Molecular Detection of \( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{SHF}} \) in Diarrhoegenic \textit{Escherichia coli} Isolated from Egyptian Children

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**Abstract**

**Introduction:** \textit{Escherichia coli} (E. coli) is a common bacterial pathogen for acute diarrhea in children. The aim of the present study was to determine the prevalence mainly of \( \text{TEM} \) and \( \text{SHF} \)-beta lactamase-encoding genes responsible for extended spectrum beta lactamase (ESBL) production amongst diarrheagenic \textit{E. coli} species as these strains are reported to have been increased in recent years.

**Methodology:** The study included children with acute diarrhea presented in Mansoura University children hospital during the period from January 2011 till Jun 2014. In the microbiological laboratory stool samples were cultured and the isolated colonies were identified by standard biochemical reactions and by multiplex polymerase chain reaction (PCR) for identification of diarrheagenic \textit{E. coli} strains. In vitro susceptibility testing of all isolates was performed using the discs diffusion method. \textit{E. coli} isolates were subjected to testing to detect the possible presence of \( \text{SHV} \), and \( \text{TEM} \) genes by conventional PCR.

**Results and Conclusion:** The study included 600 children presented as acute diarrhea. Acute diarrhea caused by \textit{E. coli} was detected in 160 samples. ESBL genes either \( \text{TEM} \) or \( \text{SHF} \) was detected in 31.1% of isolated \textit{E. coli}. The commonest gene was \( \text{TEM} \) (22.5%), then \( \text{SHF} \) (10%). Combined genes were detected only in 2 isolates, table 6.

The commonest resistance pattern of \textit{E. coli} harboring ESBL genes cefepime (100%), cefazolin (96%) and cefotaxime (96%) and for non beta lactam the commonest was for ciprofloxacin (88%), amikacin and tobramycin (20% for each), Table 7. From this study we can conclude that extended beta lactamase production is common among diarrheagenic \textit{Escherichia coli} isolated from children below 5 years. The \( \text{bla}_{\text{TEM}} \) is the common genetic mechanism for extended beta lactamase production in these isolates followed by \( \text{bla}_{\text{SHF}} \).

**Keywords:** Diarrheagenic \textit{E. coli}; ESBLs; \( \text{bla}_{\text{TEM}} \); \( \text{bla}_{\text{SHF}} \).

**Introduction**

Acute infectious diarrhea is an important cause of morbidity and mortality in children worldwide. The disease remains a health problem challenge especially in developing countries [1-3]. The causative pathogens include a wide variety of microorganisms like bacteria, viruses and parasites. [4-7]. Diarrhogenic \textit{Escherichia coli} (E. coli) represented one of the most frequent causes of acute diarrhea in children under five years old in developing countries [8-10]. In UNICEF report in Egypt based on survey conducted by Egypt demographic and health state the overall percentage of children under five who had diarrhoea in survey was conducted was 8.5%, and though antibiotics and anti-diarrhoeal medications are generally not recommended to treat diarrhoea in young children; results from the survey reported that antibiotics were given to one-third of children with diarrhea [11].

Recent reports indicated increase in the prevalence of extended beta lactamase production among diarrheagenic \textit{E. coli} species [12, 13]. The inappropriate use of antibiotics is one of the leading causes of acquisition of microbial resistance leading to severe infections. Moreover, \textit{E. coli} is known to be an important reservoir for resistance genes as described previously in several reports [14, 15].

A common mechanism of antibiotics resistance in \textit{E. coli} is the production of extended beta lactamase enzymes. Beta-lactamases are enzymes produced by some bacteria and are responsible for bacterial resistance to beta-lactam antibiotics like penicillins, cephamycins and carbapenems (ertapenem). These antibiotics have a common four-atom ring known as a beta-lactam in their molecular structure. The beta-lactamase enzyme breaks that ring open, leading to deactivating the molecule’s antibacterial properties [16]. At the beginning (ESBL) producing organisms were
isolated from outbreaks in Germany, United States and later on reported world wide [17]. ESBL organisms are commonly resistance to cephalosporines antibiotics [18].

The production of extended-spectrum β-lactamases like TEM-1, TEM-2 and SHV-1 by gram-negative bacteria renders these species resistant to penicillins, cephalosporins and aztreonam in the treatment of serious infections caused by these pathogens. Though there is no information on their molecular types [19, 20].

