International Journal of Research Studies in Microbiology and Biotechnology (IJRSMB)
Volume 2, Issue 3, 2016, PP 7-14
ISSN 2454-9428 (Online)
http://dx.doi.org/10.20431/2454-9428.0203002
www.arcjournals.org

PCR-Based Molecular Detection of ESBLs Encoding Genes *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{SHV}$ among MDR *Escherichia coli* Isolates from Diarrhoea Stool Cultures in Cairo, Egypt.

Mahmoud M. Tawfick[1, 2*], Abdel-Nasser A. El-Moghazy[1], Mohamed A. Hassan[1]

[1]Microbiology and Immunology Department, Faculty of Pharmacy, Al-Azhar University, Nasr City, Cairo, Egypt.
[2]Microbiology and Immunology Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), 6th October City, Egypt.
**mahmoud_tawfick@azhar.edu.eg*

Abstract: *Resistance to β-lactams owing to the production of extended-spectrum β-lactamases (ESBLs) enzymes has become common among diarrheagenic E. coli, especially in developing countries. This study aimed to detect ESBLs encoding genes bla$_{TEM}$, bla$_{CTX-M}$ and bla$_{SHV}$ among E. coli isolates from patients with acute infectious diarrhoea in Cairo, Egypt. A total of 60 E. coli isolates were isolated from diarrhoea stool cultures, during the period from March 2014 to December 2015, at three hospitals in Cairo. Isolation and identification of E. coli were performed using standard microbiological methods and confirmed by Vitek 2 automated system. Antimicrobial susceptibility testing was performed using Kirby-Bauer disc diffusion method against diverse classes of antimicrobial agents. Confirmatory tests for ESBLs production were performed using standard disc diffusion methods according to Clinical and Laboratory Standards Institute (CLSI). The E. coli ESBL-producer phenotypes were investigated for the presence of bla$_{TEM}$, bla$_{CTX-M}$ and bla$_{SHV}$ via polymerase chain reaction (PCR) using each gene-specific primers. Based on CLSI guidelines of E. coli isolates were multi-drug resistant (MDR) ESBLs-producer. All isolates were resistant to penicillins, third- and fourth-generation cephalosporins and aztreonam. E. coli isolates showed high resistance rates to non-β-lactams antimicrobial agents. The combination disc method for detection of ESBLs production showed positive results in 86 % of isolates. The cefoxitin-cloxacillin double disc synergy test for AmpC production showed positive results in 18 % of isolates. PCR studies revealed that bla$_{TEM}$ was the predominant ESBLs encoding determinant in 85 % of isolates, followed by bla$_{CTX-M}$ in 73.3 % of isolates; bla$_{SHV}$ was not detected in this study. All ESBLs-producing E. coli isolates possessed one or two of the PCR-positive ESBLs determinants. Each bla$_{TEM}$ and bla$_{CTX-M}$ was found alone in 63.3 % and 51.7 % of all isolates, respectively. The bla$_{TEM}$ in combination with bla$_{CTX-M}$ was detected in 21.7 % of E. coli isolates. ESBLs production is a noteworthy increasing among E. coli causing diarrhoeal diseases. The bla$_{TEM}$ is the common genetic mechanism for ESBL production in these pathogens isolated from Egypt. The limited treatment alternatives of infections caused by ESBL-producers E. coli require a public health policy on appropriate prescribing and the rational use of antimicrobial agents.*

Keywords: *E. coli, diarrhoea, ESBLs, PCR.*

1. INTRODUCTION

Diarrheagenic *Escherichia coli* is one of the most common causes of acute infectious diarrhoea (Alikhani *et al*., 2013), which is a major cause of morbidity and mortality, particularly among children (Collins, 2007; Sang *et al*., 2012).[2,3] Although the treatment of diarrhoea caused by *E. coli* has been successful using antimicrobial agents, the rapid development of multi-drug resistance has become an increasingly emerging worldwide problem with serious consequences on public health, particularly in developing countries (Sankaran, 2000; WHO, 2014).[4,5] One of these successful treatments was the β-lactam group of antimicrobial agents. They are killing the microorganism through the irreversible binding to the penicillin binding proteins (PBPs) required for the final transpeptidation step in the biosynthesis of peptidoglycan; the important stage for bacterial cell wall construction. The most clinically important members of this group are the third-generation cephalosporins, β-lactam/β-lactamase inhibitor combinations and carbapenems.[6] There are four main mechanisms by which bacteria can overcome β-lactam antimicrobial agents: production of extended spectrum β-lactamases (ESBLs) enzymes, changes in the active site of PBPs, decreased expression of outer membrane

