Detection of VT1 and VT2 genes in *Escherichia coli* isolated from Diarrhea Patients in AL-Anbar, Iraq using PCR Sequencing

Laheeb Rajab Hamad[1], Muthanna Hamid Hassan[2], Abdalwahab Bdewi Hussain[3]

[1]Lecturer, College of Applied Science, University of Fallujah, Iraq, [2]Assitance Professor, College of Sciences, University of Anbar, Iraq, [3]Professor, University of Fallujah, Iraq

Abstract

Enteric-borne diarrheal illnesses are significant causes of human morbidity and mortality. Over 2 million diarrhea-related fatalities happen annually, especially among kids under the age of five. During the period extended from February 2019 to June 2019, A total of 196 stool locally specimens from diarrheal patients samples were collected from AL-Anbar city of AL-Ramadi hospital and AL-Fallujah hospital out of 196 samples diagnosis by biochemical test , morphology and selective chromogenic medium, found 80 samples of E. coli . The aim of the study detection of VT1 and VT2 genes in Escherichia coli isolated from Diarrhea patients by using PCR Sequencing. The result showed when diagnosis by PCR for Sanger Sequencing technique for VT1 and VT2 genes E. coli O157:H7,and E. coli O25b:H4 were positive for VT1gene ,and Escherichia coli O25b:H4 ,and Escherichia coli O18:H1 positive for VT2gene.

Key words:*Escherichia coli, diarrhea patients, VT1 and VT2 genes.*

Introduction

Diarrheal illnesses are a significant the cause of low-to-middle-income morbidity and mortality nations and are predestined to be the second most important reason from death among kids < 5 years of age, resulting in 0.5 million fatalities worldwide(1). Sub-Saharan and South-East Asian areas have the largest illness burden (> 72%). In spite of the known actuality that diarrheal illnesses are transferred by stool oral route (2). It is a complicated syndrome because it includes interaction of environmental, dietary and infectious variables (3). *Escherichia coli* is extremely multilateral bacteriumthat develops its commensal and pathogenic capacity in human host. Diarrheagenic E. coli (DEC) is recorded as one of the world's major causes of gastrointestinal turmoil and is an significant public health issue(4,5,6,7). These pathotypes likewise play a significant part in the morbidity of diarrhea in the Indian people . Remarkably, distinguish DEC pathotypes offer particular, virulence arsenal which convert the prevalent repertoire available for diagnostic and therapeutic methods. DEC is moreover cataloged into different pathotypes based upon appearance from these virulence determinants participating to specific pathophysiology(8). Human and bovine STEC strains detailed two powerful phage-encoded cytotoxins called Shiga toxins (Stx1 and Stx2) or Verotoxin (VT1 and VT2) (9, 10). Moreover, Shiga-like toxin (Stx)-producing Escherichia coli (STEC) contagion is an continuing health issue that can leadership to severe complications, inclusive hemolytic uremic syndrome (HUS) and death(11).

Materials and Method

Subjects and specimen collection.

A total of 196 stool samples of patients with diarrhea aged among (2-60) yearsthrough the period from February 2019 to June 2019.Samples were collected from AL-Anbar city of AL--Ramadi hospital and AL-Fallujah hospital out of 196 samples diagnosis by biochemical test , morphology and selective chromogenic medium, found 80 samples of E. coli . Patients with soft stool as a major complications, furthermore reported another clinical appearance likewise dehydration, vomiting, Fever, common abdominal pain and mucus. Patients have been given written consent. To identify *E.coli,* stool specimens were plated into Differential media Sorbitol- MacConkey agar(SMAC), Chromo agar *E.coli* and Eosin methylene blue agar(EMB) Samples were cultured on differential agar and incubated aerobically at 37 C for 24-48 hours to isolate *E.coli*(12).

Extraction of total DNA

Genomic DNA of 80 Escherichia coli isolate was extracted by utilizing (DNA mini kit that was provided by G- spin DNA extraction kit, Korea) as instructed by the manufacturer's instructions,primers were acquired from the IDT corporation in this study.