There are so many types of ESBLs like TEM, SHV, CTX, OXA, but majority of the ESBLs are derivatives of TEM or SHV enzymes and these enzymes are most often found in E. coli and K. pneumoniae [21].

There are limited studies about the molecular mechanisms of ESBLs in diarrheagenic E.coli in Egypt related to presence of bla TEM and bla SHF genes in diarrheagenic E.coli.

The aim of the present study was to determine the prevalence mainly of bla TEM and bla SHV genes common genes responsible for ESBL production amongst diarrheagenic E. coli species isolated from the children admitted to Mansoura University children hospital.

Materials and Methods

The study included children with acute diarrhea presented in Mansoura University children hospital during the period from January 2011 till Jun 2014. Diarrhea was defined as (i) at least three loose (or watery) stools within 24 h, regardless of other gastrointestinal symptoms; (ii) two or more loose stools associated with one other symptom of gastrointestinal infection like abdominal pain, nausea, vomiting, and fever; or (iii) passage of a single loose stool with grossly evident blood and/or mucous [22].

Patients were selected according to the Centers for Disease Control and Prevention (CDC) definition of hospital acquired diarrhea [23]. The parents of each child signed and the study was approved by the ethical committee of Mansoura Faculty of Medicine, Egypt. Each child was subjected to full history taking and clinical examinations. Stool sample were obtained from each patient and subjected to full microbiological examination.

Stool samples were collected in plastic containers. In the microbiological laboratory, stool samples were spread on MacConkey at 37°C for 24 hours. The isolated colonies were identified by standard biochemical reactions by MicroScan® WalkAway diagnostic microbiology system (Siemens HealthCare Diagnostics, formerly Dade Behring, USA).

E.coli was identified by further multiplex PCR to identify the five common diarrheagenic E.coli species namely Enterotoxigenic E. coli (ETEC) Enteraggregative E. coli (EAEC) Enterohemorrhagic E. coli (EHEC), Enteropathogenic Esch. coli (EPEC) and Enteroinvasive Esch. coli (EIEC).

DNA Extraction

Ten colonies of isolated E.coli were suspended in 1ml of sterile distilled water and bacterial suspension was boiled for 10 minutes centrifuged and supernatent was used as DNA templates for all PCR procedures.

Multiplex PCR for identification of diarrheagenic E.coli species

Multiplex PCR was designed for the detection of target genes: eae for enteropathogenic E. coli (EPEC),, cae and stx for enterohemorrhagic E. coli (EHEC), ipaH for enteroinvasive E. coli (EIEC), CVD432, aspU and aggR for enteroaggregative E. coli (EAEC) and elt and est for enterotoxigenic E. coli (ETEC). The primers used and bp size were summarized in Table 1 [24]. A total volume of 50μl master mixture was used for PCR using mixtures of 10x buffer 5μl, dNTP (0.2mM each) 4μl, primer mixture (30 p mol concentration of each) 3μl, Taq polymerase (2.5 U) for 25 cycles.

Table 1. PCR primers used for detecting different Diarrheagenic E. coli strains.

| E.coli strain | Primer | bp | gene |
|--------------|--------|----|------|
| EPEC | SK1 CCC GAA TTC GGC ACA AGC ATA AGC | 881 | eae |
| EPEC | SK2 CCC GGA TCG GTG CTC CCA GTA TTC G | |
| EPEC | VTcom-u GAG CTA AAT TTA TAT GTG TAT CTA CCA AAT AC | 518 | stx |
| EPEC | VTcom-d TGA TGA TGG CAA TTC AGT AT | |
| ETEC | AL65 TTA ATG ACA CCC GGT ACA AGC AL125 CCT G | 147 | est |
| ETEC | GAC TCT TCA AAA GAG AAG AAA ATT AC | |
| ETEC | LIT LIT TCT TTA TGT GCA TAC GGA GC | 322 | elt |
| ETEC | LTR CCA TAC TGA TGG CGG CAAT T | |
| EIEC | ipaIII TGT CCT TGA CCG CCT TTC GCA GAC TGT C | 619 | ipaH |
| EIEC | CVD432 | |
| EIEC | aggR | |
| EAEC | aggR | 254 | aggR |
| EAEC | aggR | |
| EAEC | Eaggfp AGA CTG CAG GGA AGG ACT GTA ATC | 194 | CVD432 |
| EAEC | Eaggfp Eaggfp ATG GCT GTG CTC AAT AGA TGA GAA C | |
| EAEC | spU-3 GCC TTT GGC GGT GGT AGC GG | 282 | aspU |
| EAEC | aspU-2 AAC CCA TTC GGT TAG AGC AC | |
0.5μl, DNAase free water 36.5μl and DNA template (sample) 1μl. Amplification was performed in the thermal cycler at 95°C (Initialisation) 5 minutes, 95°C (Denaturation) 1 minute, 56°C (Annealing) 1 minute 30 cycles, 72°C (Extension) 1 minute 72°C (Final extension) 10 minutes [24].

