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Molecular Identification of Specific Virulence Genes in Enteropathogenic*Escherichia coli*

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This paper is part of a M.Sc. thesis of the second author.

Abstract: *A total of fifty Escherichia coli isolates were isolated from 300 clinical samples. The isolates were identified using traditional methods and polymerase chain reaction (PCR) technique. The electrophoresis analysis of PCR amplification products of specific virulence genes revealed that ten isolates (20%) werebelonged to enteropathogenicE. coli (EPEC).Of these,two isolates (4%) harboured eae, bfpAandeafgenes, but lacking stx1, stx2 and hlyA genes, these isolates identified as typical EPEC. Whereas eight isolates (16%) werecarried eae gene but did not possess bfpA, eaf, stx1,stx2 and hlyA genes, these isolates identified as atypical EPEC. Forty isolates (80%) of E. colifound do not have any one of the specific virulence genes, these isolates identified as non-EPEC. These findings indicated that theeae, bfpAandeafgenes are significant for molecular Identification of EPEC.*
Keywords*: Diarrhea,EPEC, PCR, Typical, Virulence.*

I. Introduction

Diarrheagenic or pathogenic *E. coli* offered a taxonomic challenge since for many years, their characterization was based on the virulence traits, this group of bacteria are named enterotoxigenic*E. coli* (ETEC), enteroinvasive*E.coli* (EIEC), enteroaggregative*E. coli* (EAEC), diffusely adherent *E.coli* (DAEC), enterohemorrhagic*E. coli* (EHEC) and enteropathogenic*E. coli* (EPEC)[8,14]. Generally, EPEC causes infantal and sporadic diarrhea in the world[16]. The main mechanism of EPEC pathogenesis is a lesion called attaching and effacing (A/E) which is characterized by microvilli destruction, intimate adherence of bacteria to the intestinal epithelium of small intestine, pedestal formation and aggregation of polymerized actin and elements of the cytoskeleton at sites of bacteria attachment[15]. The EPEC adherence factor plasmid (pEAF) containing an operon of 14 genes encoding for complete and functional bundle forming pilli (BFP)[7,17]. BFP are postulated to initiate a long range adhesion of bacteria with the intestinal epithelium and recruited other EPEC cells into aggregates, which result in the presence of bacterial microcolonies[17]. The *bfpA*gene, which is located on pEAF, and the *eae* gene located in the locus of enterocyte effacement pathogenicity island, have both used for subdivision of EPEC into typical and atypical strains[12]. Therefore, the strains with A/E genotype (eae^+) that harbour the pEAF($bfpA^+$) are classified as typical EPEC and the strains with A/E genotype that $bfpA^-$ are classified as atypical EPEC. Hence, this research was undertaken to focus on the detection of some virulence genes in EPEC as a rapid identificationof this group of bacteria.

II. Materials And Methods

Collection of Samples

Three hundred faecal specimens were collected from children ≤ 2 years ofage infected withdiarrhea, hospitalized in Babylon Paediatric Hospital,Iraq. The specimens were collected in 50 ml sterile containers and transferred immediately into the microbial laboratory for further experiments.

Isolation and Identification

The specimens were cultured on MacConkey agar (Himedia-India) and incubated at 37℃ for 24 hours under aerobic conditions in order to differentiate the lactose fermented bacteriafrom the non-lactose fermented bacteria. Well isolated colonies were selected and cultured on Eosin methylene blue agar (Himedia-India) to detect the*E. coli* isolates, which produce a green metallic sheen. The isolates were identified depending on morphological properties (for cells and colonies) and biochemical tests as described by Macfaddin(2000).

DNA Extraction

For molecular identification of*E. coli*isolates, whole genomic DNA was extracted using Wizard Genomic Extraction Kit (Promega, USA).

PCR primers and Conditions

The primers of PCR amplification of specific virulence genes used in this study were synthesized by Bioneer, Korea. These primers and their reaction conditions are demonstrated in Table 1.

Table 1 . PCR primers and their conditions used in this study (Bioneer, Korea).