proteins and efflux pumps.[7] Whilst, the predominant mechanism of resistance to β-lactam antimicrobial agents in *E. coli*, including penicillins, extended-spectrum cephalosporins and carbapenems, is the production of ESBLs enzymes encoded by *bla* genes. These enzymes hydrolyze the β-lactam ring in the molecular structure of β-lactam antimicrobial agents leading to inactivation of the antibacterial activity of these drugs.[8] The most commonly known ESBLs are derived from Ambler class A β-lactamases enzymes which include TEM, SHV and CTX-M.[9] Detection of theses common ESBLs encoding genes in *E. coli* can provide necessary information on their epidemiology helping for more efficient antimicrobial therapy. Therefore, this study aimed to detect ESBLs encoding genes bla_{TEM}, bla_{CTX-M} and $bla_{SHV,}$ using PCR, among MDR diarrheagenic *E. coli* isolates from patients with acute infectious diarrhoea in Cairo, Egypt.

2. MATERIALS AND METHODS

2.1. Study Population and *E. coli* Bacterial Isolates

The present study was carried out on 60 non-duplicate *E. coli* isolates recovered from diarrhoea stool cultures of inpatients with acute infectious diarrhoea at three hospitals in Cairo: Kasr El-Aini University hospital, EL-Demerdash University hospital and Abbassia Fever hospital, during the period from March 2014 to December 2015.

2.2. Collection of Specimens and Processing

The diarrhoea stool samples were collected into sterile bottles and transported to laboratory over a period of two hours in Cary-Blair transport medium.[10] All collected specimens were processed in the same day of collection.

2.3. Isolation and Identification of E. coli Isolates

Primary isolation was performed following the conventional methods for isolation of enteric bacterial Gram-negative bacilli. On arrival to the laboratory, the stool samples were directly inoculated onto MacConkey and eosin methylene blue (EMB) culture media, and plates were then incubated at 37°C for 24 - 48 hours. Isolates were identified microscopically by Gram-staining and biochemical testing methods.

2.4. Identification and Screening for ESBLs Producers by VITEK® 2 Automated System.

VITEK® 2 automated system (Biomerieux, France) was used to confirm the identification and assays the antimicrobial susceptibility of the tested isolates to screen for ESBLs production using VITEK® 2 GN and VITEK® 2 AST-N204 panels, respectively, according to the manufacturer's guidelines. Tested isolates were considered as ESBLs producers along with the Clinical and Laboratory Standards Institute (CLSI) recommendations.[11]

2.5. Antimicrobial Susceptibility Testing

Antimicrobial susceptibilities of *E. coli* isolates to different antimicrobial agents were determined manually using Kirby-Bauer disc diffusion method on Mueller-Hinton (MH) agar (Oxoid, UK) following CLSI guidelines.[11] A number of 15 antimicrobial discs, representing different classes of antimicrobial agents, were included in this study. Discs were the product of Oxoid, UK: ampicillin, piperacillin, amoxicillin-clavulanate, cefoxitin, cefuroxime sodium, cefotaxime, ceftriaxone, aztreonam, kanamycin, tetracycline, ciprofloxacin, levofloxacin, norfloxacin, trimethoprim-sulfamethoxazole and chloramphenicol. Discs were stored at 4°C and allowed to reach room temperature before being used. The inhibition zones developed around the discs measured in millimetre (mm) were interpreted as susceptible (S), intermediate (I) or resistant (R) to a particular antimicrobial agent according to CLSI.[11] Isolates that showed resistance to at least three different classes of antimicrobial agents were considered as MDR.

2.6. Detection of ESBL Phenotypes

Phenotypic confirmation of ESBL production was performed according to CLSI guidelines.[11] The antimicrobial discs used for the following ESBLs phenotypic confirmatory tests were the product of Bio-Rad, France: ceftazidime (30 µg), ceftazidime-clavulanate (30 µg/10 µg), cefotaxime (30 µg) and cefotaxime-clavulanate (30 µg/10 µg).

