Rapid Diagnosis and Characterization of Diarrheagenic *Escherichia coli* In Egyptian Children Using Multiplex PCR

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Escherichia coli (E.coli) **is the most important etiologic agent of childhood diarrhea and represents a major public health problem in developing countries. Aim of this work was to investigate the role of diarrheagenic *E.coli* in Egyptian children below 5 years age using multiplex PCR and to evaluate multiplex PCR in rapid diagnosis of enteric infections caused by diarrheagenic *E.coli* strains. Rectal swabs were taken from 83 children under 5 years age with diarrhea and 33 age-matched controls. All *E.coli* isolates were O serotyped using *E.coli* O polyvalent and monovalent antisera and subjected to multiplex PCR assay with specific primers, eae primer of *eaeA* (gene of intimin of EHEC and EPEC), primer bfpA of *bfpA* (structural gene for the bundle-forming pilus of EPEC), primers VT1 and VT2 of *vt1* and *vt2* genes (genes of shiga toxins 1 and 2 of EHEC respectively), primer LT of *eltB* (gene of labile toxin of ETEC), primer ST for *estA* (gene of stable toxin of ETEC), primer SHIG of *ial* (invasion-associated locus of the invasion plasmid found in EIEC) and primer EA of pCVD (the nucleotide sequence of the EcoRI-PstI DNA fragment of pCVD432 of EAEC).**

The study revealed that diarrheagenic *E.coli* strains were significantly isolated from patients more than control using multiplex PCR. Out of 70 *E.coli* strains isolated from patients, 17(24.3%) isolates were proved to be diarrheagenic by multiplex PCR where 53 (75.7%) isolates were non diarrheagenic. Out of 30 *E.coli* isolates recovered from control group, 1 (3.3%) isolate was proved to be diarrheagenic by multiplex PCR where 29 (96.7%) isolates were non diarrheagenic(Chi-square=18.5 & $p \leq 0.001$) as shown in table (1). As regard to serology of isolated *E.coli* strains, serologically typable strains were insignificantly isolated from both patients and controls. Out of 70 *E.coli* isolates recovered from patients, 23(32.9%) isolates were serologically typable and 47 (67.1%) isolates were serologically non-typable. Out of 30 *E.coli* isolates recovered from control group, 7(23.3%) isolates were serologically typable and 23(76.7%) isolates were serologically non-typable (Chi-square = 2.28 & $p \leq 0.20$) as shown in table (2). Matching results of multiplex PCR and results of serology revealed that multiplex PCR was significant in differentiating diarrheagenic *E.coli* strains in both patients and control. Out of the tybable 23 *E.coli* strains isolated from patients, 12(52.2%) strains were proved to be diarrheagenic by multiplex PCR where 11(47.8%) strains were non diarrheagenic. Out of the non- tybable 47 *E.coli* strains isolated from patients, 5 (10.6) strains were proved to be diarrheagenic by multiplex PCR where 42 (89.4) strains were non diarrheagenic(Chi-square = 40.17 & $p \leq 0.001$) as shown in table (3). Non of the typable 7 E.coli strains isolated from control was proved to be diarrheagenic by multiplex PCR. Out of the non- tybable 23 *E.coli* strains isolated from control, 1(4.3%) strain was proved to be diarrheagenic by multiplex PCR where 22 (95.7%)strains were non-diarrheagenic(Chi-square = 4.39 & $p \leq 0.05$) as shown in table(4). Out of the diarrheagenic *E.coli* isolated, 9(52.9%) isolates were ETEC; 5(29.4%) isolates were EPEC; and 3 (17.6%)isolates were EAEC. 4(23.5%), 3(17.6%), and 2(11.8%) isolates of ETEC showed the *eltB, estA, eltB+ estA* genotypes respectively. 4 (23.5%)& 1(5.9%) isolates of EPEC showed *eae* and *eae+bfp* genotypes respectively. The 3(17.6%) EAEC isolates showed pCVD genotype table (5). This study concluded that multiplex PCR could be used as a rapid method for rapid diagnosis and characterization of diarrheagenic *E.coli* in children and recommended that further studies must be done for the application of multiplex PCR for the rapid diagnosis of diarrheagenic *E.coli* directly from stools.

