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Molecular Characterization of Extended-Spectrum Beta-Lactamases producing *Escherichia coli* and *Klebsiella pneumoniae* Blood Isolates from Egyptian Pediatric Cancer Patients

Abeer K. Abdulall[1]*, Hadir. A. El-Mahallawy[2] and Mahmoud M. Tawfick[3]

[1]Microbiology and Immunology Department,
Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt.
[2]Clinical Pathology, Medical Oncology Department, National Cancer Institute, Cairo University, Egypt.
[3]Microbiology and Immunology Department, Faculty of Pharmacy (Boys),
Al-Azhar University, Cairo, Egypt.

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The occurrence of Extended-Spectrum Beta-Lactamases(ESBLs) producing bacteria in Egypt has been well documented. However, investigations on blood stream infections (BSIs) caused by these bacteria in pediatric cancer patients are limited. The present study was carried out to assess the prevalence of and to characterize the ESBLs types in *K. pneumoniae* and *E. coli* causing BSIs in pediatric cancer patients. Fifty four ESBL producing *K. pneumoniae* and *E. coli* blood isolates collected from pediatric patients with malignancies at the National Cancer Institute, Cairo, Egypt, were investigated for ESBLs production by phenotypic and genotypic methods. These isolates were recognized as ESBLs producers. Molecular characterization of ESBLs types revealed the presence of the Ambler class A ESBLs types. bla_{TEM} type was the predominant in all *E. coli* and in 70.8 % of *K. pneumoniae* isolates. bla_{SHV} gene was more frequent in *K. pneumoniae* as being in 87.5 % of the isolates. bla_{CTX-M} gene was detected in high percentages in both *E. coli* (93.3 %) and *K. pneumonia* isolates (91.6 %) as well. All isolates were co-producers of the ESBLs types. It is concluded that a random and/or empirical use of the antibiotics in cancer patients should be restricted as far as possible.

Key words: ESBLs, blood stream infections, *K. pneumoniae*, *E. coli.*

The extended spectrum cephalosporins, such as cefotaxime and ceftazidime, have gained widespread clinical use since the early 1980s. These antibiotics have been developed to overcome the ever-increasing prevalence of ampicillin-hydrolysing β-lactamases in *Enterobacteriaceae* and/or other Gram-negative species[1]. During the last decade, the hospital-acquired Gram-negative bacilli, particularly *K. pneumoniae* and *E.coli*, have produced mutated versions of β-lactamases enzymes; called Extended-Spectrum β-Lactamases (ESBLs) [2,3]. These Enzymes are able to inactivate ampicillins, the extended-spectrum cephalosporins, monobactams and other β-lactam antibiotics, though they are inhibited by clavulanic acid, sulbactam or tazobactam[3-5] ESBLs are encoded by genes commonly found among *Enterobacteriaceae* members. These encoding genes are carried mostly on plasmids which is an important cause of transferring multidrug resistance trait among Gram-negative bacteria worldwide[6-8]. ESBLs are derived from Ambler class A β-Lactamases based on the molecular structure,

* To whom all correspondence should be addressed.
Tel.: 24052967; Fax.: 24052968-24018119;
Mob.: 00202-01111520599;
E-mail: Khairy.abeer@yahoo.com

which include TEM, SHV and CTX-M, in addition to class D ESBL (OXA)[3,4,9,10]. The Ambler classes A and D ESBLs encoding genes bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{OXA} have been characterized in *E. coli* and *K. pneumoniae*[2,8,10,11]. The gene bla_{TEM} was first discovered in *E. coli* in the early eighties from a patient named Teminora from Greece. The gene bla_{SHV}, refers to sulphydryl variable, is commonly associated with *K. pneumoniae*. In addition, bla_{CTX-Ms}, the rapidly growing plasmid mediated ESBLs encoding genes of significant clinical impact[4,11-13]. The bla_{CTX-Ms} encoding ESBLs comprise a distinct lineage of the molecular class A β-lactamases; currently, there are more than 50 known allelic variants[4,5,12,14]. Lately, CTX-Ms are being the most prevalent ESBLs worldwide[15,16], which maybe due to having a greater ability to spread and cause outbreaks[16]. Bouchillon *et al*. (2004) findings indicated that Egypt had the highest incidence of ESBLs producing isolates compared to some European countries, Lebanon, Saudi Arabia and South Africa[17]. In addition, bla_{CTX-Ms} encoding ESBLs have been reported in *E. coli* isolates from Egypt, Middle East region and North Africa, with $bla_{CTX-M15}$ was the most common one[18,19,20,21]. In contrast to SHV and TEM ESBLs, most of the CTX-M β-lactamases have greater activity against cefotaxime than other oxyimino-beta-lactam substrates such as ceftriaxone and ceftazidime[3,10,12,13]. The class D β-lactamases, called oxacillinases, are designated OXA-1, OXA-2, etc., found in Enterobacteriaceae. Natural OXA variants have arisen with an extended substrate spectrum that includes ampicillins, imipenem and third-generation cephalosporins such as cefotaxime, ceftriaxone, and aztreonam. In addition, class D enzymes are not inhibited by mechanism-based class A inhibitors such as clavulanic acid[10,22].