2.6.1. Combination Disc Method for Detection of ESBLs

This test was performed according to Garrec *et al.*[12] An overnight culture of the test isolate was suspended to the turbidity of 0.5 McFarland and used to swab a Muller-Hinton agar plate. Disks of ceftazidime, ceftazidime-clavulanate, cefotaxime and cefotaxime-clavulanate were placed on MH

agar. Isolate was considered ESBL-producer if the inhibition zone measured around one of the combination disks after overnight incubation was at least 5 mm larger than that of the corresponding cephalosporin disc alone as recommended by the manufacturer and CLSI.

2.6.2. Cefoxitin-cloxacillin double disc synergy test (CC-DDS)

This test is used for the detection of the production of AmpC β-lactamase enzyme. The test was performed according to Polsfuss *et al*.,[13] using 0.5-McFarland of test isolate prepared from overnight culture. The suspension was used to swab a Muller-Hinton agar plate, and then discs containing 30 µg of cefoxitin and containing 30 µg of cefoxitin plus 200 µg of cloxacillin (inhibitors of the AmpC enzyme) were added. Plates were incubated at 35°C for 16 hours. A difference in the cefoxitin-cloxacillin inhibition zones minus the cefoxitin alone zones of ≥ 4 mm was considered indicative for AmpC production.

2.7. Molecular detection of ESBLs encoding genes by PCR

E. coli ESBLs phenotypes, confirmed by the CLSI selection criteria, were PCR examined for the presence of ESBLs encoding genes: *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$.

2.7.1. PCR oligonucleotide primers and DNA extraction

PCR primers used in this study are listed in **Table 1** and synthesized by Invitrogen, UK. The lyophilized powder was reconstituted using nuclease free water (Promega, USA) and the concentration of each primer was adjusted to 10 pmol/µl. Total crude 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 template DNA for PCR amplification.

Table 1. *Nucleotide sequences of PCR oligonucleotide primers.*

Gene	Primer sequence (5' – 3')	PCR product size (bp)	Source
***bla*$_{TEM}$**	**F:** 5' TCCGCTCATGAGACAATAACC 3' **R:** 5' TTGGTCTGACAGTTACCAATGC 3'	931	1
***bla*$_{CTX-M}$**	**F:** 5' TCTTCCAGAATAAGGAATCCC 3' **R:** 5' CCGTTTCCGCTATTACAAAC 3'	909	
***bla*$_{SHV}$**	**F:** 5' TGGTTATGCGTTATATTCGCC 3' **R:** 5' GGTTAGCGTTGCCAGTGCT 3'	868	

1, Kiratisin *et al*.[14]

2.7.2. PCR amplification of ESBLs encoding genes

PCR reactions were performed in total volumes of 20 µl containing 10 µl of GoTaq® Green Master 2x Ready Mix (Promega, USA), 1 µl (10 pmol concentration) of each primer, 2 µl of DNA template and the volume was completed to 20 µl by adding 6 µl of nuclease free water. The PCR program for amplification for *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{SHV}$ was as follows: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds and extension at 72°C for 1 minute; and a final extension at 72°C for 7 minutes.

2.7.3. TAE (Tris-acetate-EDTA)-agarose gel electrophoresis

Reaction aliquots of 10 µl were resolved through TAE agarose gel electrophoresis prepared in a concentration of 1 % of molecular biology grade agarose (Bioline, UK) in 1× TAE buffer. DNA fragments, stained with ethedium bromide, were visualized under UV light using UV illumination and directly photographed. DNA molecular size marker 100bp – 1500 bp (New England Biolabs, UK) was used to assess PCR product size.

3. RESULTS

In this study, based on the results of VITEK® 2 automated system, phenotypic identification and antimicrobial susceptibility testing, a total of 60 *E. coli* isolates recovered from acute diarrhoea stool samples were identified as MDR *E. coli* as they were resistant to three or more different classes of antimicrobial agents. The antimicrobial susceptibility studies of these isolates showed that all isolates (100 %) were resistant to ampicillin, piperacillin cefuroxime, cefotaxim, ceftriaxone, aztreonam and tetracycline. While there were high resistance rates to trimethoprim/sulfamethoxazole (91.7 %),

ciprofloxacin (85 %) amoxicillin/clavulanic acid (83.3 %), each levofloxacin and norfloxacin (81.7 %), kanamycin (71.7 %), cefoxitin (65 %) and finally chloramphenicol (43.3 %) (**Table 2**).