Primer	Sequence (5----------›3)		Amplicon size (bp)	Conditions (D,A and E)	Cycle No.	Source
bfpA	F	AATGGTGCTTGCGCTTGCTGC	326	94˚C/1 min 60˚C/1 min 72˚C/2 min	30	Gunzburg *et al*., 1995
	R	GCCGCTTTATCCAACCTGGTA				
bfpA	F	ATTGGTGCTTGCGCTTGCTGC	326	94˚C/30sec 56˚C/1 min 72˚C/2 min	30	Yatsuyanagi*et al*., 2002
	R	GCCGCTTTATCCAACCTGGTA				
eae	F	ACGTTGCAGCATGGGTAACTC	815	94˚C/60sec 55˚C/60sec 72˚C/60sec	30	Gannon *etal.*,1993
	R	GATCGGCAACAGTTTCACCTG				
eaf	F	CAGGGTAAAAGAAAGATGATAA	397	94˚C/60sec 57˚C/45sec 72˚C/60sec	30	Franke *et al*.,1994
	R	TATGGGGACCATGTATTATCA				
Stx1	F	AAATCGCCATTCGTTGACTACTTCT	370	94˚C/1 min 64˚C/1 min 72˚C/15 sec	35	Brian *et al*., 1992
	R	CAGTCGTCACTCACTGGTTTCATCA				
stx2	F	TGCCATTCTGGCAACTCGCGATGCA	283	94˚C/1 min 64˚C/1 min 72˚C/15 sec	35	Brian *et al*., 1992
	R	GGATATTCTCCCCACTCTGACACC				
hlyA	F	ACGATGTGGTTTATTCTGGA	166	94˚C/60sec 48˚C/180sec 72˚C/240sec	34	Nataroand Kaper, 1998
	R	CTTCACGTCACCATACATAT				

Abbreviations:D, denaturation;A, anneling ; E, extention;F, forward primer ; Reverse primer.

Preparation of Reaction Mixture

The reaction mixture was prepared according to the manufacturer instructions (Promega, USA). The total volume ofthe reaction was 25µl, consisting of 12.5 µl of Go Taq Green Master Mix, 2.5 µl of downstream primer, 2.5 µlof upstream primer, 2.5 µl of nuclease free water and 5 µlof DNA template. Negative control contains all the above contents without DNA templete was also used. The amplification reactions were performed in an automated thermocycler apparatus (Clever Scientific, UK).

Agarose Gel Electrophoresis

The amplification products of PCR were ran on horizontal agarose gel (1%) stained with ethidium bromide for 1.5 hour and 80 volt. 5 µl of amplificationproducts plus 1 µl of loading dye were loaded in the well of the gel. The DNA marker 100-1500 bp (Promega,USA) were used to detect the size of the electrophoresis fragments of amplified genes. The DNA bands were photographed by gel documentation system (Biometra-Germany)[13].

