Identification of Different Categories of Diarrheagenic Escherichia coli in Stool Samples by Using Multiplex PCR Technique

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**Abstract:** Diarrheal diseases continue to be one of the most common causes of morbidity and mortality among young children in developing countries. The objective of this study was to evaluate the multiplex PCR as a rapid diagnostic tool for simultaneous detection of four categories of diarrheagenic E. coli (ETEC, EPEC, EHEC and EAEC) in one PCR reaction using six virulent genes. During the period from June 2009 to September 2009, 50 stool samples were collected from children suffering from diarrhea in Pediatric Teaching Hospital at Sulaimani and Karkuk cities. E. coli were isolated and diagnosed using set of conventional biochemical tests and API 20E system. Two different methods of DNA extraction (salting out and boiling) were used, and then the DNA was used as a template for PCR. The multiplex PCR detected target genes of diarrheagenic E. coli in 19 out of the 50 diarrheal stools specimens (38%). Genes of ETEC (\(lt\) or st) were detected in 5/19 specimens (26.3%). Gene of EPEC (\(eae\) and/or \(bfp\)) was detected in 12/19 specimens (63.1%). Genes of EAEC was detected in 4/19 specimens (21%), two of them (10.5%) were showed EAEAC plus EPEC and EAEC plus ETEC denoting mixed infection. Genes for EHEC were not detected in any of the diarrheal specimens. Multiplex PCR 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 Diarrhoegenic E. coli in diarrheal diseases.

**Key words:** Diarrheagenic, E. coli, eae, bfp, \(lt\), \(st\), PCVD, \(stx\) gene

**INTRODUCTION**

Knowledge of the specific pathogens that cause diarrheal diseases and their epidemiology is critical for the implementation of specific intervention strategies. However, data on the etiology of diarrhea in Iraq are scarce. The causes of diarrhea include a wide range of viruses, bacteria, and parasites, among the bacterial pathogens, Escherichia coli play an important role (Tawfeek et al., 2002).

There are diarrheagenic and non-diarrheagenic E. coli among E. coli isolates from stool, and they cannot be distinguished by colony morphology or biochemical tests, nor is serogrouping of the O antigen sufficient to identify the isolated E. coli as being diarrheagenic E. coli (Kalnawakul et al., 2007). Since there is no correlation between serotype and pathotype, a genotypic determination is therefore necessary for the identification of these pathogenic strains (Prere and Fayet, 2005).

Up till now six main categories of diarrheagenic strains of E. coli have been recognized on the basis distinct epidemiological and clinical features, specific virulence determinants and association with certain serotypes; Enterohemorrhagic E. coli (EHEC), Enteropathogenic E. coli (EPEC), Enterotoxigenic E. coli (ETEC), Enteroinvasive E. coli (EIEC), Enteroaggregative E. coli (EAEC), and diffusely adherent E. coli (DAEC) (Nataro and Kaper, 1998). Cytotolethal distending toxin-producing E. coli (CDT-EC) are also said to be one of the diarrheal E. coli group (Torres et al., 2005).

Introduction of PCR methodology which depends on detection of virulence factors has provided a practical and rapid way of detecting diarrheagenic E. coli. However, conducting the separate PCR reaction that are required for the detection of the virulence factors in order to assign an isolated E. coli strains to one of the above categories is very laborious and time consuming (Wani et al., 2006).

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 E. coli (Bii et al., 2005; Vidal et al., 2005; Nessa et al., 2007).

The objective of this study was to evaluate the multiplex PCR as a rapid diagnostic tool for simultaneous detection of four categories of diarrheagenic E. coli in one PCR reaction using six virulent genes.

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MATERIALS AND METHODS

Clinical specimens: During the period from June 2009 to September 2009, 50 stool samples were collected from children suffering from diarrhea in Paediatric Teaching Hospital at both Sulaimani and Karkuk cities. Diarrhea is defined as: an increase in fluidity, volume and number of stool relative to usual habits of each individual.

Isolation: A loop full of diarrheal sample was streaked on MacConkey agar and incubated for 24 h at 37 °C, pink colonies then sub cultured on Eosin Methylene Blue (EMB) on which the colonies exhibit green metallic sheen colour, for further conformation set of biochemical tests and API 20E system were used.

DNA extraction: Isolated colonies on MacConkey agar were selected for DNA extraction. Which carried out by two different methods: salting out and Physical (boiling) methods (Epplen and Lubjuhn, 1999; Nessa et al., 2007). Then the DNA was used as template for PCR.