The 60 *E. coli* isolates were confirmed as ESBLs producers according to CLSI guidelines of being resistant to all penicillins, third- and fourth-generation cephalosporins and aztreonam (**Table 2**). Based on CLSI guidelines of cefotaxime and ceftazidime susceptibility with or without clavulanate, these isolates showed positive results of combination disk method for detection of ESBLs test in 52/60 (86 %) isolates and AmpC production with the positive results of cefoxitin-cloxacillin double disc synergy test (CC-DDS) in 11/60 (18 %) isolates (**Figure 1**).

Table 2. *Antimicrobial susceptibility percentages of MDR ESBLs producing E. coli isolates included in this study.*

Antimicrobial agent	***E. coli* (60 isolates)**	
	Resistant No. (%*)	**Sensitive No. (%*)**
Ampicillin	60 (100)	0 (0)
Piperacillin	60 (100)	0 (0)
Amoxicillin/clavulanic acid	50 (83.3)	10 (16.7)
Cefoxitin	39 (65)	21 (35)
Cefuroxime	60 (100)	0 (0)
Cefotaxime	60 (100)	0 (0)
Ceftriaxone	60 (100)	0 (0)
Aztreonam	60 (100)	0 (0)
Kanamycin	43 (71.7)	17 (28.3)
Tetracycline	60 (100)	0 (0)
Ciprofloxacin	51 (85)	9 (15)
Levofloxacin	49 (81.7)	11 (18.3)
Norfloxacin	49 (81.7)	11 (18.3)
Trimethoprim/sulfamethoxazole	55 (91.7)	5 (8.3)
Chloramphenicol	26 (43.3)	34 (56.7)

*****Percentage correlated to the total number of isolates.

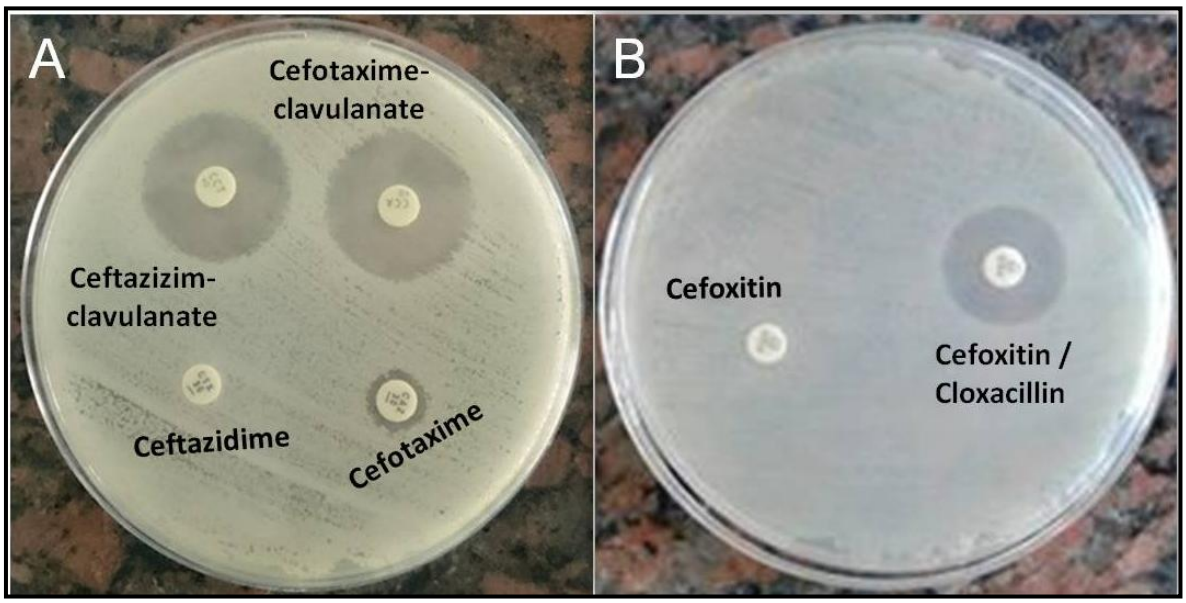

Figure 1. *Positive results of A, combination disc method for detection of ESBLs production; B, CC-DDS for AmpC β-lactamase production.*

