Journal of Medical Sciences

ISSN 1682-4474

science alert

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Research Paper

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J. Med. Sci., 7 (2): 255-262
15th February, 2007

Multiplex PCR for Detection of Diarrheagenic *Escherichia coli* in Egyptian Children

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Diarrheal diseases continue to be one of the most common causes of morbidity and mortality among young children in developing countries. The objectives of this study were to evaluate the multiplex PCR as a rapid diagnostic tool for simultaneous detection of 3 categories of diarrheagenic *E. coli* (ETEC, EPEC, EHEC) in one PCR reaction using 6 virulent genes; to determine the prevalence of these 3 categories among Egyptian children with and without diarrhea and get an idea about their antibiotic resistance pattern. The material of this study comprised stool specimens collected from two groups of children: Group I included 200 children suffering from acute diarrhea attending Alexandria University Children's Hospital while Group II comprised 100 age-matched children who are not suffering from diarrhea and served as the control group. The multiplex PCR detected target genes of diarrheagenic *E. coli* in 25 out of the 200 diarrheal stools specimens (12.5%). Genes of ETEC (*lt&st*) were detected in 16 specimens (8%). Gene of EPEC (*eae*) was detected in 7 specimes (3.5%). Genes for both ETEC and EPEC were detected in 2 specimens (1%) (*eae, bfp&st*) denoting mixed infection. Genes for EHEC were not detected in any of the diarrheal or control specimens. In the control group genes for diarrheagenic E. coli were detected in 3 specimens (3%) which is statistically much less significant than the group of diarrhea. Resistance of diarrheagenic *E. coli* strains to ampicillin (76%), trimethoprim-sulfamethoxazole (56%) and tetracycline (56%) was common, while resistance to cephalosporins and quinolone antibiotics was rarely detected. In conclusion, we recommend the use of multiplex PCR system to evaluate the role of different categories of diarrheagenic *E. coli* in Egyptian children.

Key words: Diarrheagenic *E. coli*, multiplex PCR, antimicrobial susceptibility testing

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INTRODUCTION

Diarrheal diseases continue to be one of the most common causes of morbidity and mortality among young children in developing countries (Bern *et al*., 1992; Albert *et al*., 1995). Enterotoxigenic *E. coli* has been recognized as the most common cause of infectious diarrhea in infants and children below five years in developing countries (Rao *et al*., 2003; Shaheen *et al*., 2004). Up till now six main categories of diarrheagenic strains of *E. coli* have been recognized on the basis of distinct epidemiological and clinical features, specific virulence determinants and association with certain serotypes (Nataro and Kaper, 1998): enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and diffusely adherent *E. coli* (DAEC).

The few studies conducted in Egypt have incriminated diarrheagenic *E. coli in* diarrhea but these studies have been confined to define the role of a limited categories of diarrheagenic *E. coli* by cumbersome phenotypic traits which rely on subjective tests such as tissue culture and animal assays (Massoud *et al*., 1989a, b). Later, enzyme-linked immunosorbent assay and membrane-based DNA hybridization assays, which improved speed and ease of use become popular (Rao *et al*., 2003; Shaheen *et al*., 2004; Shukry *et al*., 1986; Oyofo *et al*., 1997; Abu-Elyazced *et al*., 1999)

Introduction of PCR methodology which depends on detection of virulence factors has provided a practical and rapid way of detecting diarrheagenic *E. coli*. (Salmanzadeh-Ahrabi *et al*., 2005; Wani *et al*., 2006). However, conducting the separate PCR reaction that are required for the detection of the virulence factors in order to assign an isolated *E. coli* strain to one of the six catergories is very laborious and time consuming. Recently, various multiplex PCR methods have been developed for the simultaneous detection of several pathogenic genes in one PCR reaction. These methods showed high sensitivity and specificity for identification of human diarrheagenic *Escherichia col* (Toma *et al*., 2003; Aranda *et al*., 2004; Vidal *et al*., 2004, 2005; Bii *et al*., 2005).

The objectives of this study were to evaluate the multiplex PCR as a rapid diagnostic tool for simultaneous detection of 3 categories of diarrheagenic *E. coli* (ETEC, EPEC, EHEC) in one PCR reaction using 6 virulent genes; to determine the prevalence of these 3 categories among Egyptian children with and without diarrhea and get an idea about their antibiotic resistance pattern.