Detection of *Verotoxin* (VT1 and VT2) genes by PCR

The genes of Verotoxin (VT1 and VT2) were amplified by F (5'-CGC TGA ATG TCA TTC GCT CTG C -3') and R (5'-CGT GGT ATA GCT ACT GTC ACC -3'), sense F (5'-CTT CGG TAT CCT ATT CCC GG -3') ,anda anti sense R (5'-CTG CTG TGA CAG TGA CAA AAC GC -3')respectively [11].The PCR reaction mixture contains 5 µl of pre Master Mix , 1.5µl DNA, 1 µl of each forward and reverse primers, then the volume completed to 25 µl by deionized water.Thermo cycling conditions were as follows: initial denaturation at 5 min at 95 ° C, followed by 35 denaturation cycles at 95 ° C for 45 seconds, annealing at 58 ° C for 45 seconds, extension at 72 ° C for 45 seconds and final extension at 72 ° C for 7 minutes. A 70 volt/65 Amp current leaves the gel to run for 60 min. Visualization was performed with a UV transilluminator after electrophoresis.The sequencing of Verotoxin (VT1 and VT2) genes were performed at Macrogencompany utilizing their ABI 3730xl genetic analyzer (Applied Biosystems, US).Online at the National Center for Biotechnology Information (NCBI) at (http://www.ncbi.nlm.nih.gov)and BioEdit, homology search was conducted utilizingthe Basic Local Alignment Search Tool(BLAST) program. The outcomes were compared with information accessible online at the NCBI from the ExPASY program released by Gene Bank.

Results and Discussion

This study includes detection from the prevalence of VT1 and VT2 genes in collected samples locally from stool specimens after isolation on selective chromogenic medium and diagnosis by PCR for Sanger Sequencing technique found 1 out of 80 test samples were positive for E. coli O157:H7, 31 isolate E. coli O126:H20 , 19 isolate E. coli O25b:H4, and 29 isolate for E. coli O18:H1 .

In developing nations, toxigenic Escherichia coli has been acknowledged as the leading cause of diarrhea in both humans and animals [13].Such divergent pathogroups are often allocated using distinct terms such as "hybrid" "mixed virulence profiles" and "mixture of virulence" [14,15,16].Virulence genes in E. coli strains were connected with the coexisting STEC. In Germany, the United States and Slovakia, coli strains of human, animal and environmental origin have been recorded [17,18,19], Some of them related to human illness [19]. Previous studies in Finland recognized hybrid STEC strains from patients and livestock. [20] Nyholm performed a comparative genomics and characterization study of such strains to determine their phylogenetic position in E. Coli and the genes they harbor to define virulence[21].

The polymerase chain reaction diagnostic techniques is rapid, easy, inexpensive protocol becoming the most commonly utilized of all molecular genetics ways for detecting important toxin genes and identifying the bacteria [22]. Its elevated sensitivity, specificity methods for detect Specific sequence of nucleic acids discovered in the genome of pathogens [23]. PCR products of 302bp for VT1 gene, 850bp for VT2 gene were detected in the positive and PCR product was not seen in the negative samples as seen in figure (1,2).

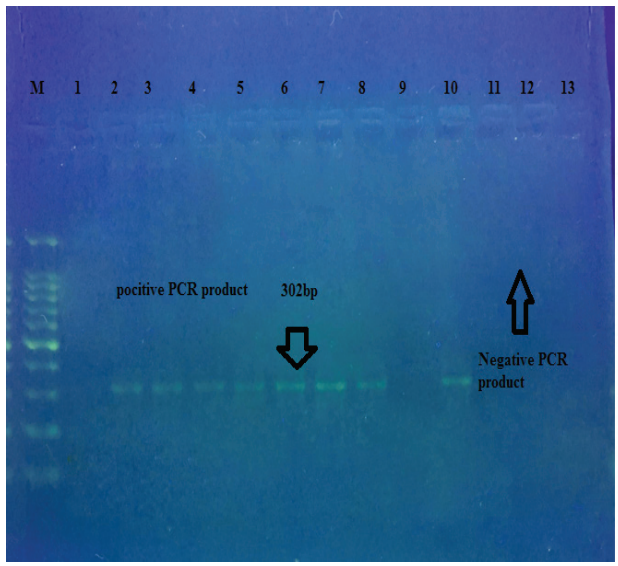

Figure (1):- Agarose gel electrophoresis forVT1gene (302bp). Bands were fractionated by electrophoresis on a 1.5% agarose gel (2 h., 5V/cm, 1X TBE) and visualized under U.V. light after staining with red stain. Lane: M (M: 100bp ladder),Lane: 1,9,11,12,13(negative PCR product) .

