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Molecular characterization of some virulence factors in multidrug resistance *Escherichia coli* O157:H7 isolates in Iraqi hospitals

Mokhtar Jawad AL- Imam[1], May Talib Flayyih[2]

AL-Rasheed University College, Department of Biology, Baghdad, Iraq[1]
Department of Biology, College of Science, Baghdad University, Iraq[2]

ABSTRACT— Two hundred and fifty specimens (stool) from children under two years for both sexes were collected from some Iraqi hospitals. All isolates were diagnosed according to morphological characteristics, biochemical tests, and results showed, 210 (84%) samples were identified as *E. coli*, (5 isolates) were identified as Enterohemorrhagic *Escherichia coli* (EHEC)serotype O157:H7. The identification was confirmed by VITEK 2 system. Susceptibility test was determined for all pathogenic E. coli isolates to 16 different antibiotics, the result showed that 100%resistant to (Ampicillin, Cefazolin, Ceftriaxone, Cefepime, Trimethoprimand Ceftazidime), but Nitrofurantoin, Cefoxitin and Gentamicin were 66%, 40%, 15% respectively, however100% of isolates were sensitive to Piperacillin, Ertapenem, Imipenem, Amikacin, Ciprofloxacin, Levofloxacin and Tigecycline. Monoplex pattern of PCR was used for detection 16SrRNA, eae, stx1,stx2,lifAand *Ler* genes in *E. coli* O157:H7, result showed isolates were positive for 16SrRNA,eae,lifA, stx1 and regulatory gene (*Ler*),while no bands appear ofstx2.

KEYWORDS: EHEC (O157:H7), Intimin, 16SrRNA, stx1, Ler

1. INTRODUCTION

Escherichia coli is a gram-negative, rod-shaped bacteria from the genus Escherichia that regularly colonizes humans. Enterohemorrhagic E. coli (EHEC) is the most prevalent cause of watery diarrhea in children in the poor world, as well as an uncommon cause of severe diarrhea in adult patients [1]. EHEC is a prevalent cause of illness in people, and some strains are shigatoxigenic (able to make shiga toxin). EHEC serotype O157:H7 has been linked to outbreaks of bloody diarrhea and hemolytic uremic syndrome all throughout the world (HUS) [2]. EHEC infections have a wide variety of symptoms, from asymptomatic to severe. Humans can be infected by the EHEC through a variety of means, including direct contact with infected animals and humans, as well as ingesting contaminated food and water. The majority of outbreaks are known to be caused by the intake of contaminated food, such as ground beef, dairy products, or fresh fruits and vegetables [3]. The generation of shiga toxin is a crucial step in the genesis and progression of EHEC infections (Stx). The possibility of releasing Stx from dead and dying bacteria, as well as the risk of acquiring resistance, have limited the antibiotic's use against EHEC. Shiga toxins are produced by both EHEC and Shigella dysenteriae type 1, which is linked to their pathogenicity [4]. The locus of enterocyte effacement (LEE) is a 35.6-kilobyte pathogenicity island found in the genomes of certain bacteria, including EHEC. LEE is made up of genes that are responsible for attaching and effacing lesions, which are lesions that play a role in bacteria's close adherence to enterocytes, resulting in a signaling cascade that destroys the brush border and microvilli, eases ions loss, and eventually causes severe diarrhea. The LEE has 41 open reading frames and five main operons, as well as codons for type three system apparatus, secreted proteins, and an adhesin known as intimin. The translocated intimin receptor is a type of intimin

receptor (Tir) [5], [6]. *Ler* is first gene in LEE1 and the master regulator of the LEE Pathogenicity Island and functions as an anti-H-NS releasing the H-NS-mediated repression and was shown to activate the transcription of the LEE2, LEE3, LEE4, LEE5, espG, grlRA, and map genesoperons [7]. This study was designed to isolate and identify *E.coli* serotype O157:H7 from individuals with diarrhea under the age of two years, to determine antibiotic susceptibility of *E.coli* isolates, and to detect the presence of some virulence determinants of *E.coli* serotype O157:H7.

2. Isolation and Identification

Two hundred and fifty specimens (stool) from children under two years for both sexes were collected in sterilized containers from Iraqi hospitals. The diagnosis of *E.coli* was achieved according to their morphological properties on macConkey agar, EMB medium, Sorbitol macConky agar, Chrome agar O157 andOxidase production, Catalase production, Methyl red test, Indole production, Urease production, VogeusProskauer tests, Kligler iron agar and Citrate utilization,motility test [8], [9].