III. Resultsand Discussion

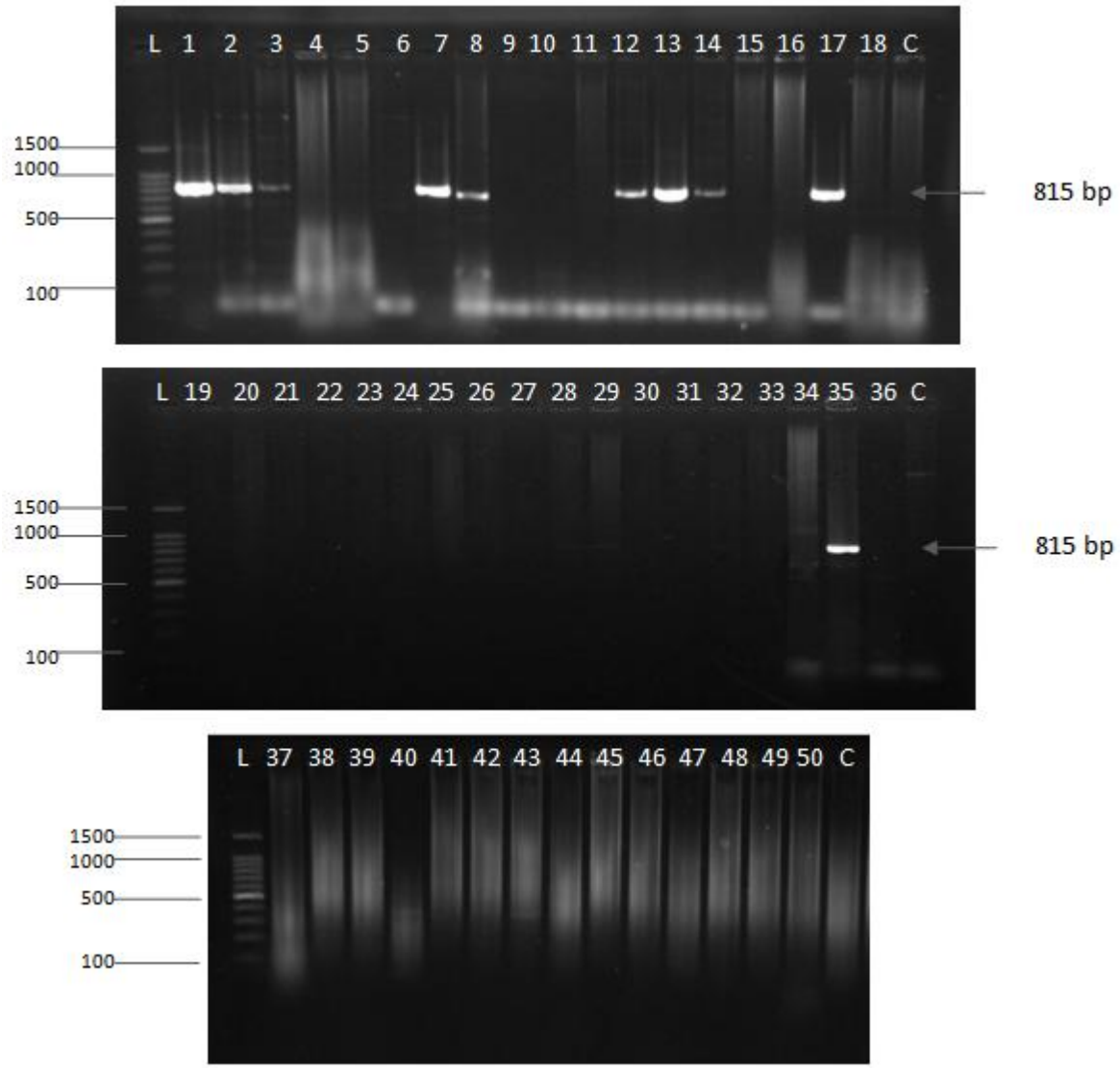
The detection of some virulence genes(*eae*,$bfpA_{ATT}$,$bfpA_{AAT}$,*eaf, stx1, stx2* and *hlyA* genes) from genomic DAN of *E. coli* isolates were investigated. A total of fifty *E. coli* isolates were isolated from 300 stool specimens of children (≤ 2 years of age) infected with diarrhea. The distribution of these isolates according to sex, age and host are summarized in Table 2.

Table 2. Distribution of *E. coli* isolates according to sex, age and host.