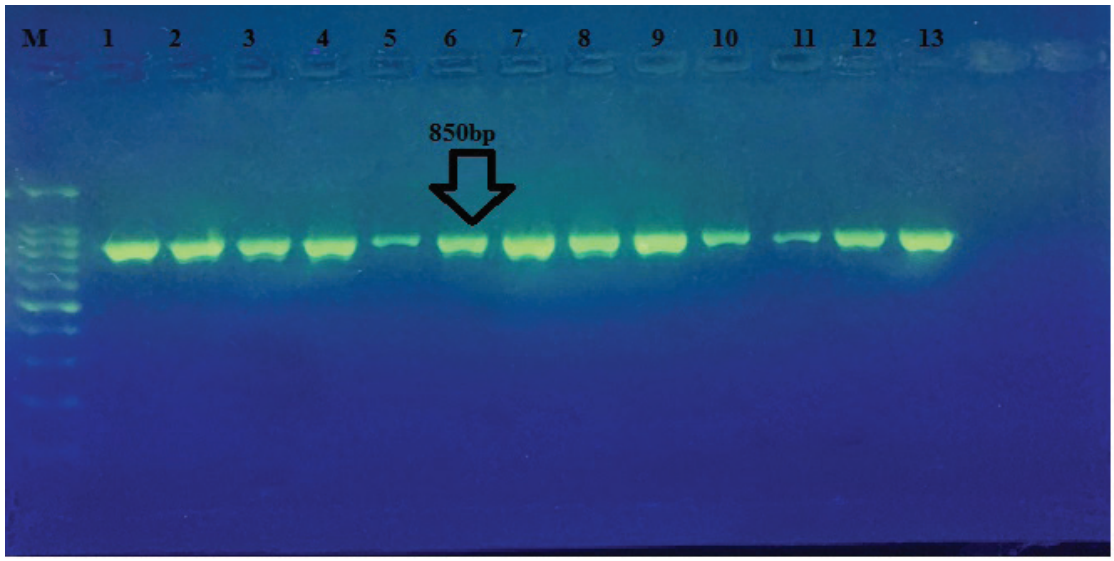

Figure (2):- Agarose gel electrophoresis forVT2gene (850bp). Bands were fractionated by electrophoresis on a 1.5% agarose gel (2 h., 5V/cm, 1X TBE) and visualized under U.V. light after staining with red stain. Lane: 1 (M: 100bp ladder).

The VT1and VT2 genes were amplified by PCR method, and sent for sequencing service to Macrogen company Korea. The sequencing result of VT1gene shows for strain Escherichia coli O25b:H4 having one Transition T>C in location (1273120nucleotide)code TTG>CTG of amino acid Leucine>Leucine and Predicted effect nonsense ,also three Transversion one in location G>C (1273126 nucleotide) code GAA>CAA amino acid change Glutamic acid to Glutamine the effect Missense ,and two T>G in location (1273131 and 1273148 nucleotide respectively code GGT>GGG of the same amino acid Glycine > Glycine, that nonsense substitution ,also code CTG to CGG transformation Leucine to Arginine that effect Missense. From the Gene Bank, found that part of VT1 gene having 99% compatibility with subject of VT1 gene in NCBI as seen in table (1) [24].

Table (1): Represent type of polymorphism ofVT1gene.

Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Sequence ID
Transition	1273120	T>C	TTG>CTG	Leucine>Leucine	nonsense	ID: CP015085.1
Transvertion	1273126	G>C	GAA>CAA	Glutamic acid> Glutamine	Missense	
Transvertion	1273131	T>G	GGT>GGG	Glycine > Glycine	nonsense	
Transvertion	1273148	T>G	CTG>CGG	Leucine> Arginine	Missense	

While the analysis of the VT1 gene for strain Escherichia coli O25b: H4 our study was coordinated by 99% having one Transvertion of Pyrimidine nucleotide C to Purine of nucleotide G, code ACG > AGG and amino acid transformation Threonine> Arginine the effect Missense in location (1273112 nucleotide), under sequence ID: CP015085.1, as shown in table(1)[24].

Compatibility of 97 percent in Gene Bank of VT1 gene as shown in table (4)under sequence ID: CP015085.1 having one Transition A to G in location (1273045nucleotide),and code ATC>GTC amino acid Isoleucine>Valine and Missense substitution ,as well three Transversion A to T in location (1273037nucleotide), the code AAT>ATT amino acid change Asparagine to Isoleucine , another location C>G (1273049 nucleotide), have code GCC>GGC and amino acid Alanine> Glycine

,and C to A in location (1273135nucleotide), the code CCT>ACT,amino acid Proline> Threonine the effect Missense respectively(24).

Table (2): Represent type of polymorphism ofVT1gene.

Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Sequence ID
Transvertion	1273037	A>T	AAT>ATT	Asparagine> Isoleucine	Missense	ID: CP015085.1
Transition	1273045	A>G	ATC>GTC	Isoleucine>Valine	Missense	
Transvertion	1273049	C>G	GCC>GGC	Alanine> Glycine	Missense	
Transvertion	1273135	C>A	CCT>ACT	Proline> Threonine	Missense	