2.1 Molecular study

DNA extracted and purified carry out using genomic DNA purification kit protocol (GeneaidExtraction Kit). From each DNA extracted sample, 2 µl was added to the specialized measuring lens of the Nano- drop system after swabbing the lens with D.W. wetted cotton swab to measure the concentration and purity of extracted DNA sample at 260 nm and 280 nm. The results were recorded computerization and the specific primerswere designed according to Bio edit program and NCBIBLAST.http://www.ncbi.nlm.nih.gov website with conserved region. (Table1). The extracted DNA, primers and PCR master mix were mixed together. PCR mixture was set up in a total volume of 20µL included 5µl of PCR Green master mix,1µL of each primer, and 2µL of template DNA have been used, the rest volume was completed with sterile de-ionized distilled water, then vortexed. De-ionized water added firstly, then primers and DNA template added at last. Negative control contained all material except template DNA, so instead that distilled water was added. PCR reaction tubes were centrifuged briefly to mix and placed into thermocycler PCR instrument where DNA was amplified. The PCR program includes 35 cycles were carried out: Initial denaturation at 95 ºC for 5min,denaturation 95 ºC for 30sec, extension 72 ºC for 40sec, final Extension 72 ºC for 5min, and annealing stage are changing,*16srRNA* at 59.2ºC for 30sec,*eae* at 58.5ºC for 40sec,*LifA*at 58ºC for 30sec,*Stx1* at 61.6ºC for 30sec, *Stx2* at 59 ºC *at* 30sec,*Ler*at60ºC for 30sec.The PCR products were analyzed by using 2% agarose gel electrophoresis in 100 ml of 1x TBE buffer and melted, then the agarose gel was cooled to 55-60ºC; the gel was stained by safe dye and 100 bp DNA ladder. Then the electric current was matched (70 volt for 1 hr).

(Table 1): The primers and their sequences used in conventional PCR

	Primer name	Sequence 5' 3'	Product length	Origin
1.	16SrRNA	F:GATGACCAGCCACACTGGAA R:GGAGTTAGCCGGTGCTTCTT	213pb	New
2.	eae	F: GGGCGGTCAGATTCAGCATA R: CCATCACTGACTGTCGCACT	741bp	New
3.	lifA	F:TGGTCGGAGTCGTCCAGTAT R:GGACGATGACCGATTTTGCG	712bp	New
4.	STX1	F:GTGTTGCAGGGATCAGTCGT R:GACTCTTCCATCTGCCGGAC	446bp	New
5.	STX2	F:TCCGGAAGCACATTGCTGAT R:CCACCGGGCAGTTATTTTGC	500bp	New
6.	Ler	F:ACCGCAATGAAGAAGGGCAGA R:TTTCTTCTTCAGTGTCCTTCAC	120bp	New

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3. Results and Discussion

The result was identified *E.coli* serotype O157:H7 according to culturing on Chrome agar O157, the isolates gave mauve colonies and recorded 5 isolates were identified as (EHEC) serotype O157:H7 in children under two years. Current study suggests that 12-24 months of age are more susceptible to than those who were younger or older, and this is an agreement with [10]. The reasons for more susceptibility of children 12-24 months than older include the use of food and water that may be contaminated and decreased exposure to milk protective factors has played a major role in increasing diarrhea rates, this agreement with [11] and with [12]. Current study demonstrated the bloody diarrhea caused by *E. coli* O157:H7 was in first two years of life and the lowest rate was in children over two years of life, Patients selection was restricted to those who had bloody diarrhea because *E.coli* O157 is mostly associated with this clinical feature. Results in the present study are agreement with some previous studies by [13] and it was disagreement with findings of [14] were they reported that *E. coli* O157:H7 was not found in any stool samples of children under two years old.

3.1 Antibiotic susceptibility of Escherichia coli O157:H7 isolates

Susceptibility test was determined for all *Escherichia coli* O157:H7 isolates to 16 different antibiotics (Ampicillin, Piperacillin, Cefazolin, Cefoxitin, Ceftazidime, Ceftriaxone, Cefepime, Ertapenem, Imipenem, Amikacin, Gentamicin, Ciprofloxacin, Levofloxacin, Tigecycline, Nitrofurantoin, and Trimethoprim). The results showed that all *E.coli* O157:H7 isolates resistant to (Ampicillin, Cefazolin, Ceftriaxone, Cefepime, Trimethoprim, Ceftazidime,100%), Cefoxitin was 40%, Gentamicin was 24%, while 100% of isolates were sensitive to Piperacillin, Ertapenem, Imipenem, Amikacin, Ciprofloxacin, Levofloxacin, Tigecycline, Nitrofurantion. The results showed the high resistant of *E.coli* isolates to Ampicillin, and Trimethoprim and the high resistant of *E.coli* isolates to Cefazolin, Ceftriaxone, Cefepime, Ceftazidime, 100%. These compatible with results of [15] and with [16]. While Cefoxitin resistance was 40%, and this percentage compatible with [17]. Resistant of *E.coli* isolates to Gentamicin reach to 24%, this compatible with results of [18], when showed the resistance to gentamicin was found in 29% of isolates. Our results showed that none of the isolates were resistant to imipenem, ciprofloxacin and amikacin, etrapenem, levofloxacin, piperacillin, tigecycline, Nitrofurantoin this compatible with results of [19].

3.2 Molecular study

The results revealed that the concentrations of DNA ranged from (90-400) ng/µl, while purity ranged from (1.8 - 2). Current results demonstrated that (100%) of *E.coli*O157:H7 isolates had *16SrRNA, eae, lifA, stx1, Ler* with213bp, 741bp, 712bp, 446bp and120bp, respectively, while all strains were negative for *stx2* gene, figures (1-4).