INTRODUCTION

Escherichia coli is the most important etiologic agent of childhood diarrhea and represents a major public health problem in developing countries.[1,8,17] Five categories of diarrheagenic *E.coli* are recognized: enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), Shiga-like toxin-producing (STEC) or enterohaemorrhagic (EHEC) and enteroaggregative (EAEC) *E.coli*.[3,6,19]

The virulence mechanisms that characterize these categories of *E.coli* are genetically encoded by chromosomal, plasmid, and bacteriophage DNAs and are represented by the following genes: *eae* (attaching and effacing lesions), *bfpA* (localized adherence), the gene encoding enteroaggregative adherence, *ipaH* (enteroinvasive mechanism), the genes encoding heat-labile toxin (LT) and heat-

stable toxin (ST), and *stx1* and *stx2* (Shiga toxins). [2]

Some assays were developed to correctly identify diarrheagenic E.coli strains differentiating them from nonpathogenic members of the normal flora. The assays available depends upon biochemical reactions, serotyping, phenotypic assays based on virulence characteristics, and molecular detection methods.[6, 24]

Some of these assays are deficient in characterizing diarrheagenic *E.coli* while other assays require special expertise (adherence assays), and are time consuming. PCR using single primer sets has been already used to detect diarrheagenic *E.coli* by detecting virulence genes in bacterial isolates, allowing the rapid diagnosis of diarrheagenic *E.coli*. [11, 23] But screening of bacterial isolates requires a large number of individual PCRs if single primer sets are used in separate reactions. Using multiplex PCR systems can reduce the number of tests needed for diagnosis of diarrheagenic *E.coli*.[10, 12, 15, 17]

Aim of this work was to study the role of diarrheagenic *E.coli* in Egyptian children below 5 years age using multiplex PCR and to evaluate multiplex PCR in characterization and rapid diagnosis of enteric infections caused by diarrheagenic *E.coli* strains.

PATIENTS, MATERIAL AND METHODS

This study was done in the Microbiology and Pediatrics departments, Faculty of Medicine, Zagazig University. Rectal swabs were taken from 83 children under 5 years age with diarrhea attending Pediatrics department of Zagazig university hospitals and 33 age-matched controls. Patients were enrolled in the study if they had diarrhea, characterized by the occurrence of three or more loose, liquid, or watery stools or at least one bloody loose stool in a 24-h period.[24] Control subjects were healthy children with no history of diarrhea for at least 1 month.

Isolation:

Rectal swabs were collected in Cary-Blair transport medium, then cultured overnight on MacConkey agar (Oxoid). 3-5 colonies of typical *E.coli* morphology were suspended in nutrient broth followed by 2-3 hr incubation at 37°C. *E.coli* was further identified by standard biochemical reactions according to collee et al.1996. [4]

Serotyping:

All isolates were O serotyped using *E.coli* O polyvalent and monovalent antisera (Remel).

Multiplex PCR

(i) DNA extraction: DNA was extracted from bacteria by suspending one bacterial colony in 50 µl of deionized water, boiling the suspension for 5 min, and centrifuging it at 10,000 rpm for 1 min. The supernatant was then used as the DNA template for PCR. [2]

