%).The majority of ESBL positive strains were community acquired (38%), while hospital acquired infection were (32%).

Antimicrobial susceptibility

There is significant difference in resistant pattern between ESBL positive and ESBL negative E. coli isolates to: Ampicillin, SXT, Cefipeme, Azetronam, Ceftazidime, Cefotaxime, Piperacillin/Tazobactam Ceftriaxone, Gentamicin, Ciprofloxacin and Ampicillin/Sulbactam, drug resistance pattern of ESBL positive and ESBL negative isolates are illustrated in (Figure 1). Carbapenemes was the most active antibiotic against ESBL E. coli (sensitivity was 97%). In CTX-M15 positive isolates there was significant difference in resistance to Gentamicin in relation to other ESBL producing isolates.

ESBL production

Evaluation of ESBL confirmatory test in comparison with PCR for detection of CTX-M15 gene (Figure 2), the highest sensitivity
was obtained with vitek2, while the highest specificity was recorded by Etest (TZL) (Figure 3), and by using DDST (CAZ) sensitivity was 82.5% and 86% for specificity.

Results of Genetic Analysis by PCR the most predominant gene is CTXM-15(60%) figure (4) followed by combination of CTXM-15 and TEM (20%), CTXM-15 and SHV (7.10%) then TEM alone (5.70%), SHV alone (4.30%) and least percent (2.9%) for combination of CTXM-15 + SHV+TEM (Figure 5). There is statistically significant difference for the distribution of different ESBL genotypes E. coli producers, CTX-M15 alone is 63.1% among community acquired infection comparing to 56.3% hospital acquired infection, the combination of CTX-M15 +TEM is 23.7% hospital acquired infection and 15.6% community acquired. There is difference in distribution of ESBL genotypes of studied E. coli isolates among clinical specimens, in urine specimens CTX-M15 alone found with high percentage (51.3%) followed by CTX-M15+TEM (22.9%) there is statistically insignificant difference between ESBL genotypes of studied E. coli isolates regarding sex of patients.

**Sequencing of CTX-M15**

Sequencing of the CTX-M15 PCR products for two tested isolates. The results of sequencing matched CTX-M15 in all the isolates.

The sequence obtained was submitted to Gen Bank under SCEC020026, REP215 and MS05 strains with accession numbers CP034956, MG844172 and JQ397663.1 and DQ485310.1 respectively. the results revealed high degree of similarity between query (sequenced genes) and subject gene (reference genes) of NCBI- BLAST, that could be demonstrated by the high percentage of identity, which range between 98- 99% these results illustrated in (Table 2).

Antimicrobial resistance is indiscriminate and growing problem across different bacterial groups and against all antibiotic classes, impacting every region and country in the world (Yasir et al., 2018).

Antimicrobial resistance in E. coli has gained much attention since resistance against beta-lactam antibiotics are increasingly documented, and several studies reported the increase of E. coli resistance to more than one drug or class of drugs (Tadesse et al., 2012).

Early identification of ESBL in due time is mandatory not only for optimal patient management but also for immediate establishment of appropriate infection control measures to prevent the spread of these pathogens and also to prevent hospital acquired infections and outbreaks in the community (Lim et al., 2019).

CTX-M has become the most commonly detected ESBL genotype. Because CTX-M family genes are present on plasmids or other mobile genetic elements, these enzymes have become predominant worldwide and are substituting for the more common SHV and TEM, the most common type of CTX-M reported in various geographic regions is CTX-M15; these strains are usually MDR and are detected not only in nosocomial infections but also in the community (Hashemizadeh et al., 2018).

In agreement with global reports, an alarming increase in resistance to the extended-spectrum subclass, among clinical E. coli isolates is highlighted by the results of this study. We found that 70 out of 80 screened E. coli isolates (87.5%) gave positive results. In Egypt these results were in agreement with
Ramadan et al., (2019) who reported (90.4%) and in partial agreement with Abdel-Moaty et al., (2016) and Fam and El-Damarawy (2008) which was carried out in Intensive Care Unit. On the contrary, our prevalence rate was higher than that recorded by Abdallah et al., (2015), Fadil et al., (2017) and Khater and Sherif (2014) where prevalence rate was 54.5%, 61.1% and 53.3% respectively. Our results showed a high prevalence rate compared to many European countries according to (antimicrobial resistance/database/Pages/database.aspx)

This discrepancy may be attributed to the geographical distribution, selection criteria and sample size of screened population where some studies conducted on specific groups of patients e.g. blood stream infection by Abdallah et al., (2015).

Regarding gender, our study showed that there was no statistically significant difference between males (83%) and females (91%) regarding frequency of ESBL positive strains.

In agreement with Akanbi et al., (2013) who reported that no differences were apparent between ESBL-producing E. coli with gender distribution, but on the other hand Fody et al., (2017) had shown that females had a higher prevalence rate than males.

In this study the distribution of suspected ESBL positive E. coli strain was highest in urine (95%), followed by blood (81%) and this agreed with Maina et al., (2017), Abdallah et al., (2015) and Raut et al., (2015).