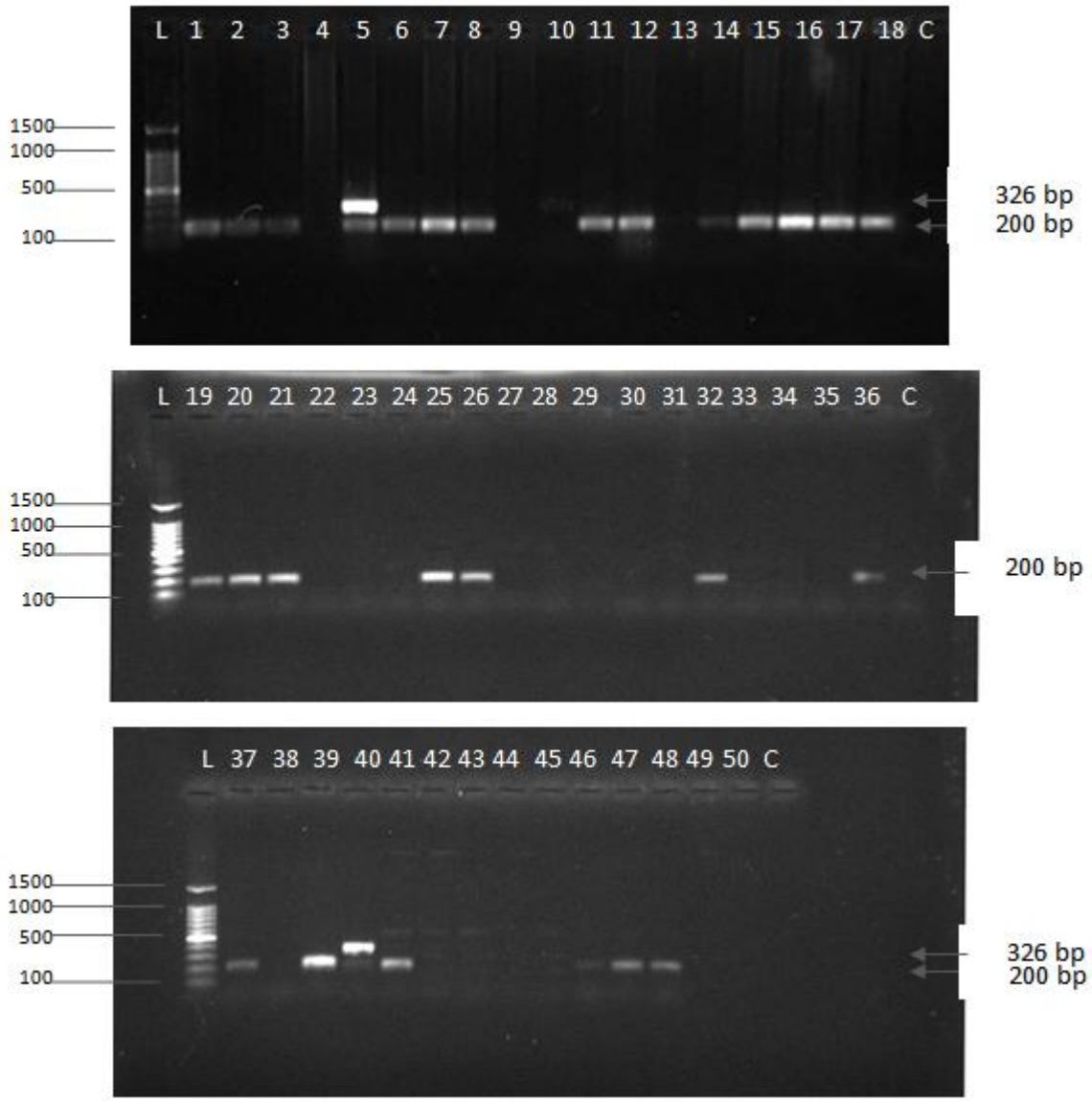
Sex	Age		Host		EPEC		Non-EPEC
	1-12 month	1-2 year	Rural	Arban	tEPEC	aEPEC	
Male	20	10	23	07	01(2%)	06(12%)	23(46%)
Femal	14	06	14	06	01(2%)	02(4%)	17(34%)
Total	34	16	37	13	02(4%)	08(16%)	40(80%)

The isolates were identified using morphological (microscopically andcultural) properties and biochemical tests (data not shown).The electrophoresis results ofPCR amplification products of virulence genes showed that theisolates EC10,EC11, EC29,EC31,EC38,EC39, EC40, EC42, EC44 and EC50 (20%) were belonged to EPEC (Figure 1 and Table 3). Of these, EC39 and EC40 isolates (4%)were harboured the *eae*, $bfpA_{ATT}$,$bfpA_{AAT}$and*eaf*genes, but lacking the *stx1, stx2* and *hlyA* genes, these isolates identified as typical EPEC. Whereas the isolates EC10,EC11, EC29, EC31, EC38, EC42, EC44 and EC50 (16%) were carried the