The PCR-based investigation of the ESBLs encoding genes among these ESBLs-producing *E. coli* confirmed phenotypes revealed the presence of TEM and/or CTX-M Ambler class A ESBLs encoding genes in all isolates. The bla_{TEM} type ESBL was the most frequent as found in 51 (85 %), followed by $bla_{CTX\text{-}M}$ gene in 44 (73.3 %) of *E. coli* isolates. The bla_{SHV} gene was not detected in the isolates included in this study (**Table 3, Figure 2**). All isolates harboured the ESBLs encoding genes; either one only or two genes co-existed together. The genes $bla_{CTX\text{-}M}$ and bla_{TEM} were found together in 71.6 % of *E. coli* isolates (**Table 4**).

Table 3. *Prevalence of CTX-M, SHV and TEM encoding genes among E. coli ESBLs phenotypes.*

ESBLs phenotypes	ESBLs encoding genes		
	bla*$_{TEM}$ No. (%)	***bla*$_{CTX-M}$ No. (%)***	***bla*$_{SHV}$ No. (%)***
***E. coli* (55 isolates)**	51 (85)	44 (73.3)	0 (0)

*****Percentage correlated to the total number of isolates.

Table 4. *Genotypic profiles of E. coli ESBLs phenotypes.*

ESBLs encoding genes	No. of *E. coli* ESBLs phenotypes (%)*
***bla*$_{TEM}$ alone**	38 (63.3)
***bla*$_{CTX-M}$ alone**	31 (51.7)
***bla*$_{CTX-M}$ + *bla*$_{TEM}$**	13 (21.7)

*****Percentage correlated to the total number of isolates.

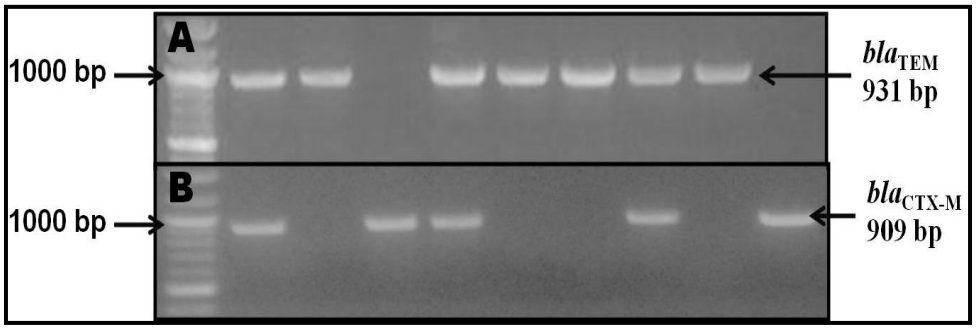

Figure 2. *Representative agarose gel (1 %) electrophoresis of PCR products of ESBLs encoding genes bla$_{TEM}$ and bla$_{CTX-M}$. M, DNA molecular weight marker; Lanes 1 – 10 in each panel, E. coli isolates.*

4. DISCUSSION AND CONCLUSION

Diarrheagenic *E. coli* is the most common cause of infectious diarrhoea worldwide, especially in developing countries.[15] It is occupying the second most important cause of mortality among childhood.[16] Seriously, the treatment of the *E. coli* caused diarrhoeal diseases and/or infections are increasingly becoming difficult because of the development of multiple-resistance to diverse antimicrobial agents.[17] Resistance to β-lactam antimicrobial agents in *E. coli* is most commonly occurred through the production of ESBLs enzymes.[8] ESBLs exhibit a high degree of diversity and encoded by diverse determinants distributed in different geographic regions of the world (Livermore *et al*., 2007).[18] ESBL testing may still be useful for epidemiological and infection control purposes.[11] Thus, in this study, a total of 60 *E. coli* isolated from faecal specimens of patients with acute infectious diarrhoea were investigated for the most common ESBLs encoding genes *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{SHV}$.