On the other hand, some studies detected a high rate of ESBL-producing E. coli in blood as Zhang et al., (2014) and Fody et al., (2017), while Fadil et al., (2017) reported that suspected ESBL positive E. coli strain from sputum was higher than in other samples.

The frequency distribution of ESBL detected by DDST-CAZ positive results was higher in this study than that detected by DDST -CTX, these results are similar to Sujatha et al., (2017). Many studies agreed with our study in reporting the high sensitivity of VITEK 2 in relation to the specificity as Kumar et al., (2018), Liu et al., (2015) and El-Jade et al., (2016).

Phenotypic testing of ESBL E. coli by Etest when using PCR as the reference method, our results indicate that Etest TZL is characterized by high sensitivity and specificity, this interpretation is reflected clearly in a study laid down by Kałużn et al., (2014) particularly with regard to E. coli.

This difference may reflect the different types of ESBLs in our isolates, and emphasizes the importance of testing more than one cephalosporin.

In the present study, Molecular characterization revealed high frequency of CTX-M15 containing isolates and this agreed with previous reports on CTX-M15 carried in Egypt as Ramadan et al., (2019), Abdallah et al., (2015) and Khalaf et al., (2009).

Yasir et al., (2018), Guiral et al., (2018) and Maina et al., (2017) support our results as high rate of CTX-M 15 E. coli, while SHV and TEM had lower percentage.

CTX-M15 has shown to be a continuously increasing problem in several countries; Germany, Cameroon, Saudi-Arabia, and Lebanon, CTX-M-15 is considered the most frequent ESBL (Pietsch et al., 2015; Valenza et al., 2015; Djuikoue et al., 2017; Mashwal et al., 2017; Al-Agamy et al., 2014).

The high prevalence of CTX-M15 worldwide could be attributed to the successful dissemination of genetic elements and the
clonal expansion of *E. coli* (Rogers *et al.*, 2011). Apart from the environment animals that are a food source serve as reservoirs for CTX-M15 spread (Ali *et al.*, 2016), also the powerful ability of CTX-M15 gene products to hydrolyze ceftazidime, cefotaxime and aztreonam, which probably offers a selective pressure on the bacteria especially when multiple antibiotics are concomitantly or consecutively prescribed (Ramadan *et al.*, 2019).

In contrast to our results, low prevalence rate of CTX-M15 was observed in the study conducted by Dasgupta *et al.*, (2018) and Bajpai *et al.*, (2017) revealed that the gene predominated was TEM followed by CTX-M15 and SHV. Also, all ESBL producing *E. coli* strains were CTX-M 15, while no strain of *E. coli* had TEM or SHV as shown by Djuikoue *et al.*, (2017).

This difference may be attributed to the variation in the frequency and predominant type of ESBL genes from region to region and even between institutions within the same region.

In the present study percentage of ESBL positive strain of studied *E. coli* isolates regarding department of ICUs was highly significant in internal medicine ICU was 98% which is alarming, this finding is in agreement with Chakraborty *et al.*, (2013).

There was statistically significant difference for the distribution of CTX-M15; in our study it was 63.1% among community acquired infection compared to 56.3% hospital acquired infection (ICUs). Implying the increased spread in the community, in concordance with our results, Abrar *et al.*, (2017); one of Indian study Chakraborty *et al.*, (2013) and Yasar *et al.*, (2018) reported that the majority of the ESBL-positive *E. coli* CTXM-15 were of community-onset.

Among 19 antibiotics tested in this study, *E. coli* ESBL isolates have high percentage of multidrug resistance while ESBL positive *E. coli* isolates were still sensitive to Imipenem, Meropenem however, it will not be long until extended exposure to carbapenams will have its impact on selecting for bacteria that are resistant to this last-resort antibiotic (Li *et al.*, 2018) have already described the emergence of carbapenem resistance in Enterobacteriaceae

Our findings are in agreement with Ouedraogo *et al.*, (2015) showed a susceptibility of 100% for imipenem and Mohajeri *et al.*, (2014) reported high resistance to various antimicrobial classes.

The high susceptibility of ESBL-producing bacteria to carbapenams may be attributed to the fact that carbapenams are highly stable to ESBL hydrolytic activity in addition their penetration to the outer bacterial membrane is excellent due to their compact molecular size (Harada *et al.*, 2008).

There were (48%) of the CTX-M15 producing isolates resistant to Gentamicin which is similar to reports from other studies Kanamori *et al.*, (2011); Strahilevitz *et al.*, (2009) and Fallah *et al.*, (2016).

Molecular characterization of isolates encoding CTX-M15 from *E. coli* involved in outbreaks in different countries has demonstrated that they additionally carried other antibiotic resistance genes Fallah *et al.*, (2016).

The results of genotyping tests showed that 63 isolates which had been detected for the prevalence of CTX-M15 genes by PCR, all are matched by sequencing technique as CTX-M15 in all the isolates the results showed high identity (98-100%) with reference protein of gene bank with CTX-M15 of NCBI-BLAST.
These results were in agreement with Wickramasinghe et al., (2012) for Accession NO. (DQ485310.1). Similarly, our results agreed with results obtained by Lopes et al., (2017), Tacão et al., (2012) for Accession NO (MG844172.1) and Accession NO (JQ397663.1) respectively, as concerning protein sequence.