Primer selection: The DNA sequences of the primers, the size of PCR and function of these genes are shown in Table 1.

PCR conditions: Each multiplex PCR assay was performed in 0.5 mL eppendorfs, each containing a total volume of 25 μL including 12.5 μL PCR master mix, 10 pmol for each primer and 2 μL of the extracted DNA. The amplification was performed in a Thermal Cycler (Genius, Techne, UK.). After an initial denaturation cycle of 2 min at 95°C, the reaction mixes were subjected to 35 amplification cycles of 45 sec at 93°C and 30 sec at 58°C and 45 sec at 72°C, and final extension of 7 min at 72°C.

DNA from amplified PCR reaction mixes was analyzed after electrophoresis on 1.5% agarose gel at 90 volts for 1.5 h and stained with ethidium bromide, a molecular marker (100bp DNA ladder, fermentas) was used to determine the size of the amplicons (Lopez-Saucedo et al., 2003; Vidal et al., 2005).

Identification of diarrheagenic E. coli by multiplex PCR: The sample was considered negative if the multiplex PCR was negative for diarrheagenic E. coli, if the multiplex PCR was positive, the sizes of bands on the gel was compared with those of marker bands in order to identify certain kinds of diarrheagenic E. coli strains in the stool sample. The minimum criteria for determination of diarrheagenic E. coli were defined as follows: the presence of lt and/or st for ETEC, the presence of stx for EHEC, the presence of pCVD for EAEC and the presence of eae (for atypical-EPEC) and eae and bfp (for typical-EPEC).

Specimens that revealed diarrheagenic E. coli were subjected to uniplex PCR for more conformation of the mixed infection and also for conformation of the specificity of the test.

RESULTS

A total of 50 stool specimens were collected from children with diarrhea under the age of 10 years. There were 26 (52%) males and 24 (48%) females with the mean age of 15.44 month (ranged from 2 months to 8 years), Table 2.

E. coli isolated and identified by using conventional biochemical methods and API 20E system, the DNA was extracted by two different methods (salting out method and boiling method) and amplified by PCR under the

<p>| Table 1: Primers used in the multiplex PCR for amplification of diarrheagenic E. coli genes |
|---------------------------------------------|----------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primers sequences (5-3)</th>
<th>Amplicon size (bp)</th>
<th>Function</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>eae</td>
<td>5'-TCAATGCAGTCCCGTTACATCAGTT-3'</td>
<td>482</td>
<td>Structural gene for intimin of EHEC and EPEC</td>
<td>Vidal et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>5'-GTAAGTCCGTTACACCGTC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lt</td>
<td>5'-GCCACACGGAGCCTCCATAGCTG-3'</td>
<td>218</td>
<td>Heat labial toxin of ETEC</td>
<td>Vidal et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>5'-AGAGGACCATGACATTTCCTC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>st</td>
<td>5'-AGGAGCTACATTGCCC-3'</td>
<td>521</td>
<td>Heat stable toxin of ETEC</td>
<td>NCBI*</td>
</tr>
<tr>
<td></td>
<td>5'-GACTACTACATGAGGCGT-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx</td>
<td>5'-AGTTAATGTTGGTGGAAGCG-3'</td>
<td>306</td>
<td>Shiga toxin of EHEC</td>
<td>NCBI*</td>
</tr>
<tr>
<td></td>
<td>5'-TGATGAAATACGCAAGCG-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCVD</td>
<td>5'-CTGAGCAAGACTGTATCAT-3'</td>
<td>630</td>
<td>encoding the enteraggregative gene of E.coli</td>
<td>Bueris et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>5'-CAATGTTAGAATACCGCTT-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bfp</td>
<td>5'-AGTTGCTTGCGCTGCTG-3'</td>
<td>324</td>
<td>structural gene for the bundle-forming pilus of EPEC</td>
<td>Lopez-Saucedo et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>5'-TACAGGGTTGATAAAGCAG-3'</td>
<td></td>
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</tr>
</tbody>
</table>


<p>| Table 2: Age and sex distribution among patients group |
|-----------------------------------------------------|----------------|----------------|----------------|
| Male 26 (52%)                                       | Female 24 (48%) | Total 50       |</p>
<table>
<thead>
<tr>
<th>Range</th>
<th>Mean</th>
<th>Range</th>
<th>Mean</th>
<th>Range</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>2M – 2.7Y</td>
<td>14.5M</td>
<td>4M – 8Y</td>
<td>16.73 M</td>
<td>2M – 8Y</td>
<td>15.44 M</td>
</tr>
<tr>
<td>M: Months; Y: Years</td>
<td></td>
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</tbody>
</table>
same conditions, as illustrated in Fig. 1, salting out method of extraction showed stronger band of amplified DNA than those extracted by boiling method.