(ii) Primers: The DNA templates were subjected to multiplex PCR with specific primers, as described previously;[22, 24] primer eae to detect *eaeA* (structural gene for intimin of EHEC and EPEC) 5'-CAC ACG AAT AAA CTG ACT AAA ATG-3' and 5'-AAA AAC GCT GAC CCG CAC CTA AAT-3'; primer bfpA of *bfpA* (structural gene for the bundle-forming pilus of EPEC) 5'-TTC TTG GTG CTT GCG TGT CTT TT-3' and 5'-TTT TGT TTG TTG TAT CTT TGT AA-3'; primers VT1 and VT2 of *vt1 and vt2* genes (genes of Shiga toxins 1 and 2 of EHEC respectively) VT1, 5'-GAA GAG TCC GTG GGA TTA CG-3' and 5'-AGC GAT GCA GCT ATT AAT AA-3'; VT2, 5'-ACC GTT TTT CAG ATT TTG ACA CAT A-3' and 5'-TAC ACA GGA GCA GTT TCA GAC AGT-3'; primer LT of *eltB* (gene of labile toxin of ETEC) 5'-TCT CTA TGT GCA TAC GGA GC-3' and 5'-CCA TAC TGA TTG CCG CAA T-3'. Primer ST for *estA* (gene of stable toxin of ETEC) 5'-GCT AAA CCA GTA GAG GTC TTC AAA A-3' and 5'CCC GGT ACA GAG CAG GAT TAC AAC A-3'; primer SHIG of *ial* (invasion-associated locus of the invasion plasmid found in EIEC) 5'-CTG GTA GGT ATG GTG AGG-3' and 5'-CCA GGC CAA CAA TTA TTT CC-3'; and primer EA of pCVD (the nucleotide sequence of the EcoRI-PstI DNA fragment of pCVD432 of EAEC) 5'-CTG GCG AAA GAC TGT ATC AT-3' and 5'-CAA TGT ATA GAA ATC CGC TGTT-3'. The products of amplification were 322bp, 147bp, 130bp, 298bp, 376bp, 320bp, 367, and 630 for the genes *eltB, estA, vt1, vt2, eaeA, ial, bfpA,* and pCVD, respectively.

The minimum criteria for determination of diarrheagenic *E.coli* were defined as follows: the presence of *eltB* and/or *estA* for ETEC, the presence of *vt1* and/or *vt2* for EHEC (the additional presence of *eaeA* confirms the detection of a typical EHEC isolate), the presence of *bfpA* and *eaeA* for typical EPEC (but the presence of only *eaeA* for atypical EPEC), the presence of *ial* for EIEC and the presence of pCVD for EAEC. [24]

(iii) DNA amplification: PCRs were performed with a 50µl reaction mixture containing 5 µl of template DNA, 5 µl of 10x PCR buffer, 4 µl of a 1.25 mM mixture of deoxynucleoside triphosphates, 4 µl of 25 mM MgCl2, 0.5 µl of 5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer) per µl, and a 0.2 µM concentration of each primer except primer VT1, which was used at a concentration of 0.4 µM. Amplifications were carried out in a thermal cycler (Perkin-Elmer cetus type (480). The thermocycling conditions were as follows: 96°C for 4 min, 94°C for 20 s, 55°C for 20 s, and 72°C for 10 s for 30 cycles, with a final 7-min extension at 72°C. [24]

PCR products (20 µl) were evaluated with a 1.5% (wt/vol) agarose gel at 120 mV for 30 min. A molecular marker (1-kb DNA ladder; Fermentans) was run concurrently. The DNA bands were visualized and photographed under UV light after the gel was stained with ethidium bromide.

Statistical Methods

All data were coded, entered and analyzed using EPI-INFO(2000) software computer package. The chi-square test was used to determine the statistical significance of the data. P value <0.05 was considered significant.

RESULTS

Out of 83 rectal swabs taken from patients, 70 *E.coli* strains were isolated from 70 patients. Out of 32 rectal swabs taken from control group, 30 *E.coli* strains were isolated.

Table(1)Results of multiplex PCR of *E.coli* isolates of patients and control groups

No of Isolates	PCR +VE	PCR –VE	Total	Significance
Patients	17(24.3%)	53(75.7%)	70	Chi-square = 18.5
Control	1(3.3%)	29(96.7%)	30	$p \leq 0.001$

Diarrheagenic *E.coli* strains were significantly isolated from patients more than control using multiplex PCR. Out of 70 *E.coli* strains isolated from patients, 17(24.3%) strains were proved to be diarrheagenic by multiplex PCR where 53 (75.7%) strains were non diarrheagenic. Out of 30 *E.coli* isolates recovered from control group, 1 (13.3%) isolate was proved to be diarrheagenic by multiplex PCR where 29(96.7%) isolates were non diarrheagenic (Chi-square = 18.5 $p \leq 0.001$) as shown in table(1).

Table(2): Results of serology of *E.coli* isolates of patients and control groups

No of isolates	Typable	Non- Typable	Total	Significance
Patients	23(32.9%)	47(67.1%)	70(100%)	Chi-square = 2.28
Control	7(23.3%)	23(76.7%)	30(100%)	$p \leq 0.20$.