**Antimicrobial susceptibility testing and ESBL detection**

*In vitro* susceptibility testing of all isolates to a wide range of antimicrobials, including both beta-lactams and non-beta-lactams, was performed using the disc diffusion method. Isolates reported as ESBL positive, were designated as ESBL screen-positive and were further subjected to a confirmatory test. Confirmation of the ESBL phenotype was performed using the combination disk method based on the inhibitory effect of clavulanic acid according to the CLSI criteria [25]. Antimicrobial disks used were obtained from BD BBL Sensi-Disc (Becton Dickinson, Sparks, MD, USA). The antibiotic susceptibility profiles of the isolates were determined to antimicrobials including amikacin 30 mcg, gentamicin 10mcg, ciprofloxacin 5mcg, tobramycin 10mcg, amoxicillin/clavulanic acid 20/10mcg, ceftoxime 30mcg, cefazidime 30 mcg, cefepime 30mcg, cefazolin 30mcg and imipenem 10mcg.

**PCR for the detection of TEM and SHV genes**

E.coli isolates were subjected to testing to detect the possible presence of *bla*-SHV, and *bla*-TEM genes by conventional PCR. The primers used in multiplex PCR are listed in Table 2. A single colony of the isolated bacteria was emulsified in the 50μl reaction mix, which contained 10 pmol of each primer, 10mM dNTPs mix (Qiagen, Hilden, Germany) and 2.5 U of Taq polymerase (Qiagen, Hilden, Germany) in 1x Taq polymerase buffer.

Amplification reactions were performed under the following conditions: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds with an extension at 72°C for 50 seconds, and a final extension for one cycle at 72°C for 5 minutes. The PCR product was then run on a 1.5% agarose gel stained with ethidium bromide, and visualized under UV transillumination for detection of the amplified fragment.

**Results**

The study included 600 children who presented with acute diarrhea in the period from January 2011 till Jun 2014. *E.coli* was detected in 160 samples.

Table 3 summarizes the demographic and clinical data of children with acute diarrhea caused by *E.coli*. The study included 600 children with mean±SD age 3.3±1.8 years complaining of acute diarrhea during the period of the study. *E.coli* was isolated for diarrhea from 160 children (26.7%). One hundred patients were non breast feed mainly with hospital acquired diarrhea.

Isolated diarrheagenic species were mainly EAEC (31%) and EPEC (25%) Table 4.

The rate of antibiotics susceptibility of isolated *E.coli* for beta-lactams antibiotics was for cefepime (96.2%), cefazolin (97.5%), followed by amoxicillin/clavulanic acid (28.8%) and imipenem (28.8%). For non beta lactams antibiotics the commonest resistance rate was for ciprofloxacin (90%), Table 5.

ESBL genes either TEM or SHF were detected in 31.1% of isolated *E.coli*. The commonest gene was TEM (22.5%), then SHF (10%). Combined genes were detected only in 2 isolates, Table 6.

The commonest resistance pattern of *E.coli* harboring ESBL genes cefepime (100%), cefazolin (96%) and ceftoxime (96%) and for non beta lactams the commonest was for ciprofloxacin (88%), amikacin and tobramycin (20% for each), Table7.