In order to detect four different categories of diarrheagenic \textit{E. coli} simultaneously, a mixture of six primer pairs specific for target genes were used in one PCR reaction. The multiplex PCR detected targeted genes of diarrheagenic \textit{E. coli} in 19 out of 50 (38\%) diarrheal samples.

As shown in Fig. 2 and 3, gene of ETEC (\textit{lt} and/or \textit{st}) were detected in 5/19 (26.3\%) samples, gene for EPEC (\textit{eae} and/or \textit{bfp}) was detected in 12/19 (63.1\%) samples, gene of EAEC (\textit{pCVD}) was detected in 4/19 (21\%), two of them (10.5\%) showed mixed infection as genes for ETEC plus EAEC and EPEC plus EAEC were detected.

No shiga toxin producing \textit{E. coli} (Enterohemorrhagic \textit{E. coli}) were detected, as the results of multiplex PCR did not showed any band of 306 bp which is the size of \textit{stx} gene.

The distribution of ETEC according to the toxin produced is shown in Table 3. Two out of 5 strains (40\%) were \textit{lt} producer, 3 out 5 strains were \textit{st} producers (60\%).

In the present study we divided the EPEC into typical and atypical according to the presence of the virulence genes, Only 3 (25\%) strain of EPEC revealed two genes...
Table 3: Distribution of ETEC and EPEC according to the type of enterotoxin and virulence genes, respectively

<table>
<thead>
<tr>
<th>ETEC 5/19 (26.3%)</th>
<th>EPEC 12/19 (63.1%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>lt only</td>
<td>st only</td>
</tr>
<tr>
<td>2/5 (40%)</td>
<td>3/5 (60%)</td>
</tr>
<tr>
<td>eae (a-typical)</td>
<td>eae and bfp (typical)</td>
</tr>
<tr>
<td>9/12 (75%)</td>
<td>3/12 (25%)</td>
</tr>
<tr>
<td>Total (19)</td>
<td></td>
</tr>
<tr>
<td>2/19 (10.5%)</td>
<td>3/19 (15.7%)</td>
</tr>
<tr>
<td>9/19 (47%)</td>
<td>3/19 (15.7%)</td>
</tr>
</tbody>
</table>

Fig. 3: Multiplex PCR results of the remaining tested clinical samples
Lane 1: Sample No. 3 (eae 482 and bfp 324), Lane 2: Sample No. 11 (st 512 and PCVD 630), Lane 3: Sample No. 6 (st 512), Lane 4: Sample No. 8 (lt 218 and st 512)

Fig. 4: Uniplex PCR results of some tested clinical samples
Lane 1 and 2: Sample No. 1 and 2 (lt, 218bp), Lane 3: Sample No. 14 (PCVD, 630bp), Lane 4 and 5: Sample No. 20 and 23 (negative) Lane 6: Sample No. 17 (PCVD, 630bp), Lane 7: Sample No. 7 (PCVD, 630bp), Lane 8: Sample No. 7 (eae, 482bp)
(eae and bfp) while other 9 (75%) strains revealed only one gene (eae), theses considered as atypical EPEC (Table 2). For further conformation a uniplex PCR assay was performed for detection of mixed infection, Fig. 4.

**DISCUSSION**

Diarrhoeagenic *E. coli* has been identified as an important cause of infantile and young childhood diarrhea in all the developing countries, but the incidence has varied in different studies from more than 40% in Bangladesh (Albert et al., 1995) to less than 30% in Jordan (Shehabi et al., 2003). The role of these pathogens in most probably underestimated due to inappropriate diagnostic methods in clinical practice (Abu-Elyazeed et al., 1999).