Bloodstream infections (BSIs) caused by ESBLs producing *Enterobacteriaceae*, especially producing *E. coli* and *K. pneumoniae*, have become a serious clinical concern worldwide. The ESBLs related BSIs have been associated with higher rates of treatment failure due to significant reductions in susceptible antibiotics particularly in case of nosocomial infections, although recently in the community settings as well. In addition, these infections are leading cause of high mortality rates and increasing the hospital costs[23,24]. Indeed, the increased prevalence of ESBLs phenotypes in *E. coli* and *K. pneumoniae* clinical isolates has been reported from different geographic areas which is significantly hampering management of nosocomial infections especially in immunocompromised patients[10, 25, 26, 27]. In many published reports, mortality of bloodstream infections caused by ESBL producing *E. coli* or *K. pneumoniae* was higher than that of infections caused by ESBL negative ones, especially in patients with underlying malignant settings[23,24,28,29,30]. That could be explained that in cancer patients there are factors that enhance the opportunity for ESBL producing bacteria, particularly those of endogenous origin, such as *E. coli* and *K. penumoniae*, to cause infections[29, 31]. In addition, the widespread of these microorganisms associated infections in the lower age group is of a particular concern since the therapeutic options, other than β-lactams antibiotics, such as floroquinolones will risk potential toxicity[32].

Detection of the common ESBLs encoding genes such as bla_{CTX-M},$bla_{TEM}$$bla_{SHV}$ and bla_{OXA} by molecular methods in ESBLs producing bacteria and their antimicrobial resistance patterns can provide valuable information about their epidemiology and can aid a more rational antimicrobial therapy. Accordingly, the aim of this study was to determine the pattern of antimicrobial resistance and to determine the molecular profile of the predominant ESBLs types in *E. coli* and *K.pneumoniae* ESBL phenotypes associated with BSIs in Egyptian pediatric cancer patients.

MATERIALS AND METHODS

Bacterial isolates

The present study was carried out on 54 non-duplicate *K.pneumoniae* (24 isolates) and *E. coli* (30 isolates) isolated from blood cultures of febrile neutrupenic pediatric patients with hematological malignancies hospitalized in the National Cancer Institute (NCI), at Cairo University, Cairo, Egypt.

Identification and screening of ESBL producers by Phoenix™ Automated System

The identification of the 54 isolates and determination of their antimicrobial susceptibility patterns were carried out by Phoenix™ Automated Microbiology System (version 4.05W) using the

GN Combo UNMIC/ID-62 panels according to the manufacturer's recommendations. The Phoenix ESBL detection panel includes testing for cefotaxime and ceftazidime susceptibility with or without clavulanate. Tested isolates were considered as ESBL producers in accordance with the Clinical and Laboratory Standards Institute recommendations.

Antimicrobial susceptibility testing and confirmation of ESBL phenotypes

Manual antimicrobial susceptibility testing was carried out by the agar disk diffusion method on Mueller-Hinton agar(Oxoid, England) according to CLSI (2008)[33]. The tested antibiotics included ampicillin (10µg), amoxicillin/clavulanate (20/10µg), aztreonam (30µg), cefoperazone (30 µg), cefotaxime (30µg), ceftazidime (30µg), ceftriaxone (30µg), cefepime (30µg), amikacine (30µg), gentamicin (10µg), imipenem(10µg), meropenem (10µg) and ciprofloxacin (5µg)(Oxoid Ltd., Basingtoke, Hampshire, England). Interpretation of results was according to CLSI guidelines and *E. coli* ATCC 25922 was used as standard strain[33]. Double disk approximation test (DDAT) was performed to confirm the production of ESBL by the disk diffusion assay. Disks containing aztreonam, ceftazidime, cefepime, and cefotaxime, 30 µg each, were placed at a distances of 20 mm from center to center around a disk containing amoxicillin 20 µg/clavulanic acid 10 µg on Mueller-Hinton agar plates inoculated with 0.5 McFarland of the tested isolate. Enhancement of the inhibition zone toward the amoxicillin-plus- clavulanate disk was confirmatory for ESBL production[34, 35].