MATERIALS AND METHODS

Population and study features: The material of this study comprised stool specimens collected from two groups of children whom their stool culture did not reveal any of the known bacterial pathogens: Group I: 200 children suffering from acute diarrhea attending Alexandria University Children's Hospital at El-Chatby during the period from March to December 2005. Group II :100 age-matched controls attending the same hospital. These children attended the hospital for nondiarrheal illnesses during the same time as the children with diarrhea. They had not had diarrhea or antibiotic therapy in the preceeding 2 weeks.

This study was approved by Alexandria University Ethical Committee and an informed consent was obtained from all parents.

The age of patients ranged between 2 and 36 months. All patients were subjected to thorough history taking at the time of specimen collection to obtain data regarding demographics, breast feeding, severity of illness and previous antibiotic use.

Diarrhea is defined as; an increase in the fluidity, volume and number of stools relative to the usual habits of each individual.

Stool culture: Faeces of diarrheal and control children were inoculated on Salmonella-Shigella (S-S) agar for the selection of Shigella and Salmonella, MacConkey agar and sorbitol MacConkey agar for isolation of *E. coli*, TCBS Cholera Medium for the selection of Vibrio and Skirrow's agar for isolation of Campylobacter as well as selenite-F broth and alkaline peptone water. All the samples were tested for Vibrio, Shigella, Salmonella and Campylobacter by using colony morphology, standard biochemical reactions and agglutination with specific sera (Duguid *et al*., 1989). Cultures which revealed one of the known pathogens (Salmonella, Shigella, Campylobacter, Vibrio) were excluded.

Reference strains: Diarrheagenic *E. coli* reference strains EHEC (stx_1 stx_2 *eae*), EPEC (*eae*, *bfp*) and ETEC (*st* and *lt*) were used as positive controls. The strains were grown on MacConkey agar plates at 37°C. DNA was extracted from bacteria by the method described by Schmitz *et al*. (1998). The supernatant was then used as the DNA template for PCR. Having confirmed the specificity of each primer by monoplex PCR, the DNA templates were subjected to multiplex PCR with specific primers as described previously (Vidal *et al*., 2004; Cebula *et al*., 1995), for the detection of the following virulence markers: *eaeA*, *bfpA*, *stx*1, *stx*2, *lt* and *st*II. The DNA sequences of the primers,

Table 1: Primers used in the multiplex PCR for amplification of diarrheagenic *E. coli* genes

Gene	Primer sequence	Size of product (bp)	Function
eae	TCA ATG CAG TTC CGT TAT CAG TT GTA AAG TCC GTT ACC CCA ACC TG	482	Structural gene for intimin of EHEC and EPEC
bfp	GGA AGT CAA ATT CAT GGG GGT AT GGA ATC AGA CGC AGA CTG GTA GT	254	Structural gene for the bundle-forming pilus of EPEC
*stx*1	CAG TTA ATG TGG TGG CGA AGG CAC CAG ACA ATG TAA CCG CTG	348	Shiga toxin 1 of EHEC
*stx*2	ATC CTA TTC CCG GGA GTT TAC G GCG TCA TCG TAT ACA CAG GAG C	584	Shiga toxin 2 of EHEC
lt	GCA CAC GGA GCT CCT CAG TC TCC TTC ATC CTT TCA ATG GCT TT	218	Heat-labile toxin of ETEC
stII	AAA GGA GAG CTT CGT CAC ATT TT AAT GTC CGT CTT GCG TTA GGA C	129	Heat-stable toxin of ETEC

the sizes of PCR products and the function of these genes are shown in Table 1. Each multiplex PCR assay was performed in 0.5 mL eppendorfs, each containing a total volume of 50 μL including 25 μL Taq PCR master mix (QIAGEN), 50 pmol primer and 100 ng of the extracted DNA. The amplification was performed in a Thermal Cycler (Genius, Techne, UK). After an initial denaturation cycle of 5 min at 94°C, the reaction mixes were subjected to 35 amplification cycles of 1.5 min at 64°C, 1.5 min at 72°C and 1.5 min at 94°C. DNA from the amplified PCR reaction mixes was analysed after electrophoresis on a 1.5% agarose gel at 80 volts for 1 h and stained with ethidium bromide. A molecular marker (100 bp DNA ladder., Fermentas) was run concurrently.