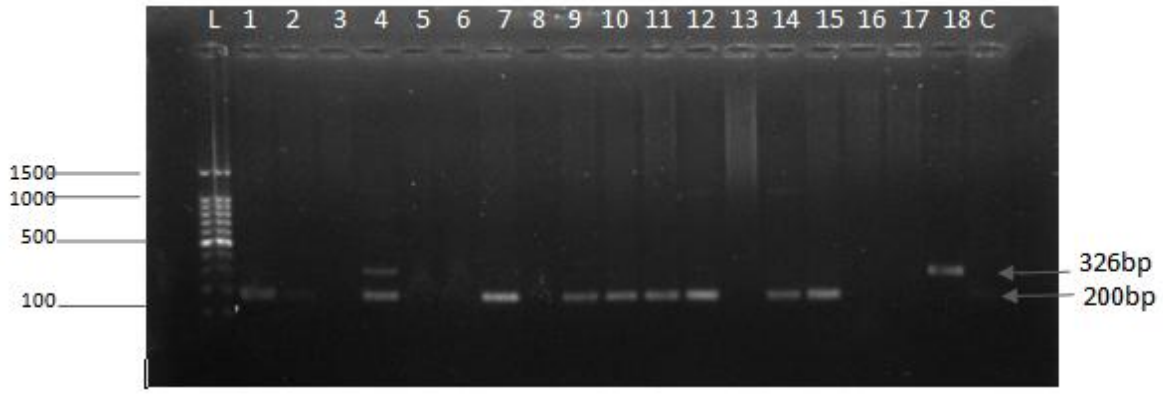
eae gene but did not possess the*bfpA*$_{ATT}$,*bfpA*$_{AAT}$, *eaf, stx1, stx2* and *hlyA* genes, these isolates identified as atypical EPEC. Fourty isolates (80%) of *E. coli* found do not have any one of the specific virulence genes, these isolates identified as non-EPEC (Figures 1, 2, 3, 4 and Table 3). It was shown that some of *E. coli* isolates were carriedthe *bfpA*gene approximately, 200bp which represent the non-specific genes coding for localized adherence like pattern (LAL) and this gene considered as negative result for identification of this bacteria as reported by Carneiro*et al*. (2003). The present results showed that all EPEC isolates were harboured the *eae*gene, and in addition to this gene, the typical EPEC isolates possessed the*bfpA*and *eaf*genes. It has previously been reported that 71 EPEC isolated by Blanco *et al*.(2006) and 19 EPEC isolated by Moura *et al*.(2012) werecarried *eaeA*gene. Mitra*et al*.(2011) reported that 51 of 178 *E. coli* isolates (28.6%) were EPEC and their frequency were higher in children with the age ofless than five years. Similar to the results of Ghosh and Ali (2010), all *E. coli* isolates in the present study were negative for*stx1, stx2* and *hlyA* genes.