Molecular detection of ESBLs encoding genes by PCR

E. coli and *K. pneumoniae* ESBLs phenotypes were screened for the presence of the Ambler class A and D ESBLs encoding genes: bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{OXA} by PCR technique. The ESBLs phenotypes confirmed by both the CLSI selection criteria and DDAT test were investigated using the previously described specific primers with minor modifications listed in Table 1. PCR products in this study were generated using 2×Dream Taq Green PCR Master Mix (ThermoScientific®–K1082), as directed by the manufacturer instructions. DNA template preparation was performed as follows. Total DNA was extracted from all isolates by heating bacterial cells suspension in sterile distilled water at 95°C for 10 min, followed by removal of cellular debris by centrifugation at 14,000 rpm for 1 min. The supernatant was collected and used as a source of template for PCR amplification. PCR reactions were performed in total volumes of 25µl containing 12.5µl of Dream Taq Green PCR Master Mix 2x Ready mix, 15pmol concentrations of each primer and 1.5 µl of DNA template. PCR amplification reactions were carried out under the following conditions: initial denaturation at 95°C for 3 min, followed by 30 cycle of denaturation at 95°C for 40 s, annealing at 58°C for bla_{TEM}, bla_{SHV} and $bla_{CTX-M,}$ and at 60°C for bla_{OXA} for 30 s and extension at 72°C for 1 min, and a final extension at 72°C for 7 min. Reactions aliquots were resolved through 0.8 % agarose gel electrophoresis in 0.5 × TBE buffer with expected amplicons' lengths of 403 bp, 293 bp, 569 bp and 701 bp for bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA} respectively. GeneRuler™ DNA molecular weight marker (100 x 10,000 bp) (Fermentase) was used to assess PCR product size.

RESULTS

The genotypic profiles of 30 *E.coli* and 24 *K. pneumoniae* ESBLs phenotypes associated with BSIs in pediatric patients suffering from malignancies were investigated by PCR. These isolates were recognized as ESBLs producers by the Phoenix™ Automated Microbiology System and were phenotypically confirmed as ESBL producers according to CLSI guidelines of being resistant to all penicillins, third- and fourth-generation cephalosporins and aztreonam (Table 2), in addition to the DDAT test results. Despite 86.7% and 79.1% of ESBLs producing *E.coli* and *K. pneumonia* isolates were respectively resistant to ciprofloxacin, only two *E.coli* isolates (6.6%) and one *K. pneumoniae* isolate(4.2 %) were resistant to carbapenems (meropenem and imipenem), respectively. It was found that 3.3% of *E.coli* isolates and 4.2% of *K. pneumoniae* isolates were resistant to amikacin. In addition, 70% of *E. coli* isolates and 62.5 % of *K. pneumoniae* isolates were resistant to gentamicin (Table 2).

Molecular characterization of *E. coli* and *K. pneumonia*e isolates in this study revealed the presence of the Ambler class A ESBLs encoding genes in all isolates. Nevertheless, none of the

isolate had the class D ESBL encoding gene(bla_{OXA}). In general, bla_{TEM} type ESBL was found in all (100%) of *E. coli* isolates and in 70.8 % of *K. pneumoniae* isolates. In addition, bla_{SHV} gene was more frequent in *K. pneumoniae* as being in 87.5 % of the isolates but was less frequent among *E. coli* as detected in 23.3% of the isolates. Almost equally, bla_{CTX-M} gene was detected in high percentages in both *E. coli* (93.3 %) and *K. pneumoniae* isolates (91.6 %) (Table 3, Fig. 1 and 2).