**Discussion**

Diarrheagenic *E.coli* is known as common causative bacteria that...
| Yes | 86 (53.8%) |
| No | 74 (46.3%) |

| Vomiting | Yes | 60 (37.5%) |
| No | 100 (62.5%) |

| Breast feeding | Yes | 40 (25%) |
| No | 120 (75%) |

| Hospital acquired diarrhea | 120 (75%) |

**Table 4. Diarrheagenic E.coli species isolated.**

| E.coli species | No (n=160) | % |
|---------------|-----------|---|
| EPEC          | 40        | 25% |
| EHEC          | 0         | 0% |
| EIEC          | 35        | 21.9% |
| EAEC          | 50        | 31.25% |
| ETEC          | 35        | 21.9% |

**Table 5. Antibiotics resistance among isolated E.coli.**

| Antibiotics                        | Resistance % |
|------------------------------------|--------------|
| Amikacin                           | 23.80%       |
| Gentamycin                         | 18.80%       |
| Ciprofloxacin                      | 90%          |
| Amoxicillin/clavulinic acid        | 28.80%       |
| Cefotaxime                         | 37.50%       |
| Ceftazidime                        | 27.50%       |
| Cefepime                           | 96.20%       |
| Cefazolin                          | 97.50%       |
| Imipenem                           | 28.80%       |
| Tobramycin                         | 25%          |

**Table 6. Genotypes of ESBLs among E.coli.**

| No. (%) |
|---------|
| Total isolates with positive ESBLs genes | 50 (31.3%) |
| TEM | 36 (22.5%) |
| SHF | 16 (10%) |
| Mixed TEM,SHF | 2 (1.3%) |

**Table 7. Pattern of antibiotics resistance among E.coli carrying TEM or SHF genes.**

| Antibiotic | No. | % |
|------------|-----|---|
| Cefepime   | 50  | 100 |
| Cefazolin  | 48  | 96% |
| Cefotaxime | 48  | 96% |
| Ceftazidime| 20  | 40% |
| Amikacin   | 10  | 20% |
| Amoxicillin/clavulinic acid | 27 | 54% |
| Imipenem   | 27  | 54% |
| Ciprofloxacin | 44 | 88% |
| Gentamycin | 8   | 16% |
| Tobramycin | 10  | 20% |
cause diarrhea among children world wide [26-28]. Different reports have been investigating the role of E.coli as diarrheagenic cause in different countries. The frequency of isolated E.coli ranged from 30% up to 40% in previous studies [29, 30]. In Egypt, in limited number of children it was 5.2% Out of 134 patients [31].

In the present study, of the 600 stool samples examined, DEC strains were isolated from (26.7%), of cases mainly under 5 years of age. This finding is online with the concept that diarrheagenic E.coli strains are the most common causes of diarrheal diseases in developing countries [32].

It is well known that E.coli diarrhea has mild course and it is usually self-limited and rehydration is the most effective treatment. The use of antibiotics in general has no role and has many limitations on the grounds of drug toxicity and the risk of increased wide-spread antimicrobial resistance [33, 34].

Isolated diarrheagenic species were mainly EAEC (31%) and EPEC (25%). These results on line to the findings in other study carried in Egypt recently where EAEC was the commonest species [35].

There is increase in antimicrobial resistance due multiple factors one of them is the inappropriate use of antibiotics therapy in developing countries [34]. In the present study, the rate of antibiotics susceptibility of isolated E.coli for beta-lactams antibiotics was for cefepime (96.2%), cefazolin (97.5%), followed by amoxicillin/clavulenic acid (28.8%) and imipenem (28.8%). This finding has been observed also in an earlier study [36]. In another study, antibiotic resistance rates among Egyptian diarrheagenic E.coli isolates were 68.2%, 57.2% and 24.2% for ampicillin, trimethoprim-sulfamethoxazole and ampicillin-sulbactam, respectively [37].

Many studies indicate that multidrug resistant E. coli are widespread among the DEC strains and occurrence of resistant DEC could be because of environmental conditions, including transmission of resistant isolates from adults to children, or from animals to humans [38]. The main factors for dissemination of resistance genes are mobilizable plasmids, self-transmissible plasmids and conjugative transposons [39].

The effective beta-lactam/beta-lactamase inhibitor combination was amoxicillin/clavulenic acid combination. The rate of resistance to beta-lactam/beta-lactamase inhibitor this in study was nearly similar to that recorded by Díaz et al. (2010) [40], who reported that 69.4% were susceptible to amoxicillin/clavulanes. Other study reported complete failure of clavulanate to retain the activity of amoxicillin against ESBL-producers derived from Egyptian medical institute [41]. Lower resistance rates (77%) to amoxicillin/clavulenic acid than our finding showed by hospitals and community derived E. coli isolates in medical institute in Cairo, Egypt [42]. This could be explained by the difference in the beta lactamase types among different studies. It is well known that clavulanate is a potent inhibitor of beta lactamase.