Application of multiplex PCR for detection and identification of diarrheagenic *E. coli* strains from stool samples: The stool samples collected were inoculated onto the surface of MacConkey plates. After incubation at 37°C overnight, a smear from the first area of a MacConkey plate was taken for DNA extraction. The DNA template was subjected to the multiplex PCR. If the result was negative, the sample was considered negative for diarrheagenic *E. coli*. If the multiplex PCR was positive, the sizes of the bands on the gel was compared with those of the marker bands in order to identify certain kinds of diarrheagenic *E. coli* strains in the stool sample (Nguyen *et al*., 2005).

The minimum criteria for determination of diarrheagenic *E. coli* were defined as follows: the presence of *lt* and/or *stII* for ETEC, the presence of *stx1* and/or *stx2* for EHEC, the presence of *bfpA* and *eaeA* for typical EPEC (Nguyen *et al*., 2005). specimens that revealed diarrheagenic *E. coli* were subjected to single PCR.

Single PCR: Another smear of the same area on MacConkey was taken and cultured on a fresh MacConkey plate to get separate colonies. These were subjected to standard biochemical reactions to confirm their identity as *E. coli*. After incubation at 37°C overnight up to 3 colonies with typical *E. coli* morphologies were streaked onto fresh plates. Each colony was independently tested by PCR with a primer specific for a suspected diarrheagenic *E. coli* isolate from the multiplex PCR.

Antimicrobial susceptibility testing: The 25 diarrheogenic *E. coli* isolated were tested by the disk diffusion method (Bauer *et al*., 1966) and were interpreted according to National Committee for Clinical Laboratory Standards guidelines (Wikler *et al*., 2005; Miles and Amres, 1996) as susceptible, intermediate or resistant. For analysis, the intermediate and resistant categories were grouped together as non-susceptible. Multiresistance was defined as non susceptibility to at least three families of antibiotics, including ampicillin, trimethoprim-sulphamethoxazole and tetracycline. *E. coli* ATCC 25922 was used as quality control strain. The antibiotic test panel included ampicillin, ampicillin-sulbactam, trimethoprim-sulphamethoxazole, tetracycline, amikacin, aztreonam, cefepime, ceftriaxone, gentamicin, nalidixic acid, ciprofloxacin, ceftazidime, imipenem.

Statistics: Data were fed to SPSS/Win. Analysis of data was done using arithmetic mean, standard deviation, count and percentage.

RESULTS

Two hundred children with diarrhea were included in the study. In order to detect three different categories of diarrheagenic *E. coli* simultaneously, a mixture of six primer pairs specific for the target genes was used in one PCR reaction. Figure 1 shows the PCR products derived from pure cultures of reference strains of EPEC, ETEC and EHEC, respectively. The multiplex PCR detected target genes of diarrheagenic *E. coli* in 25 out of the 200 diarrheal specimens (12.5%).

Genes of ETEC (*lt* and *st*) were detected in 16 specimens (8%). Gene of EPEC (*eae*) was detected in 7 specimes (3.5%). Genes for both ETEC and EPEC were

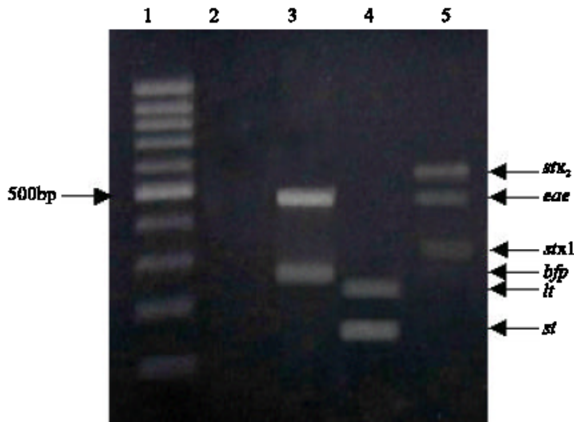

Fig. 1: Multiplex PCR of reference strains. Bands corresponding to *eae*, *bfp*, *stx*2, *stx*1, *lt* and *stII* are indicated. Lane 1, 100-bp size ladder; lane 2, reagent control; lane 3, enteropathogenic *E. coli* (*eae, bfp*); lane 4, enterotoxigenic *E. coli* (*lt, st*); lane 5, enterohemorrhagic *E. coli* (*stx*1, *stx*2, *eae*)

Table 2: Distribution of the ETEC strains according to the type of enterotoxin produced

Type of enterotoxin produced	No. of strains	(%)
ST only	4	25
LT only	10	62.5
ST and LT	2	12.5

detected in 2 specimens (1%) (*eae*, *bfp* and *st*) denoting mixed infection. Genes for EHEC were not detected in any of the diarrheal or control specimens.