Table 1. Nucleotide sequences of PCR primers used to amplify ESBLs genes

Gene	Primer sequence (5' – 3')	Expected amplicon size (bp)	Source
bla_{SHV}	F: 5‘ CGCCTGTGTATTATCTCCCT 3‘ R: 5‘ CGAGTAGTCCACCAGATCCT 3‘	293	1
bla_{TEM}	F: 5' TTTCGTGTCGCCCTTATTCC 3' R: 5' ATCGTTGTCAGAAGTAAGTTGG 3'	403	
bla_{CTX-M}	F: 5' CGCTGTTGTTAGGAAGTGTG 3' R: 5' GGCTGGGTGAAGTAAGTGAC 3'	569	
bla_{OXA}	F: 5' ATGGCGATTACTGGATAGATGG 3' R: 5' AGTCTTGGTCTTGGTTGTGAG 3'	701	

1, Bali *et al*. (2010)[2]

Table 2. Antimicrobial resistance rates of the tested *E. coli* and *K. pneumoniae* blood isolates from BSIs in pediatric patients with hematological malignancies

Antimicrobial agent	*E. coli*(30 isolates)		*K. pneumonia* (24 isolates)		Total (54)
	No.	%	No.	%	%
Ampicillin	30	100	24	100	100
Amoxicillin-clavulanic acid	30	100	24	100	100
Aztreonam	30	100	24	100	100
Ceftazidime	30	100	24	100	100
Cefotaxime	30	100	24	100	100
Cefoperazone	30	100	24	100	100
Cefepime	30	100	24	100	100
Imipenem	2	6.6	1	4.2	5.6
Meropenem	2	6.6	1	4.2	5.6
Ciprofloxacin	26	86.7	19	79.1	83.3
Gentamicin	23	70	15	62.5	70.4
Amikacin	1	3.3	1	4.2	3.7

Table 3. Prevalence of CTX-M, SHV and TEM encoding genes in *E. coli and K. pneumoniae* ESBL phenotypes

ESBL phenotypes (No.)	ESBL encoding gene		
	bla_{CTX-M}No. (%)	bla_{TEM}No. (%)	bla_{SHV}No. (%)
E. coli (30)	28 (93.3)	30 (100)	7 (23.3)
K. pneumonia (24)	22 (91.6)	17 (70.8)	21 (87.5)
Total (54)	42 (77.8)	45(83.3)	28 (51.9)

Table 4. Genotypic profiles of *E. coli and K. pneumoniae* ESBL phenotypes

ESBL genes	*E. coli* (30)No. (%)30	*K. pneumonia* (24)No. (%)	Total (54)No. (%)
bla_{CTX-M}+bla_{TEM} + bla_{SHV}	7 (23.3)	12 (50)	19 (35.2)
bla_{CTX-M}+ bla_{TEM}	21 (70)	3 (12.5)	24 (44.4)
bla_{CTX-M}+ bla_{SHV}	0	6 (25)	6 (11.1)
bla_{TEM} + bla_{SHV}	0	2 (8.3)	2 (3.7)
bla_{TEM}	2 (6.7)	0	2 (3.7)

All isolates were co-producers of the ESBLs encoding genes; either two or all the three genes co-existed together. The genes bla_{CTX-M}, bla_{TEM} and bla_{SHV} were found all together in 50% of *K. pneumoniae* isolates and 23.3% of *E. coli* isolates. The most prevalent genetic profile for *E.coli* isolates was the bla_{CTX-M} together with bla_{TEM} genes; it was in 70% of the isolates. While for *K. pneumoniae*, the most prevalent profile was bla_{CTX-M} together with bla_{SHV} genes; it was detected in 25% of *K. pneumoniae* isolates (Table 4).

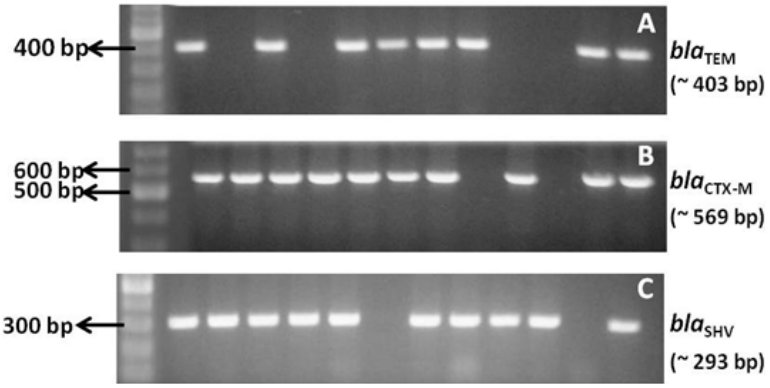

Fig. 1. PCR products of ESBLs encoding genes of *K. pneumonia* isolates. Agarose gel (0.8 %) resolved PCR amplicons of ESBLs encoding genes: First lane, GeneRuler DNA M.W. marker (100 " 10,000 bp). , A, bla_{TEM}; B, bla_{CTX-M}; C, bla_{SHV}