Out of 70 *E.coli* strains isolated from patients, 23(32.9%) isolates were serologically typable and 47 (67.1%) isolates were serologically non-typable. Out of 30 *E.coli* isolates recovered from control group, 7(23.3%) isolates were serologically typable and 23(76.7%) isolates were serologically non-typable (Chi-square = 2.28 & $p \leq 0.20$) as shown in table (2).

Table(3): Relation of PCR to serology results of E coli isolated from patients

	Typable	Non-Typable	Significance
PCR +VE	12(52.2%)	5(10.6%)	Chi-square = 40.17
PCR –VE	11(47.8%)	42(89.4%)	$p \leq 0.001$
Total	23(100%)	47(100%)	

Out of the tybable 23 *E.coli* strains isolated from patients, 12(52.2%) strains were proved to be diarrheagenic by multiplex PCR where 11(47.8%) strains were non diarrheagenic. Out of the non- tybable 47 *E.coli* strains isolated from patients, 5 (10.6%) strains were proved to be diarrheagenic by multiplex PCR where 42 (89.4%) strains were non diarrheagenic(Chi-square = 40.17 & $p \leq 0.001$) as shown in table 3

Table(4): Relation of PCR to serology results of *E.coli* isolated from control

	Typable	Non Typable	Significance
PCR +VE	0(0%)	1(4.3%)	Chi-square = 4.39
PCR –VE	7(100%)	22(95.7%)	$p \leq 0.05$
Total	7(100%)	23(100%)	

Out of 30 *E.coli* strains isolated from control group, 7 strains were serologically typable and 23 strains were serologically non-typable. Non of these typable 7 *E.coli* strains was proved to be diarrheagenic by multiplex PCR. Out of the non- tybable 23 *E.coli* strains isolated from control, 1(4.3%) strain was proved to be diarrheagenic by multiplex PCR where 22 (95.7%)strains were non diarrheagenic (Chi-square = 4.39 & $p \leq 0.05$) as shown in table(4).

Table(5): Pathotypes of diarheagenic *E.coli* isolated from children with diarrhea.

Type of E.coli	Genotype	No(percentage)	
ETEC	*estA*	3(17.6%)	Total ETEC = 9(52.9%)
	eltB	4(23.5%)	
	estA + eltB	2(11.8%)	
EPEC	*eae*	4(23.5%)	Total EPEC = 5(29.4)
	eae+bfp	1(5.9%)	
EAEC	pCVD	3(17.6)	3(17.6)
EIEC	-------	0	0
EHEC	-------	0	0
Total	6	17(100%)	

Out of the diarrheagenic *E.coli* isolated, 9(52.9%) isolates were ETEC; 5(29.4%) isolates were EPEC; and 3 (17.6%)isolates were EAEC. 4(23.5%), 3(17.6%), and 2(11.8%) isolates of ETEC showed the *eltB, estA, eltB+ estA* genotypes respectively. 4 (23.5%)& 1(5.9%) isolates of EPEC showed *eae and eae+bfp* genotypes respectively. The 3(17.6%) EAEC isolates showed pCVD genotype (table 5). The diarrheagenic *E.coli* strain isolated from control was genotypically related to EAEC (This result not shown in tables).

DISCUSSION

Escherichia coli is the predominant facultative anaerobe of the human colonic flora. The organism typically colonizes the infant gastrointestinal tract within hours of life.[6] Diarrheagenic *E.coli* represents a major public health problem in developing countries.[1,8,18] Diarrheagenic *E.coli* strains are classified into five main pathotypes according to the presence of different virulence genes.

