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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 EAEC 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 Diarrhoeagenic *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* (Kalnauwakul *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 diarreheagenic 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). Cytolethal 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 diarreheagenic *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 diarreheagenic *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 diarreheagenic *E. coli* in one PCR reaction using six virulent genes.

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

Targetgenes	Primers sequences (5-3)	Amplicon size (bp)	Function	Ref.
eae	5'-TCAATGCAGTTCCGTTATCAGTT-'3 5'-GTAAAGTCCGTTACCCCAACCTG-'3	482	Structural gene for intimin of EHEC and EPEC	Vidal *et al.* (2005)
lt	5'-GCACACGGAGCTCCTCAGTC-'3 5'-TCCTTCATCCTTTCAATGGCTTT-'3	218	Heat labial toxin of ETEC	Vidal *et al.* (2005)
st	5'-AGGAACGTACATCATTGCCC-'3 5'-CAAAGCATGCTCCAGCACTA-3	521	Heat stable toxin of ETEC	NCBI*
stx	5'-AGTTAATGTGGTGGCGAAGG-'3 5'-TGTGAAAAATCAGCAAAGCG-'3	306	Shiga toxin of EHEC	NCBI*
pCVD	5'-CTGGCGAAAGACTGTATCAT-'3 5'-CAATGTATAGAAATCCGCTGTT-'3	630	encoding the enteroaggregative gene of E.coli	Bueris *et al.* (2007)
bfp	5'-AATGGTGCTTGCGCTTGCTGC-'3 5'-TACCAGGTTGGATAAAGCGGC-3'	324	structural gene for the bundle-forming pilus of EPEC	Lopez-Saucedo *et al.* (2003)

*http://www.ncbi.nlm.nih.gov/

Table 2: Age and sex distribution among patients group

Male 26 (52%)		Female 24 (48%)		Total 50	
Range	Mean	Range	Mean	Range	Mean
2M – 2.7Y	14.5M	4M - 8Y	16.73 M	2M– 8Y	15.44 M

M: Months; Y: Years

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. Diarreah is defined as: an increase in fluidity, volume and number of stool relative to usual habits of each individual.

Isolation: A loop full of dairrheal sample was streaked on MacConkey agar and incubated for 24 h at 37 ºC, 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 95ºC, the reaction mixes were subjected to 35 amplification cycles of 45 sec at 93ºC and 30 sec at 58ºC and 45 sec at 72ºC, and final extension of 7 min at 72ºC. 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

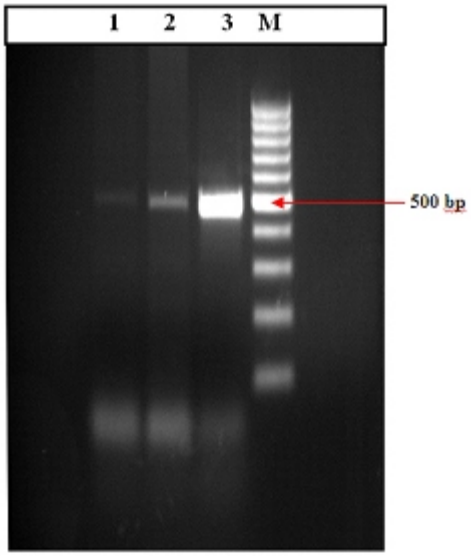

Fig. 1: Comparison between two different DNA extracted methods
Lane 1: DNA extracted by boiling method (15 min boiling)
Lane 2: DNA extracted by boiling method (10 min boiling)
Lane 3: DNA extracted by salting out method
M: 100bp DNA ladder

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 *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 *E. coli* in 19 out of 50 (38%) diarrheal samples.

As shown in Fig. 2 and 3, gene of ETEC (*lt* and/or *st*) were detected in 5/19 (26.3%) samples, gene for EPEC (*eae* and/or *bfp*) was detected in 12/19 (63.1%) samples,gene of EAEC (*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 toxine producing *E. coli* (Enterohemorregic *E. coli*) were detected, as the results of multiplex PCR did not showed any band of 306 bp which is the size of *stx* gene.

The distribution of ETEC according to the toxin produced is shown in Table 3. Two out of 5 strains (40%) were *lt* producer, 3 out 5 strains were *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