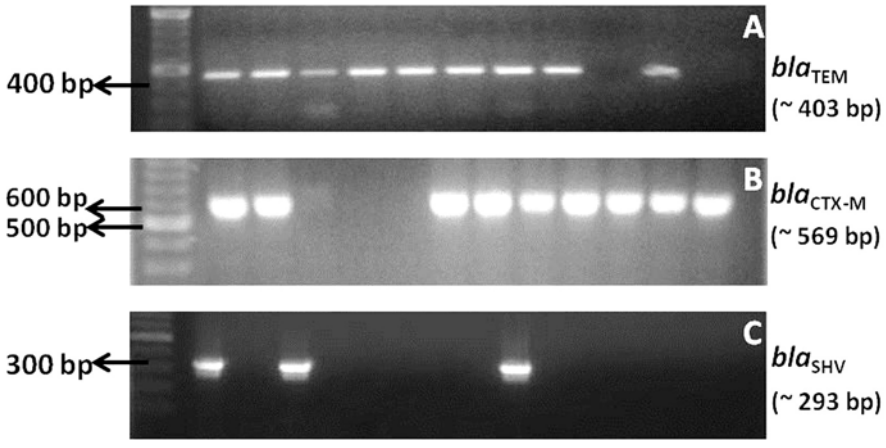

Fig. 2: PCR products of ESBLs encoding genes of *E. coli* isolates. Agarose gel (0.8 %) resolved PCR amplicons of ESBLs encoding genes: First lane, GeneRuler DNA M.W. marker (100 - 10,000 bp)., A, blaTEM; B, blaCTX-M; C, blaSHV.

DISCUSSION

The extensive use of antibiotics in hospitals and/or for empirical treatment of immunocompromised patients has led to selection of multidrug-resistant organisms; one of most concern is ESBLs producing Gram-negative bacilli[36, 37].

ESBLs are a heterogeneous group of enzymes responsible for the resistance of organisms to broad-spectrum β-lactam antibiotics[24, 28]. Infections caused by ESBLs producing Gram-negative bacilli, especially ESBLs producing *E. coli* and *K. pneumoniae*, have become an emerging global issue. Because these infections are frequently associated with clinical problems particularly in developing countries like Egypt, where high endemic rates of ESBL producing *Enterobacteriaceae*, of up to 70%, were reported[16, 17,24]. Importantly, bloodstream infections due to ESBL producing Gram-negative microorganisms are associated with high mortality, particularly in immunocompromised patients[24]. In addition, the prevalence of ESBLs producing *E. coli* and *K.pneumoniae* was found to be high in patients with malignancies, particularly patients with hematological malignancies, who have been hospitalized for a long period and/or who have repeatedly received cephalosporins in the struggle to control their febrile neutropenic episodes[23,29,30,38, 39]. This study aimed to characterize prevalent genetic determinants of TEM, SHV, CTX-M and OXA ESBLs encoding genes in *K.pneumoniae* and *E. coli* blood isolates collected from febrile neutrupenic pediatric patients with hematological malignancies hospitalized in the NCI at Cairo University.

In the present study, we found that 100 % of *E. coli* and *K.pneumoniae* blood stream isolates were ESBL phenotypes. They were resistant to all penicillins, aztreoname and extended spectrum cephalosporins; particularly cefotaxime, cefoperazone, ceftazidime and cefepime. ESBLs production for all ESBL phenotypes identified by the Phoenix™ Automated Microbiology System was confirmed by the DDAT. In addition, they harbored ESBLs Ambler class A encoding genes detected by PCR. This finding showed that Phoenix™ automated system was a sensitive and reliable tool for detection of ESBLs producers. However, laboratory phenotypic detection of ESBLs producers is recommended to confirm ESBLs productions when an organism is reported by a semi-automated system. In addition, more than one kind of third-generation cephalosporins should be used in combination with clavunulate for the detection of ESBL production[35]. Notably, the prevalence rate of ESBL phenotypes detected in this study (100%) was high comparing to previous reports from Egypt of 80 %[36]. Moreover, it was higher than the rates of prevalence of ESBLs producing Enterobacteriaceae in other geographic regions[16, 40, 41]. That can be explained that many risk factors have been reported for BSIs caused by ESBL producing *Enterobacteriaceae*. One of the most important risk factors is recent antibiotic therapy, particularly with β-lactams. Other factors include previous invasive procedures and/or presence of devices, in addition to admission from long-term care facilities[23,24,29,30]. In addition, prevalence of ESBLs producing *E. coli* and *K.pneumoniae* is high in febrile neutropenic patients with underlying malignancy settings, particularly patients with leukemia, who have been stayed in the hospital for long time and/or have repeatedly received cephalosporins[39].