having eight Transition showed three C to T in sites (1478626,1478533,and 1478368 nucleotide) having a code and not changing amino acid ACC>ACT Threonine> Threonine, TCC>TCT Serine> Serine ,and CCC>CCT Proline>Proline they have no impact nonsense,also one transition T to C in site (1478620 nucleotide) the code TTT>TTC ,and then the same amino acid Phenylalanine > Phenylalanine,as well as three Transition G to A in sites (1478554,1478293,and 1478224 nucleotide), having the code CAG>CAA , GCG>GCA,and CAG>CAA , the acids of the amino respectively Glutamine to Glutamine, Alanine to Alanine, and Glutamine to Glutamine that nonsense substitution, While A > G is in place(1478416 nucleotide), the code CAA > CAG and the amino acid Glutamine to Glutamine has an effect nonsense. Two Transversion as displaying one A > C (1478524 nucleotide), code GCA > GCC amino acid not altering Alanine > Alanine, and predicted nonsense impact, finally appeared G toT in location(1478405nucleotide) , the code AGC>ATC ,and amino acid alteration Serine> Isoleucine the Predicted effect Missense. From the Gene Bank, part of the VT1 gene forstrainEscherichia coli O157:H7 was found to be 98% compatible with the NCBI standard, as shown in table(2).

Table (3): Represent type of polymorphism ofVT1gene.

Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Sequence ID
Transition	1478626	C>T	ACC>ACT	Threonine> Threonine	nonsense	ID: CP040572.1
Transition	1478620	T>C	TTT>TTC	Phenylalanine > Phenylalanine	nonsense	
Transition	1478554	G>A	CAG>CAA	Glutamine> Glutamine	nonsense	
Transition	1478533	C>T	TCC>TCT	Serine> Serine	nonsense	
Transvertion	1478524	A>C	GCA>GCC	Alanine> Alanine	nonsense	
Transition	1478416	A>G	CAA>CAG	Glutamine> Glutamine	nonsense	
Transvertion	1478405	G>T	AGC>ATC	Serine> Isoleucine	Missense	
Transition	1478368	C>T	CCC>CCT	Proline>Proline	nonsense	
Transition	1478293	G>A	GCG>GCA	Alanine> Alanine	nonsense	
Transition	1478224	G>A	CAG>CAA	Glutamine> Glutamine	nonsense	

Another part of sequencing for VT2 gene to strain Escherichia coli O25b:H4 the result shows Compatibility of 100% in Gene Bank of VT2 gene for Escherichia coli O25b:H4 under sequence ID: CP015085.1,so no recorded change noticed from the Gene Bank in VT2 gene [25].

Amplification of the VT2 gene for Escherichia coli O18:H1 with one Transition T > C in intron place (1902685 nucleotide) and two Transition T to C place (1903016,and 1903166 nucleotide), code ATT > ATC, and CGT > CGC amino acids respectively Isoleucine > Isoleucine, Arginine > Arginine, and predicted nonsense impact.

The sequencing analysis of VT2 gene for Escherichia coli O18:H1 as seen in table(3). The our study was coordinated 99% having two Transvertion T to A in place (1902482 nucleotide)code TAA>AAA and amino acid alteration Leucine to Phenylalanine, another Transvertion from Pyrimidine nucleotide C to Purine of nucleotide G in place (1902494nucleotide) that code CTT to GTT, amino acid alteration Lysine to Asparagine the effect Missense, also one Transition T to Cin intron,and two Transition Tto C in locations (1903016 and 1903166 nucleotide),code TTG>TCG ,and CGT>CGC ,the amino acid not altering Isoleucine> Isoleucine, and Arginine> Arginine respectively and nonsense substitution. under sequence ID: CP028320.1.

Table (4): Represent type of polymorphism ofVT2gene.

Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Sequence ID
Transvertion	1902482	T>A	TAA>AAA	Leucine> Phenylalanine	Missense	ID: CP028320.1
Transvertion	1902494	C>G	CTT>GTT	Lysine> Asparagine	Missense	
Transition	1902685	T>C	INTRON			
Transition	1903016	T>C	TTG>TCG	Isoleucine> Isoleucine	nonsense	
Transition	1903166	T>C	CGT>CGC	Arginine> Arginine	nonsense	

For the first time in Iraq, this study explored VT1 and VT2 genes using Sanger Sequencing method where few research was conducted to detect those samples through sequencing. Few studies have assessed or compared easy DNA extraction techniques to enable and improve the sensitivity of fecal samples to PCR detection of enteric pathogens [26, 27].

Conclusions

In the present study in stool samples of patients with diarrhea when diagnosis by PCR for Sanger Sequencing technique for VT1 and VT2 genes E. coli O157:H7,and E. coli O25b:H4 were positive for VT1gene ,and Escherichia coli O25b:H4 ,and Escherichia coli O18:H1 positive for VT2gene.

Conflict of Interest: There is no conflict of interest among the authors.

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Ethical Clearance: This study is ethically approved by the Institutional ethical Committee.

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