E. coli isolates were identified based on conventional cultural characteristics and biochemical testing, in addition to confirming the identification using the fully automated VITEK® 2 system. Based on the records of the antimicrobial susceptibility testing performed in this study, all *E. coli* isolates were considered as MDR and demonstrated high resistance rates to the tested antimicrobial agents. Previous reports revealed good antibacterial activity of ampicillin, chloramphenicol, tetracycline and aminoglycoside against enteric bacterial pathogens including *E. coli*.[19] The antimicrobial susceptibility patterns in this study showed that all isolates were resistant to ampicillin and tetracycline. In addition, isolates were highly resistant to kanamycin (71.7 %) and gentamicin (55.9 %), and chloramphenicol (43.3 %). In addition, there were higher resistance percentages of *E. coli* isolates of 100 % to piperacillin cefuroxime, cefotaxim, ceftriaxone and aztreonam, 66.7 % to trimethoprim-sulfamethoxazole. In addition, there were high resistance rates to trimethoprim/sulfamethoxazole (91.7 %), ciprofloxacin (85 %) amoxicillin/clavulanic acid (83.3 %) and levofloxacin or norfloxacin (81.7 %). These records indicated that these antimicrobial agents are not fit for initiation of empirical antimicrobial therapy of diarrhoea in the study hospitals when infection with an ESBL-producing *E. coli* is suspected. This high resistance rates may be explained by the misuse and/or overuse of these antimicrobial agents in Egypt and/or this region of this country.[20]

All isolates in this study were described as MDR *E. coli* based on the definition of being resistant to three or more different classes of antimicrobial agents.[21] This could be attributed to the extensive use of antimicrobial agents in developing countries, like Egypt, which has led to the emergence of MDR bacteria causing diseases.[22] Thus, regular antimicrobial susceptibility examination is necessity before treating infectious diarrhoea in a country like Egypt.

E. coli isolates included in this study were subjected to screening for ESBLs production by the fully automated VITEK® 2 system, antimicrobial susceptibility testing and confirmatory phenotypic assays for ESBLs production. The *E. coli* isolates included in this study exhibited the resistance patterns typical of ESBL producers following the CLSI guidelines as all isolates were resistant to penicillins (ampicillin, piperacillin), third- and fourth-generation cephalosporins (cefotaxime, cefuroxime, ceftriaxone) and aztreonam.[11] In addition, the confirmatory phenotypic disc diffusion test for detection of ESBLs according to CLSI, using both cefotaxime and ceftazidime alone and in combination with clavulanate, showed positive results in 86 % of isolates. Moreover, the AmpC production test using cefoxitin-cloxacillin double disc synergy test showed positive results in 18 % of isolates. This might well reflect the high rates of ESBLs production in *E. coli* and faecal colonization in a country like Egypt. AmpC β-lactamases, are mainly chromosomally encoded in Enteorbacteriaceae and they confer resistance to cephalothine, cefazoline, cefoxitin, most penicillins and to β-lactamase inhibitor like clavulanic acid.[9]

Notably, the finding of this study that ESBLs-producers *E. coli* being MDR is consistent with the fact that ESBLs-producing *E. coli* often display resistance to non-β-lactam antimicrobial agents. Indeed, a high rate of co-resistance to potentially active antimicrobial agents of divers classes is a common feature of ESBL-producing *E. coli* strains,[23] which was also found in the current study. This is explained by that genes coding for ESBLs and those conferring resistance to other antimicrobial agents often reside within the same conjugative plasmids. The conjugational transfer of plasmid-mediated ESBLs occurs efficiently in the intestinal tract, where enteric rods, in particular *E. coli*, often act as a reservoir of self transmissible resistance markers that can be exchanged between different species and/or strains of the Enterobacteriaceae family (Bonnet, 2004; Franiczek *et al*., 2012).[24,25] in one study, it was found that 39 % of children had commensally gut flora strains, including *E. coli*, which were resistant to all antimicrobial agents tested and 70 % of the children had MDR resistant strains. These bacteria could act as reservoirs for transmission of resistance markers to other enteric pathogens.[26]