**Table 1** Sequences of primers used in PCR

| Gene    | Primer   | Sequence (5’ to 3’)                                      | Amplicon size (bp) | Ref                              |
|---------|----------|---------------------------------------------------------|--------------------|----------------------------------|
| CTXM15  | Ctxm15F  | CAC ACG TGG AAT TTA GGG ACT                              | 995 bp             | Muzaheed et al., 2008            |
|         | CTXM15R  | GCC GTC TAA GGC GAT AAA CA                               |                    |                                  |
| TEM     | TEMF     | ATG AGT ATT CAA CAT TTC CGTG                            | 840 bp             | De Oliveria 2008                 |
|         | TEMR     | TTA CCA ATG CTT AAT CAG TGAG                            |                    |                                  |
| SHV     | SHVF     | ATG CGT TAT ATT CGC CTG TG                              | 776 bp             | Schlesinger et al., 2006         |
|         | SHVR     | AGA TAA ATC ACC ACA ATG CGC                             |                    |                                  |

**Table 2** The BLAST results of CTX-M15 *E. coli* in the gene bank, and compatibility of DNA sequences obtained from NCBI

| Gene    | Nucleotide sequence of query and subject | Score | Expect value | Identities | Gaps          | Accession NO. |
|---------|------------------------------------------|-------|--------------|-------------|---------------|---------------|
| CTXM15  |                                          | 1537 bits(832) | 0.0          | 896/915 (98%) | 22/915 (2%)   | CP034956.     |
| CTXM15  |                                          | 1607 bits(870) | 0.0          | 922/944 (98%) | 15/944 (1%)   | JQ397663.1    |

**Nucleotide sequence of query and subject**

| Score | Expect value | Identities | Gaps | Accession NO. |
|-------|--------------|------------|------|---------------|
| 1663 bits(900) | 0.0          | 928/940 (99%)   | 8/940 (0%) | DQ485310.1    |
| 1607 bits(870) | 0.0          | 915/934 (98%)   | 13/934 (1%) | MG844172      |
Fig. 1 Drugs resistance pattern of ESBL positive and ESBL negative isolates

Fig. 2 Comparison of confirmatory phenotypic tests for detection of ESBL positive *E. coli* isolates harboring CTX-M15 gene using PCR as reference method

Fig. 3 Positive ESBL *E. coli* using E test ESBL (TZL): Ceftazidime (TZ) / Ceftazidime +clavulanic acid (TZL) shows deformation of the TZ inhibition ellipse indicative of ESBL
**Fig. 4** Gel electrophoresis of PCR for detection of CTX-M15 gene

Lane 1: molecular mass marker (100 bp DNA ladder), lane 1, 3, 4, 5, 6 and 7 show positive *E. coli* isolates for CTXM15 gene at 995 bp, lane 2 shows negative *E. coli* isolate for CTXM15 gene

**Fig. 5** Prevalence of ESBL genotyping among studied *E. coli* isolates

This study had some limitation as relatively small sample size of 80 *E. coli* might be little to obtain significant results; the present study was performed at a single institution in Egypt. Thus, the study results may not reflect the actual prevalence and epidemiology of other geographic areas within the country. Furthermore, epidemiological typing to assess clonality of the isolates was not performed, and this could have added value to the understanding of the epidemiological spread of ESBL genes.

Multicenter molecular based epidemiological studies of *ESBL E. coli* with longer surveillance duration are recommended for better understanding of the prevalence and distribution of the ESBL genes especially CTX-M15, that can help in prevention of the spread of these resistance bugs in Egyptian hospitals and support the determination of priorities for local intervention actions.

In conclusion, this study highlights the threat of extended spectrum beta lactamases
producing *E. coli* and their potential multidrug resistance considered a serious problem. CTX-M15 is a predominant gene conferring ESBL-production in *E. coli* clinical isolates causing both hospital- and community-acquired infections in our setting. Early detection of ESBL *E. coli* strains harboring CTXM-15 is necessary because of uncontrolled dissemination of these plasmid mediated resistant genes. Regarding phenotypic confirmatory tests, Etest (TZL) has higher sensitivity and specificity than other methods (double disc synergy method DDST and Vitek 2) although; Consequently, Vitek 2 system is a reliable semi-automated microbiology system which may be used for routine, accurate and rapid in our clinical settings. Carbapenems are still a good choice treatment option against ESBL *E. coli* infections.

**Conflict of interest**

The authors declare no conflict of interest this research did not receive any specific grant from funding agencies in the public, commercial or not-for- profit sector

**Abbreviations**

ESBL: extended spectrum beta lactamases MALDI-TOF MS: Matrix-assisted laser desorption ionization–time of flight mass spectrometry, MDR: multidrug-resistant, ctxm15: cefotaxime hydrolyzing capabilities-Munich, DDST: double disc synergy, E test: epsilometer test.

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