The age distribution of the children with diarrhea showed that they were mostly infants (less than or equal to 12 months) which was 11/19 (57.8%), similar results were commonly observed in Iraq (Makkia et al., 1988; Al-Saffar, 1992; Ali and Al-Sadoon, 1997; Akbar, 2008). Our data are also in accordance with those from neighbouring countries. Reports from Bahrain (Krishnamurthy, 1990) and Saudi Arabia (Qadri et al., 1990) showed that 50 and 43.4%, respectively of their children hospitalized with diarrhea were below 1 years of age. Reports from Bangladesh with 53.6 % of infected infants (Albert et al., 1995) also support our results. The age-specific differences suggest that infants as well as having immature immune systems may be exposed to contaminated formula of milk, foods or environment or may have not been protected completely by breast feeding (Akbar, 2008).

Shegatoxine producing *E. coli* (Verotoxin-producing *E. coli* or EHEC) was not isolated in our study. This result was not in accordance to previous studies in Baghdad-Iraq, which was the first report of EHEC O157:H7 in Iraq. Since Shebib et al. (2003) isolated EHEC O157 (11.5 %) among 200 samples of bloody diarrhea (they collected only the samples which were bloody diarrhea), the poor hygienic measures in Baghdad-Iraq during 1999 could have been associated with an increase in the incidence of *E. coli* O157 (Shebib et al., 2003).

The absence of EHEC (O157:H7) in the present study was in agreement with reports from Iraq, Kalar town, (Akbar, 2008) and other countries, such as Iran (Alikhani et al., 2006; Alikhani et al., 2007), Turkey (Güney et al., 2001), Libya (Ghenghesh et al., 2008), Bangladesh (Albert et al., 1995), Gabon (Presterl et al., 2003), Vietnam (Nguyen et al., 2005), East Africa (Raji et al., 2006), and Uruguay (Torres et al., 2001). 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* strains, such as ETEC or EPEC strains (Nataro and Kaper, 1998).

EAEC in our study was found in 4/19 (21%) of the patients, two of them (10.5%) were co-infection with EPEC (one case) and ETEC (one case). Hien et al. (2007) also reported that 7% of cases showed co-infection (EPEC and EAEC). The EAEC has been implicated as the etiological agent of diarrhea not only in developing countries but also as a cause of gastroenteritis outbreaks in some industrialized countries. It has been reported as the cause of a massive outbreak of gastrointestinal illness in school children in Japan, as the cause of persistent diarrhea in children in Brazil, and in other developing countries (Nessa et al., 2007).

The second most common type of diarrheagenic *E. coli* was the ETEC (5/19, 26.3%). Our finding is approximately similar to those reported by Qadri et al. (1990) and Nessa et al. (2007), in which they found that the rate of ETEC was 18.5 and 18%, respectively. The *st* genes of ETEC were found in 3/5 (60%) of patients compared to the *lt* genes in 2/5 (40%) of patients. Other studies also showed predominance of *st* producing ETEC (Rao et al., 2003; Shaheen et al., 2004). The ETEC has been attributed as the common cause of infections among the tourists visiting Asia, Africa and South America; and also as a common diarrhoeal pathogen in children in many developing countries of Asia, Africa and South America (Nessa et al., 2007).

Two out of nineteen of our patients showed co-infection with EAEC plus ETEC and EAEC plus EPEC that accounted for (10.5%) of the cases. There are also reports of co-infection with EAEC and ETEC (Adachi et al., 2001; Nessa et al., 2007).

The prevalence of EPEC (typical and atypical) in our study was 12/19 (63.1%) among all patients. Our result was higher than those reported in other parts of Iraq, 48% by Al-Obсид and Al-Delaimi (1999), 12.5% by Al-Kaissi et al. (2006) and 13 % by Tawfeek et al. (2002). This may be due to that they depended only on serological test for diagnosis of EPEC and they hadn’t used molecular techniques such as PCR and they also recorded only EPEC, while in our study we depended on PCR for detecting the virulence gene. Furthermore, we divided the EPEC to t-EPEC and a-typical EPEC according to the presence of *eae* and *eae* plus *bfp* gene, and as our knowledge, this is the first study that reports the typical and atypical EPEC in Iraq, so we were unable to compare our result with others conducted in our country. This high rate of EPEC in our study was possibly an indication of poor hygiene, or contamination of water supplies and overcrowding. The rates of isolation of enteric pathogens reported in different studies are related to socioeconomic, health, and weather conditions (Regua et al., 1990).

In conclusions, the development of 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 diarrhoeagenic E. coli in diarrheal diseases.

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REFERENCES