CTX-M-type β-lactamases are the most prevalent ESBLs in *Enterobacteriaceae* in many geographical areas.[25] However, the PCR investigation of Ambler class A ESBLs (TEM, CTX-M, SHV) encoding genes revealed that bla_{TEM} was the predominant ESBLs encoding determinant in 85 % of isolates, followed by $bla_{\text{CTX-M}}$ in 73.3 % of isolates, however bla_{SHV} was not detected in this study. All ESBLs-producing *E. coli* isolates in this study possessed one or two of the PCR-positive ESBLs determinants. Each bla_{TEM} and $bla_{\text{CTX-M}}$ was found alone in 63.3 % and 51.7 % of all isolates, respectively. The bla_{TEM} in combination with $bla_{\text{CTX-M}}$ was detected in 21.7 % of *E. coli* isolates. The results of this study is consistent with the study of Zaki *et al*.,[27] which was carried out on diarrheagenic *E. coli* isolated from acute diarrhoea patients in Mansura Governorate, Egypt. They reported that the commonest gene among diarrhoeagenic *E. coli* was bla_{TEM}, however they detected bla_{SHV} with low percentage. This may indicate the most common carriage of TEM-type ESBL among diarrhoea causing *E. coli* in Egypt. Although, the study of Fernandes *et al.* in Portugal reported also that that TEM-type ESBL was the most prevalent type followed by CTX-M.[28] Moreover, a study from Iran by Ghorbani-Dalini *et al*.,[29] reported clearly that bla_{TEM} was the most common gene in diarrhoeagenic ESBLs-producer *E. coli*. The origin of SHV is the chromosome of *Klebsiella* spp. and has only a narrow β-lactam hydrolyzing activity conferring resistance to penicillin and ampicillin. In addition, SHVs are more prevalent in Europe,[9] which may explain the absence of bla_{SHV} in this study.

In conclusion, ESBLs production is a noteworthy increasing among *E. coli* causing diarrhoeal diseases. The bla_{TEM} is the common genetic mechanism for ESBL production in these pathogens isolated from Egypt, followed by $bla_{\text{CTX-M}}$ Egypt. The limited treatment alternatives of infections caused by ESBL-producers *E. coli* require a public health policy on appropriate prescribing and the rational use of antimicrobial agents.

REFERENCES

[1] Alikhani MY, Hashemi SH, Aslani MM, Farajnia S. Prevalence and antibiotic resistance patterns of diarrheagenic *E. coli* isolated from adolescents and adults in Hamedan, Western Iran. Iranian journal of microbiology 2013; 5(1): 42-7.

[2] Collins S. Treating severe acute malnutrition seriously. Archives of disease in childhood 2007; 92(5): 453-61.

[3] Sang WK, Oundo V, Schnabel D. Prevalence and antibiotic resistance of bacterial pathogens isolated from childhood diarrhoea in four provinces of Kenya. The Journal of Infection in Developing Countries 2012; 6(07): 572-8.

[4] Nyvall RF. Microbes and People: An AZ of Microorganisms in Our Lives. Journal of Natural Resources and Life Sciences Education 2001; 30: 135.

[5] World Health Organization. Antimicrobial resistance global report on surveillance: 2014 summary; http://www.who.int/drugresistance/ documents/surveillance report/en/last reviewed February 10, 2016.

[6] Shaikh S, Fatima J, Shakil S, Rizvi SM, Kamal MA. Antibiotic resistance and extended spectrum β-lactamases: Types, epidemiology and treatment. Saudi journal of biological sciences 2015; 22(1): 90-101.

[7] Drawz SM, Bonomo RA. Three decades of β-lactamase inhibitors. Clinical microbiology reviews 2010; 23(1): 160-201.

[8] Bush K, Jacoby GA. Updated functional classification of β-lactamases. Antimicrobial agents and chemotherapy 2010; 54(3): 969-76.

[9] Kocsis B, Szabo D. Antibiotic resistance mechanisms in Enterobacteriaceae. Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education, Formatex Research Centre, Badajoz 2013; 251-7.

[10] Cheesbrough M. District laboratory practice in tropical countries. Cambridge university press 2006.

[11] CLSI. Performance Standards for Antimicrobial Susceptibility Testing; 24th informational Supplement. CLSI document 2014; M100-S24: 50-57.

[12] Garrec H, Drieux-Rouzet L, Golmard JL, Jarlier V, Robert J. Comparison of nine phenotypic methods for detection of extended-spectrum β-lactamase production by Enterobacteriaceae. Journal of clinical microbiology 2011; 49(3): 1048-57.

[13] Polsfuss S, Bloemberg GV, Giger J, Meyer V, Böttger EC, Hombach M. Practical approach for reliable detection of AmpC beta-lactamase-producing Enterobacteriaceae. Journal of clinical microbiology 2011; 49(8): 2798-803.