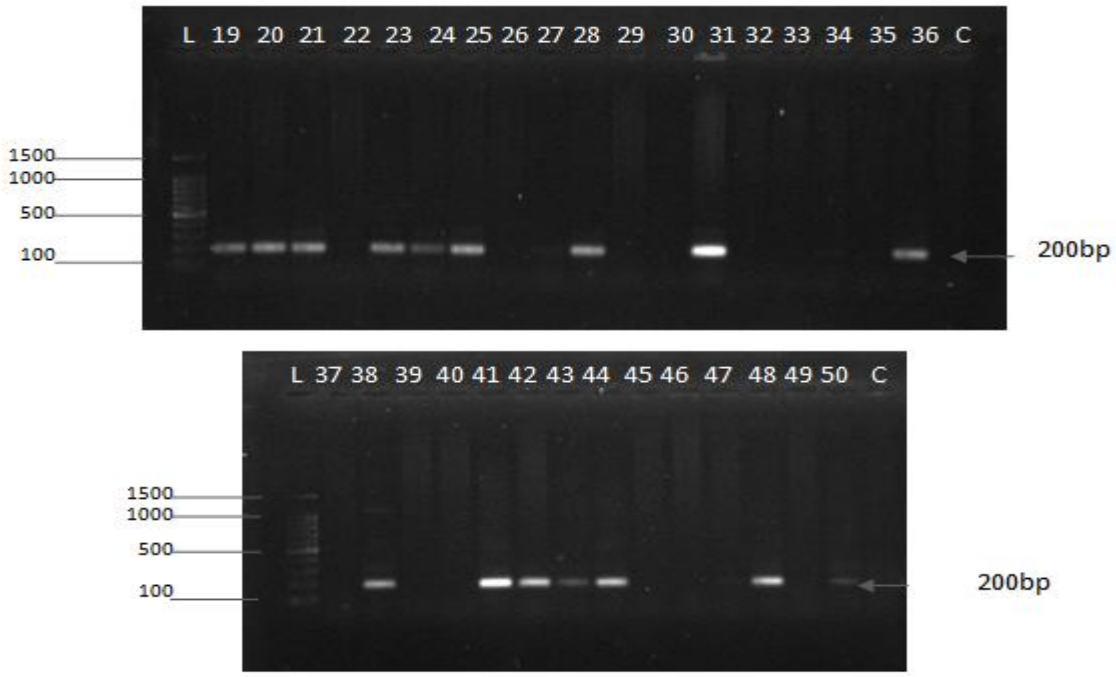


Figer 1.Electrophoresis of*eae* gene amplification products from genomic DNAof *E. coli* isolates on (1%) agarose gel for 90 min. Lane L: ladder, 1.5 Kb; Lanes: 1, 2, 3, 7, 8, 12,13, 14, 17, and 35 represent the positive results (815 bp) of the isolates EC39, EC31, EC40, EC10, EC38, EC29, EC11, EC42, EC44 and EC50, respectively; Lane C: negative control.