ESBLs producers are usually multidrug-resistant[3-5,27,28]. Here, about 65% of ESBL producers *E.coli* and *K.pneumonaie* showed multidrug resistance to more than two classes of antimicrobial agents. These isolates were resistant to all β-lactams tested, except for imipenem and meropenem, and to gentamicin and ciprofloxacine. Their genotypic profile showed Ambler class A ESBLs (CTX-M, TEM, SHV) encoding genes. All had $bla_{\text{CTX-M}}$in combination with either bla_{TEM}, which was most predominantly present in *E.coli* isolates (70%) and/or in combination with bla_{SHV} that was predominant in *K.pneumoniae* isolates (25%). Moreover, we had 35.2% of the ESBL producer isolates showing $bla_{\text{CTX-M}}$ together with both bla_{TEM} and bla_{SHV} ESBL encoding genes. Although, these multidrug-resistant isolates were apparently sensitive to imipenem and meropenem, they may revert to resistance due to newer types of class A ESBLs recently rooming the globe and this should be of most concern. Although, imipenem and meropenem were apparently more effective than other antimicrobial agents, we had two *E.coli* and one *K.pneumoniae* isolates totally resistant

to all tested antimicrobial agents. Their genotypic profile showed presence of CTX-M, TEM and SHV ESBL types together. Thus, we should be concerned about dissemination of such notorious isolates that will compromise the efficiency of the currently available antimicrobial agents in the near future.

The high prevalence of CTX-M-type extended-spectrum β-lactamases in Enterobacteriaceae and the global dissemination of bla_{CTX-M} ESBL-encoding gene is of great concern, since CTX-M expression is often associated with coresistance that critically reduces treatments options[42-44]. In this study CTX-M encoding gene was frequently detected in ESBL producers *E.coli* (93.3%) and *K.penumoniae* (91.6%) isolates that were associated with blood stream infections. A finding that is most consistent with the phenomenal spreading of CTX-M ESBLs[13-16,18-21,42,43,45-50], particularly CTX-M-14 and CTX-M15[48], which were detected in an earlier study from Egypt [21].

In this study, it was found 86.7% and 79.1% of ESBLs producing *E.coli* and *K. pneumonia* isolates were respectively resistant to ciprofloxacin, and 70% of ESBL producing *E.coli* and 62.5% of *K. pneumonia* were resistant to gentamicin. These isolates had CTX-M ESBL encoding gene in their molecular profiles. Our results are consistent with some other studies where much of the CTX-M ESBL producing isolates were resistant to aminoglycosides and fluoroquinolones[51-53]. It is believed that fluoroquinolone or multidrug-resistance gives advantage to CTX-M for maintenance due to co-selection process[43,53,54].

Some of the CTX-M producers particularly CTX-M 15, have been found to be resistant to carbapenem, but no mutations are involved in this resistance. This is often due to co-production of other β-lactamases or loss of porins[43,54]. In this study, the two *E.coli* isolates with reduced susceptibility to imipenem and meropenem harbored CTX-M encoding gene and they were multidrug-resistant being susceptible only to amikacin.

Our results clearly demonstrated the dominance of both multidrug-resistance and ESBL cefotaximases genetic determinants in our blood isolates and it should be emphasized that It's of at most concern to be aware of the dissemination of CTX-M ESBLs when considering an appropriate antibiotic policy in our hospital. In addition, the emergence of multi-resistant pathogens and spreading of resistance genes should enforce the application of strict prevention strategies, including hygiene measures, changes in antibiotic treatment regimens and control of horizontal transmission of nosocomial organisms. In addition, physicians must be keenly aware of the infection risks, diagnostic methods, and antimicrobial therapies required for management of febrile patients through the neutropenic period**.**

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