Diarrheagenic *E.coli* strains were among the first pathogens for which molecular diagnostic methods were developed. Substantial progress has been made in the development of nucleic acid-based technologies especially PCR methods.[6] Multiplex PCR represents a major advance in the molecular diagnosis of diarrheagenic *E.coli*. Instead of performing several PCRs with different primers specific for different pathotypes genes, in a multiplex PCR we can combine many primer pairs specific for different pathotypes in a single reaction thus reducing time and effort. [24]

In this study using multiplex PCR, diarrheagenic *E.coli* strains were isolated

significantly more often from children with diarrhea (24.4 % of the isolated strains) compared to healthy controls (3.3%) (Chi-square = 18.5 & $p \leq 0.001$) as shown in table(1). This result agrees with other literatures as that of Trung et al 2005 [24] who recovered 22.5 %, 12.5% ($p \leq 0.001$)diarrheagenic strains from children with and without diarrhea respectively using multiplex PCR with one set of primers. According to the study done by Aranda et al 2004 [2] in Brazil on 36 children with diarrhea, 19.4 % of the *E.coli* strains recovered were diarrheagenic using two multiplex primer sets. Ratchtrachenchai et al 2004[16] in Thailand recovered 17.8 % diarrheagenic *E.coli* strains from children with diarrhea using two multiplex primer sets. This lower percentage may be due to his inclusion of children up to age 12 years in his study. However other investigators has seen low prevalence of ETEC in children with diarrhea[7].

The most prevalent pathotype of diarrheagenic *E.coli* isolated in this study was ETEC(52.9%). This result agrees with many literatures which denotes that ETEC is the most important cause of acute childhood diarrhea in developing countries. [5&21] This result also agrees with a study done in Egypt by Rao et al 2003[14] who concluded that ETEC was the most common cause of diarrhea in rural Egyptian children. This result disagrees with other studies done in Vietnam,[24] Brazil,[2] and Thailand [16] who denoted that EAEC , EPEC & EAEC respectively were the diarrheagenic *E.coli* most frequently isolated from children. This difference can be accepted as the studies were done in different countries.

The ETEC virulence gene most frequently detected was *eltB*. This result agrees with other studies done in other countries. [24, 13] This result disagrees with the study of Ratchtrachenchai, 2004.[16] This difference can be attributed to geographical variation. It also disagrees with the study of Rao et al 2003[14] and Shaheen et al 2004[20]who denoted that stable toxin ETEC was more prevalent than labile toxin ETEC as a cause of ETEC diarrhea in Egyptian children under 36 month age. The disagreement between our study and both studies may be due to difference in age and number of the children included.

In the present study, we did not isolate any EHEC strains from any of the groups of children. Similarly, no child with diarrhea was infected with EHEC in other studies [9, 22, 24] which concurs with an interesting phenomenon in developing countries, in which EHEC is much less frequently isolated than other diarrheagenic *E.coli* pathotypes, such as ETEC or EPEC strains. [6]

Comparing the results of serotyping of the isolated *E.coli* strains to multiplex PCR results signifies that significant number of isolates would have been misidentified by serotyping based diagnosis. Out of the tybable 23 E coli strains isolated from patients, 12(52.2%) strains were proved to be diarrheagenic by multiplex PCR where 11(47.8%) strains were non diarrheagenic. Out of the non- tybable 47 *E.coli* strains isolated from patients, 5 (10.6%) strains were proved to be diarrheagenic by multiplex PCR where 42 (89.4%) strains were non diarrheagenic(Chi-square = 40.17 & $p \leq 0.001$) as shown in table 3. Non of the typable 7 *E.coli* strains isolated from control was proved to be diarrheagenic by multiplex PCR. Out of the non-tybable 23 *E.coli* strains isolated from control, 1(4.3%) strain was proved to be diarrheagenic by multiplex PCR where 22 (95.7%)strains were non diarrheagenic (Chi-square = 4.39 & $p \leq 0.05$) as shown in table(4). This result agrees with Ratchtrachenchai et al 2004 [16] who reported that about 71%of EAEC, 54%of EPEC, 45%of ETEC and 33% of EIEC strains were non-typable while 24% of nondiarrhoeagenic *E.coli* strains were typable and indicated that although only about 9% of serogroups were identified exclusively in single pathotypes, more than 60% of serogroups tested were not restricted to any pathotype, signifying the unrestricted nature of serogroups among different pathotypes. These results confirm the assumption that serology of stools *E.coli* isolates can be used as a presumptive diagnosis of diarrheagenic *E.coli* but sure diagnosis of diarrheagenic *E.coli* necessitates using other specific and sensitive methods like PCR .