[14] Kiratisin P, Apisarnthanarak A, Laesripa C, Saifon P. Molecular characterization and epidemiology of extended-spectrum-β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates causing health care-associated infection in Thailand, where the CTX-M family is endemic. Antimicrobial agents and chemotherapy 2008; 52(8): 2818-24.

[15] Davidson G, Barnes G, Bass D, Cohen M, Fasano A, Fontaine O, Guandalini S. Infectious diarrhoea in children: working group report of the First World Congress of Pediatric Gastroenterology, Hepatology, and Nutrition. Journal of pediatric gastroenterology and nutrition 2002; 35: S143-50.

[16] Boschi-Pinto C, Velebit L, Shibuya K. Estimating child mortality due to diarrhoea in developing countries. Bulletin of the World Health Organization 2008; 86(9): 710-7.

[17] Mathur P, Kapil A, Das B, Dhawan B. Prevalence of extended spectrum (beta) lactmase producing gram negative bacteria in a tertiary care hospital. Indian Journal of Medical Research 2002; 115: 153.

[18] Livermore DM, Canton R, Gniadkowski M, Nordmann P, Rossolini GM, Arlet G, Ayala J, Coque TM, Kern-Zdanowicz I, Luzzaro F, Poirel L. CTX-M: changing the face of ESBLs in Europe. Journal of Antimicrobial Chemotherapy 2007; 59(2): 165-74.

[19] Brooks G, Butel JS, Ornston LN, Jawetz E, Melnick JL, Adelberg EA. Enteric Gram-negative rods (Enterobacteriaceae). Jawetz, Melnick & Adelberg's Medical Microbiology 1991: 215-20.

[20] Nguyen TV, Van Le P, Le CH, Weintraub A. Antibiotic resistance in diarrheagenic *Escherichia coli* and *Shigella* strains isolated from children in Hanoi, Vietnam. Antimicrobial agents and chemotherapy 2005; 49(2): 816-9.

[21] Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson- Liljequist B, Paterson DL. Multidrug- resistant, extensively drug- resistant and pandrug- resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical microbiology and infection 2012; 18(3): 268-81.

[22] Temu MM, Kaatano GM, Miyaye ND, Buhalata SN, Shushu ML, Kishamawe C, Changalucha JM. Antimicrobial susceptibility of *Shigella flexneri* and *S. dysenteriae* isolated from stool specimens of patients with bloody diarrhoea in Mwansa, Tanzania. Tanzania Journal of Health Research 2007; 9(3): 186-9.

[23] Tängdén T, Cars O, Melhus Å, Löwdin E. Foreign travel is a major risk factor for colonization with *Escherichia coli* producing CTX-M-type extended-spectrum β-lactamases: a prospective study with Swedish volunteers. Antimicrobial agents and chemotherapy 2010; 54(9): 3564-8.

[24] Bonnet R. Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. Antimicrobial agents and chemotherapy 2004; 48(1): 1-4.

[25] Franiczek R, Sobieszczańska B, Turniak M, Kasprzykowska U, Krzyzanowska B, Jermakow K, Mokracka-Latajka G. ESBL-producing *Escherichia coli* isolated from children with acute diarrhoea-antimicrobial susceptibility, adherence patterns and phylogenetic background. Adv Clin Exp Med. 2012; 21(2): 187-92.

[26] Shears P, Suliman G, Hart CA. Occurrence of multiple antibiotic resistance and R plasmids in Enterobacteriaceae isolated from children in the Sudan. Epidemiology and infection 1988; 100(01): 73-81.

[27] Zaki, EA; Mansour, AK. Molecular Detection of bla_{TEM} and bla_{SHF} in Diarrhoegenic *Escherichia coli* Isolated from Egyptian Children. *Int J Microbiol Adv Immunol* 2015; 3(1): 49-54.

[28] Fernandes R, Amador P, Oliveira C, Prudêncio C. Molecular characterization of ESBL-producing Enterobacteriaceae in Northern Portugal. The Scientific World Journal 2014.

[29] Ghorbani-Dalini S, Kargar M, Doosti A, Abbasi P, Sarshar M. Molecular Epidemiology of ESBL Genes and Multi-Drug Resistance in Diarrheagenic *Escherichia coli* strains Isolated from Adults in Iran. Iranian journal of pharmaceutical research: IJPR 2015; 14(4): 1257.