The rates of resistance that have been demonstrated by clinical E. coli isolates against the non-beta-lactam antimicrobials was for ciprofloxacin (90%), amikacin and tobramycin (20% for each) reflecting the high resistance rates against the different classes of antimicrobials and the limited therapeutic option for the treatment of the infections that are caused by these E. coli. The rate of resistance against quinolones in this study was similar to that reported previously [41], but greatly higher than that recorded by Mohamed Al-Agamy et al., (41.3%) (2006) [42]. Strong relation between resistance to quinolones and other types of ESBLs like CTX-M-15 production has been documented [43]. The rates of resistance against aminoglycosides, in general, in our study were higher than the previously reported rates [42, 44]. The resistance for both quinolones and ESBL producing E. coli strains may be associated with conjugative transfer of the same plasmids carrying resistance genes for both [45].

The bla-TEM and bla-SHF was detected in 31.1% from 160 E.coli isolates. The detection rates of ESBLs genes in various studies range from 4% up to 50% according to the geographical region of the isolates [46]. The commonest gene was TEM (22.5%), then SHF (10%). These rates are similar to previous findings in Iran where bla-TEM was reported in 24% of isolated E.coli and 6% was blao-SHF and 3% had both genes.

The shortage of this study is the lack of identification of CTX-M genotype that might be responsible for ESBL activity.

From this study we can conclude that extended beta lactamase production is common among diarrheagenic Escherichia coli isolated from children below 5 years. The bla-TEM is the common genetic mechanism for extended beta lactamase production in these isolates followed by bla-SHF. Antibiotics use should be restricted in diarrhea in children to restrict the spread of antibiotics resistance.

References
[1]. Usein CR, Taru-Chitou D, Cionea S, Conedi M, Damian M (2009) Escherichia coli pathotypes associated with diarrhea in Romanian children younger than 5 years of age. Jpn J Infect Dis. 62(4): 289-93.
[2]. Ochoa TJ, Ruiz J, Molina M, Del Valle LJ, Vargas M, et al., (2009) High frequency of antimicrobial drug resistance of diarrheagenic Escherichia coli in infants in Peru. Am J Trop Med Hyg. 81(2): 296–301.
[3]. Jafari F, Hamidian M, Rezaeddinshahi M, Doyle M, Salzmanzadeh-Ahbari S, et al., (2009) Prevalence and antimicrobial resistance of diarrheagenic Escherichia coli and Shigella species associated with acute diarrhea in Tehran, Iran. Can J Infect Dis Med Microbiol.; 20(3): e56-62.
[4]. Wilson G, Earov JM, Mukhopadhyay C, Shivananda PG (2009) Child Health Epidemiology Reference Group., WHO estimates of the causes of death in children. Lancet. 365(9465): 1147-52.
[5]. Niehaus MD, Moore SR, Patrick PD, Derr LL, Lonzet B, Lima AA, et al., (2002). Early childhood diarrhea is associated with diminished cognitive function 4 to 7 years later in children in a northeast Brazilian shanty town. Am J Trop Med Hyg. 66(5): 590–3.
[6]. Nguyen TV, Le PV, Le CH, Weintraub A (2005) Antibiotic resistance in diarrheagenic Escherichia coli and Shigella strains isolated from children in Hanoi, Vietnam. Antimicrob Agents Chemother. 49(2): 816–9.
[7]. Yang CM, Lin MF, Lin CH, Huang YT, Hsu CT, Liou ML (2009) Characterization of antimicrobial resistance patterns and integrons in human fecal Escherichia coli in Taiwan. Jpn J Infect Dis. 62(3): 177–81.
[8]. Vila J, Vargas M, Casals C, Urassa H, Mshinda H, Schellemberg D, et al., (1999) Antimicrobial resistance of diarrheagenic Escherichia coli isolated from children under the age of 5 years from Izkara, Tanzania. Antimicrob Agents Chemother. 43(12): 3022–4.
[9]. Mansouri S, Sharestif S (2002) Antimicrobial resistance pattern of Escherichia coli causing urinary tract infections, and that of human fecal flora, in the southeast of Iran. Microb Drug Resist. 8(2): 123–8.
[10]. Mansouri S, Shareifi S (2002) Molecular Detection of blaTEM and blaSHF in Diarrheogenic Escherichia coli Isolated from Egyptian Children. Int J Microbiol Adv Immunol. 03(1), 49-54.