This study concludes that mutiplex PCR can be used as a rapid method for diagnosis of diarrheagenic *E.coli* in children and recommends that further studies must be done for the application of mutiplex PCR for

the rapid diagnosis of diarrheagenic *E.coli* directly from stools.

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التمييز و التشخيص المبكر لأيشريشيا كولاى المسببة للإسهال فى الأطفال المصريين باستخدام التفاعل المتسلسل لأنزيم البلمرة التعددى

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إن الأيشريشيا كولاى هو أهم ميكروب مسبب لإسهال الأطفال و يمثل مشكلة صحية كبرى فى البلدان النامية.

كان الغرض من الدراسة تقييم التفاعل المتسلسل لأنزيم البلمرة التعددى فى التشخيص المبكر للعدوى المعوية بالأيشريشيا كولاى المسببة للإسهال.

تم أخذ مسحات شرجية من٨٣ طفلا مصابا بالإسهال تحت سن ٥ سنوات و من ٣٣ طفل سليم كمجموعة ضابطة و تم إخضاع كل معزولات الأيشريشيا كولاى للفحص السيرولوجى و التفاعل المتسلسل لأنزيم البلمرة التعددى لتمييز الأيشريشيا كولاى المسببة للإسهال عن غيره.

و كانت النتائج كالأتي:

١. تم عزل الأيشريشيا كولاى المسببة للإسهال من المرضى أكثر من المجموعة الضابطة بفرق هام إحصائيا.
٢. كان ٢٤,٣ % و ١٣,٣ % من الأيشريشيا كولاى المعزولة من المرضى و المجموعة الضابطة على التوالي من نوع الأيشريشيا كولاى المسببة للإسهال.
٣. كان ٣٢,٩ % و ٢٣,٣ % من الأيشريشيا كولاى المعزولة من المرضى و المجموعة الضابطة على التوالي مصنفا بالتحليل السيرولوجى.
٤. ثبت أن نسبة ٥٢,٢ % من الأيشريشيا كولاى المعزولة من المرضى و المصنفة بالتحليل السيرولوجى كانت من نوع الأيشريشيا كولاى المسببة للإسهال و ذلك باستخدام التفاعل المتسلسل لأنزيم البلمرة التعددى.
٥. ثبت أن نسبة ١٠,٦ % من الأيشريشيا كولاى المعزولة من المرضى والغير مصنفة بالتحليل السيرولوجى كانت من نوع الأيشريشيا كولاى المسببة للإسهال و ذلك باستخدام التفاعل المتسلسل لأنزيم البلمرة التعددى.
٦. ثبت أنه لم يكن أي من الأيشريشيا كولاى المعزولة من المجموعة الضابطة و المصنفة بالتحليل السيرولوجى هي من نوع الأيشريشيا كولاى المسببة للإسهال و ذلك باستخدام التفاعل المتسلسل لأنزيم البلمرة التعددى.
٧. ثبت أن نسبة ٤,٣ % من الأيشريشيا كولاى المعزولة من المجموعة الضابطة والغير مصنفة بالتحليل السيرولوجى كانت من نوع الأيشريشيا كولاى المسببة للإسهال و ذلك باستخدام التفاعل المتسلسل لأنزيم البلمرة التعددى.
٨. ثبت أن نسبة الأنواع **ETEC** و **EPEC** و **EAEC** كانت ٥٢,٩ % و٢٩,٤% و ١٧,٦ % على التوالي في الأيشريشيا كولاى المسببة للإسهال المعزولة من المرضى و ذلك باستخدام التفاعل المتسلسل لأنزيم البلمرة التعددى.

خلص البحث إلى إن التفاعل المتسلسل لأنزيم البلمرة التعددى يمكن أن يستخدم كطريقة سريعة للتشخيص المبكر للعدوى المعوية بالأيشريشيا كولاى المسببة للإسهال فى الأطفال. وأوصى البحث بعمل مزيد من الدراسات لتطبيق التفاعل المتسلسل لأنزيم البلمرة التعددى فى التشخيص المبكر للأيشريشيا كولاى المسببة للإسهال مباشرة من عينات البراز.