The distribution of ETEC according to the toxin produced is shown in Table 2. Ten strains (62.5%) were LT producers, 4 strains (25%) were ST producers and two strains (12.5%) produced both LT and ST.

In the single PCR only one strain of EPEC revealed 2 genes (*eae*, *bfp*) while the other 6 strains revealed only one gene (*eae*) in multiplex PCR as well as in the single PCR. These were considered as atypical EPEC.

The mean age of the diarrheagenic *E. coli* infected patients was 10.3 months. They were all found to be formula fed. Seventy two percent of them were from rural area and 28% from urban area. While the incidence of ETEC diarrhea was more frequent during the warmer season between May and August, EPEC diarrhea occurred at similar levels throughout the period of study. The demographic characteristics of the diarrheogenic *E. coli* infected group is shown in Table 3.

In the control group genes for diarrheagenic *E. coli* were detected in 3 specimens. Genes for ETEC and EPEC were detected in 1 and 2%, respectively with significant difference between both groups of children ($p<0.05$).

Antimicrobial susceptibility testing of diarrheagenic *E. coli* isolates revealed high rate of resistance to ampicillin (76%), trimethoprim-sulfamethoxazole (56%), tetracycline (56%) and ampicillin-sulbactam (unasyn) (36%) (Table 4). Few of the isolates displayed resistance to nalidixic acid (4%) and aztreonam (4%), while universal susceptibility to aminoglycosides (amikacin and gentamicin), cephalosporins (ceftazidime, ceftriaxone and cefipime), quinolones (ciprofloxacin) and carbapenems (imipinem) was demonstrated (Table 4).

Table 3: Demographic characteristics of the diarrheogenic *E. coli* infected group

Characteristic	Diarrheogenic infected group (n = 25) No.	(%)
Age (months)	3-20 months	
Min-Max Mean±SD	10.3±5.162	
Sex		
Male	15	60
Female	10	40
Feeding		
Breast fed	0	0.0
Formala fed	25	100
Residence		
Rural	18	72
Urban	7	28

Table 4: Antibiotic resistance pattern found in diarrheogenic *E. coli* isolates (n = 25)

Antibiotic	Resistance No.	(%)
Ampicillin	19	(76)
Ampicillin-sulbactam	9	(36)
Ceftazidime	0	(0.0)
Ceftriaxone	0	(0.0)
Cefepime	0	(0.0)
Imipenem	0	(0.0)
Amikacin	0	(0.0)
Gentamicin	0	(0.0)
Tetracycline	14	(56)
Aztreonam	1	(4)
Trimethoprim-Sulphamethoxazole	14	(56)
Nalidixic acid	1	(4)
Ciprofloxacin	0	(0.0)

DISCUSSION

Diarrheagenic *E. coli* has been identified as an important cause of infantile and young childhood diarrhea in all the developing countries where it has been looked for, but the incidence has varied greatly in different studies. An incidence higher than 40% has been reported in Bangladesh (Albert *et al.*, 1995, 1999). A lower incidence of about 30% has been reported in Jordan (Shehabi *et al.*, 2003).

The role of these pathogens is most probably underestimated due to inappropriate diagnostic methods in clinical practice (Abu-Elyazeed *et al.*, 1999). The development of various multiplex PCR methods for the simultaneous detection of several pathogenic genes in one PCR reaction will save time and effort involved in

analyzing various virulence factors and will help investigators to clarify the role of diarrheogenic *E. coli* in diarrheal diseases (Rappelli *et al*., 2001; Lopez-Soucedo *et al*., 2003; Kimata *et al*., 2005)

In Egypt, previous study conducted in the same hospital in 1981 on infants and children below 2 years of age suffering from diarrhea reported EPEC and ETEC in 5.4 and 11.8%, respectively while another study conducted in the same hospital over 5 years (1982 up to 1987) using animal and tissue culture assay reported EPEC and ETEC in 12% and 12.6, respectively in children with diarrhea below 5 years of age. In our study genes for EPEC and ETEC were detected in 3.5 and 8%, respectively. This might be attributed to the difference in the age groups of children. Alternatively, this decline in prevalence of *E. coli* in our study might be attributed to encouragement of breast feeding by the National Ministry of Health guidelines following WHO recommendations in the last few years (Tompson *et al*., 2002).