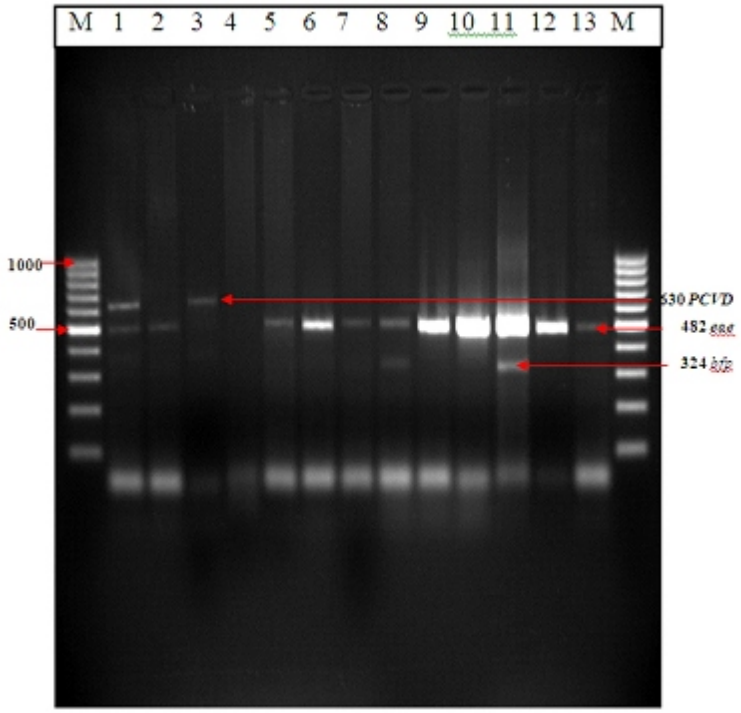

Fig. 2: Multiplex PCR results of some clinical samples
M: 100bp DNA ladder; Lane1: Sample (No. 7) (*eae* 482 *bp* and PCVD 630bp), Lane 2: Sample (No.12) (*eae* 482bp), Lane 3: Sample (No. 14) (PCVD 630bp), Lane 4: Sample (No. 5)(negative), Lane 5,6,7: Samples (No. 13),(No.1) and (No.15) (*eae* 482bp), Lane 8: Sample (No. 16) (*eae* 482bp and *bfp* 324bp), Lane 9 and 10: Samples (No. 4) and (No.18) (*eae* 482bp), Lane 11: Sample (No. 19) (*eae* 482bp and *bfp* 324bp), Lane 12 and 13: Sample (No. 2) and Sample (No.10). (*eae* 482bp)

Table 3: Distribution of ETEC and EPEC according to the type of enterotoxin and virulence genes, respectively

	ETEC 5/19 (26.3%)		EPEC 12/19 (63.1%)	
	lt only	*st* only	*eae*(a-typical)	*eae* and *bfp* (typical)
	2/5 (40%)	3/5 (60%)	9/12 (75%)	3/12 (25%)
Total (19)	2/19 (10.5%)	3/19 (15.7%)	9/19 (47%)	3/19 (15.7%)

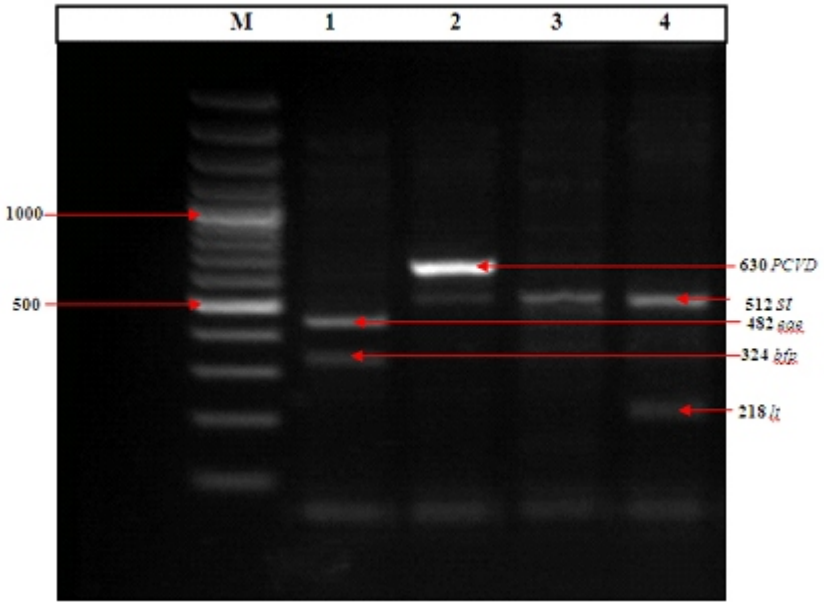

Fig. 3: Multiplex PCR results of the remaining tested clinical samples
Lane 1: Sample No. 3 (*eae* 482 and *bfp* 324), Lane 2: Sample No. 11(*st* 512 and PCVD 630), Lane 3: Sample No. 6(*st* 512), Lane 4: Sample No. 8 (*lt* 218 and *st* 512)

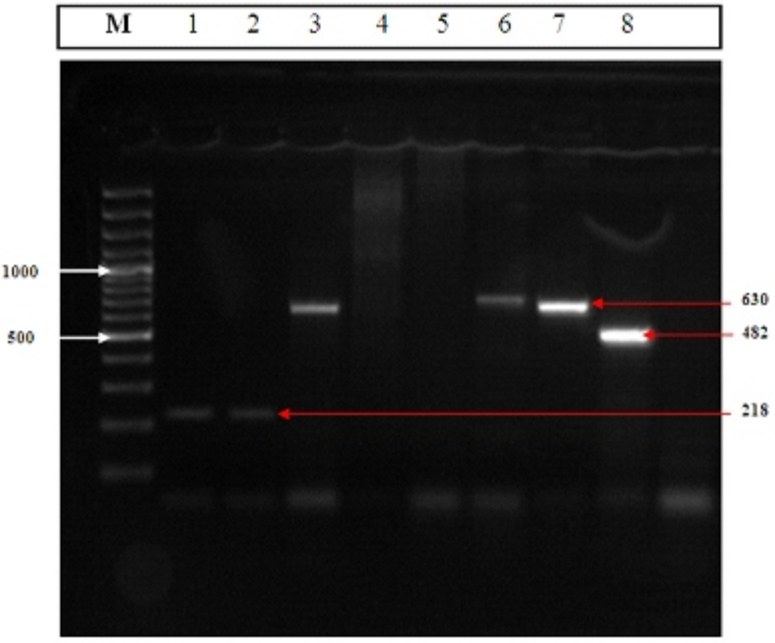

Fig. 4: Uniplex PCR results of some tested clinical samples
Lane 1and 2: Sample No.1 and 2 (*lt*, 218bp), Lane 3: Sample No.14 (PCVD, 630bp), Lane 4 and 5: Sample No .20and 23 (negative)
Lane 6: Sample No.17 (PCVD, 630bp), Lane 7: Sample No.7 (PCVD, 630bp), Lane 8: Sample No.7 (*eae*, 482bp)

(*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* (Verotoxine producining *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-Obsidi 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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