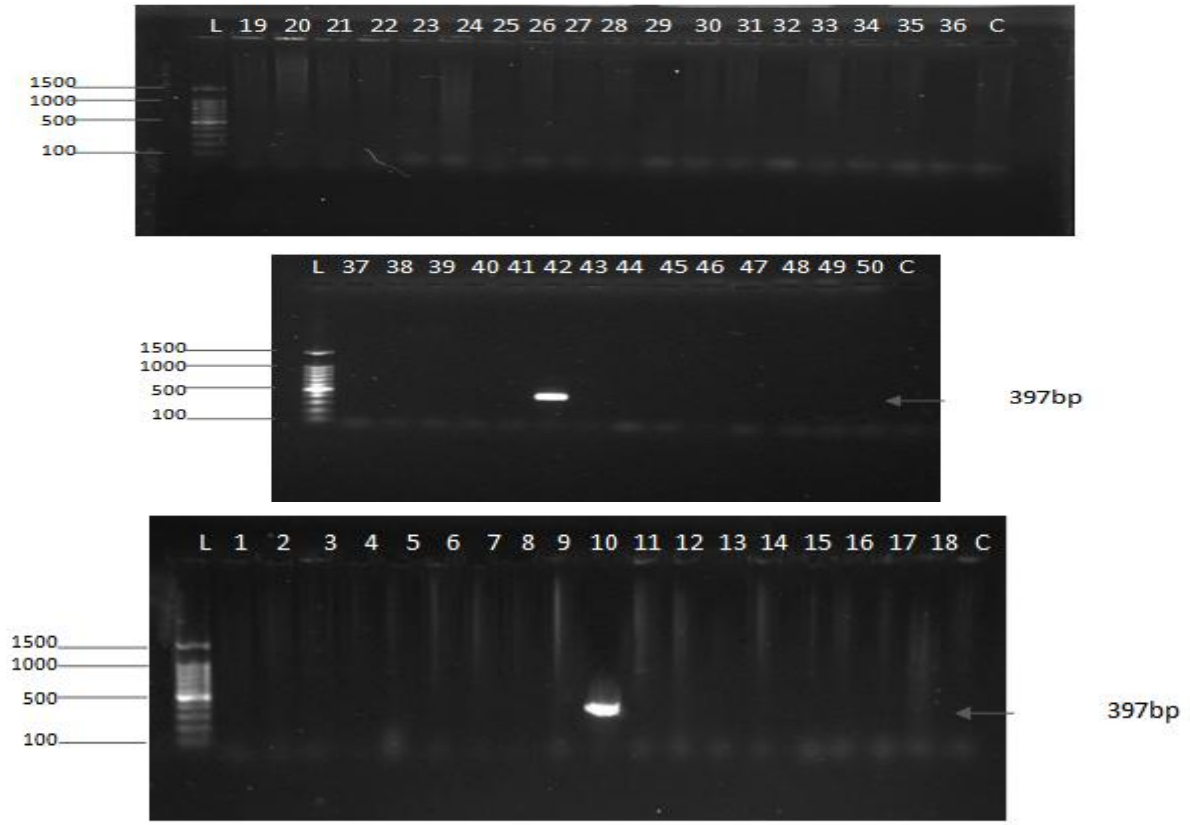


Figer2. Electrophoresis of *fpA*$_{ATT}$gene amplification products from genomic DNA of*E. coli*isolates on (1%) agarose gel for 90 min. Lane L: ladder, 1.5 Kb; Lanes: 5 and 40 represent the positive results (326 bp) of theisolates EC40 and EC39, respectively ; The amplified*bfpA*$_{ATT}$ gene (200bp) represent the non-specific genes coding for localized adherencelike pattern(LAL) ; Lane C: negative control.





Figer3. Electrophoresis of*bfpA*$_{AAT}$ gene amplification products from genomic DNA of*E. coli* isolates on (1%) agarose gel for 90 min. Lane L: ladder, 1.5 Kb; Lanes: 4 and 18 represent the positive results (326 bp) of the isolates EC40 and EC39, respectively ; The amplified*bfpA*$_{ATT}$ gene (200bp) represent the non-specific genes coding for LAL ; Lane C: negative control.



Figer4. Electrophoresis of*eaf* gene amplification products from genomic DNA of*E.coli* isolates on (1%) agarose gel for 90 min. Lane L: ladder, 1.5 Kb; Lanes: 10 and 42 represent the positive results (397bp) of the isolates EC40 and EC39, respectively ; Lane C: negative control.

Table 3.Frequency of the EPEC and their virulence genes.

Isolate No.	Virulence gene							Pathotype
	eae	$bfpA_{ATT}$	$bfpA_{AAT}$	*eaf*	*Stx1*	*Stx2*	*hlyA*	
EC10	+	-	-	-	-	-	-	atypical EPEC
EC11	+	-	-	-	-	-	-	atypical EPEC
EC29	+	-	-	-	-	-	-	atypical EPEC
EC31	+	-	-	-	-	-	-	atypical EPEC
EC38	+	-	-	-	-	-	-	atypical EPEC
EC39	+	+	+	+	-	-	-	typical EPEC
EC40	+	+	+	+	-	-	-	typical EPEC
EC42	+	-	-	-	-	-	-	atypical EPEC
EC44	+	-	-	-	-	-	-	atypical EPEC
EC50	+	-	-	-	-	-	-	atypical EPEC
Remainder	-	-	-	-	-	-	-	Non-EPEC

IV. Conclusion

PCR is a highly sensitive and specific molecular technique for the detection of target DNA in various clinical specimens; it can help to differentiate EPEC from those of the normal florain stool samples. Thus, it is concluded thatthe*eae, bfpA*and*eaf*genes appear to be essential for molecular identification of EPEC and for subdivision of this group of bacteria into typical and atypical pathotypes.

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