The present study showed that children who were infected with diarrheagenic *E. coli* were all formula fed. It has been suggested that breast feeding might have a protective action against ETEC diarrhea. Factors in the milk such as specific secretory immunoglobulin A antibodies and receptor analogues as well as other innate and anti-inflammatory properties might all contribute to decrease the infection (Holmgren *et al*., 1983).

Genes for EPEC and ETEC *E. coli* were detected simultaneously in two specimens (*eae*, *bfp* and *st*) denoting the possibility of mixed infections, while none of the children in the control group was colonized with more than one category of diarrheagenic *E. coli*. This was also reported by other investigators (Albert *et al*., 1995; Nguyen *et al*., 2005; Echeverria *et al*., 1994; Presterl *et al*., 1999).

The distribution of enterotoxin genes among ETEC isolates in this study is not similar to the previously reported studies in Egypt (Rao *et al*., 2003; Shaheen *et al*., 2004; Shukry *et al*., 1986; Oyofo *et al*., 1997), where ST-only ETEC predominated. In the present study, LT-only ETEC represented 62.5% of the isolates. This was also demonstrated in other developing countries (Cravioto *et al*., 1990; Black, 1993 and Viboud *et al*., 1999). STLT strains were detected in the same ratio in our study as well as previous studies.

Six of the EPEC strains isolated from the studied patients were *eae* PCR positive but were negative for *bfp*A (they were all atypical EPEC isolates). *bfp*A is the structural gene encoding BFP (the bundle-forming pilus). These fimbriae are produced only under certain culture conditions (Nataro and Kaper, 1998). This could be the reason for the failure to identify them in our study, as shown also in other studies by others (Nguyen *et al*., 2005; Scotland *et al*., 1983; Alikhani *et al*., 2006).

EPEC and EHEC share *eae*A (the intimin structural gene), but the major virulence factor defining the characteristics of EHEC is *stx*. In the present study, we did not detect any EHEC strains from any of the studied children. Some studies have suggested that there is an interesting phenomenon in developing countries in which EHEC is much less frequently isolated than other diarrheagenic *E. coli*, such as ETEC or EPEC strains. (Rappelli *et al*., 2005). The result of the present study coincides with that found in other developing countries (Albert *et al*., 1999; Nguyen *et al*., 2005; Shehabi *et al*., 2003).

As found previously in Egypt (Rao *et al*., 2003), the incidence of ETEC diarrhea was higher during the warmer season. Infections with EPEC were prevalent throughout the year, with no particular seasonal pattern. A similar result has been reported in a previous study in Bangladesh (Albert *et al*., 1999).

The role of antibiotics in the treatment of childhood diarrhea is limited because of their limited benefits and potential side effects (Alam and Ashraf, 2003). Yet, knowledge about antibiotic sensitivity pattern of these strains may be beneficial for hospital treatment of severe and complicated cases (Pickering and Snyder, 2004). It might also be of benefit for prophylaxis and treatment of traveler's diarrhea as ETEC seems to be the most frequent cause of traveler's diarrhea in Europeans visiting developing countries (Ericsson, 2003). A large proportion of isolates (56%) were Multi Drug Resistant (MDR) showing resistance to SXT, ampicillin and tetracycline antibiotics. The same finding has been reported in many other studies in Egypt (Shaheen *et al*., 2004; Putnam *et al*., 2004) and different parts of the world (Okeke *et al*., 2000; Hoge *et al*., 1998; Shapiro *et al*., 2001; Turner *et al*., 1998). There is an increasing isolation rate of MDR strain in Nigeria (Okeke *et al*., 2000), Thailand (Hoge *et al*., 1998), Kenya (Shapiro *et al*., 2001) and Israel (Turner *et al*., 1998). All the strains were susceptible to ciprofloxacin, therefore, it is still the drug of choice for the treatment of traveler's diarrhea as reported by other investigators (Ericsson *et al*., 2003; Dupont, 2006).

In conclusion, this study has shown that diarrheagenic *E. coli* are still major pathogens in infantile diarrhea in Egypt especially in rural areas, though their incidence has declined. The use of multiplex PCR system distinguishes the different categories of diarrheagenic *E. coli* with greater precision. We recommend extending the study to target more virulence genes in a large group of underline children with diarrhea. This can greatly facilitate the process of development of a vaccine against diarrheogenic *E. coli* which should be considered a public health priority in Egypt as well as other developing countries.

ACKNOWLEDGMENTS

The authors are grateful to Professor Boussaina Massoud for her comments during writing this manuscript. This work has been supported by a research fund in Faculty of medicine, Alexandria University, Egypt.

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