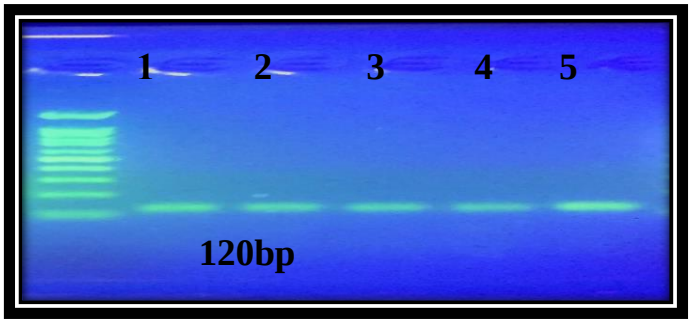

Figure (1): Gel electrophoresis of amplified Ler (120bp)

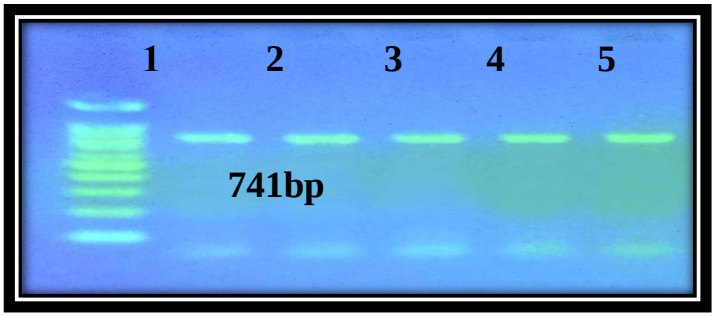

Figure (2): Gel electrophoresis of amplified eae (741bp)

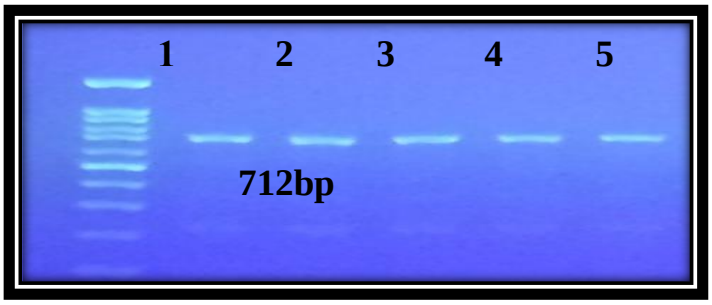

Figure (3): Gel electrophoresis of amplified lifA (712bp)

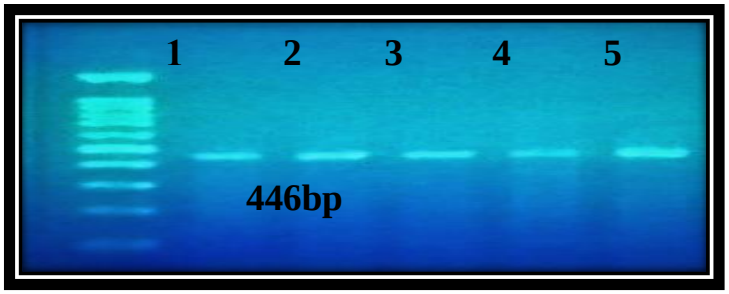

Figure (4) Gel electrophoresis of amplified Stx1 (446bp)

The Iraqi study by [20] compatible with our result when detection *Escherichia coli* O104: H4 by *16SrRNA*. In gene sequencing technique, the *16SrRNA* has several advantages over phenotypic and biochemical identification. The usage of *16SrRNA* advantage includes: the presence of the gene in all bacteria, extensive mutations are lack, therefore; using *16SrRNA* is more accurate than bacteriological and biochemical assays. The genotypic characterization methods are generally, more accurate than the phenotypic methods. The reason behind that is because the later tends to be changeable due to the environmental and growth conditions as well as the temperature and the pH level [21]. Current study compatible with study in Northern Ireland about four-year 1997–2000, demonstrated that 80% of *E.coli* O157 isolates possessed the *eae* gene [22]. Other study Using universal primers and probe to detection *eae* gene in *E.coli* O157:H7 isolates and demonstrated the *eae* gene was detected in 73.3% of the samples [23]. Enterohemorrhagic *E. coli* (EHEC) strains also possess an *eae* gene and are capable of intimate attachment and microvillus effacement in vitro and in animal models, and some studies have proven the role of the EHEC *eae* gene in intimate attachment when constructed an *eae* deletion/insertion mutation in wild-type EHEC O157:H7 strain 86-24 by using linear electroporation of a recombinant allele. EHEC has *lifA* gene that act to inhibition lymphocyte with strongest statistical association with diarrhea. Current results with high significant percentage of strains have *lifA* gene compatible with [24] when demonstrated a strong statistical association between the presence of *lifA*-positive pathogenic *E.coli* and the presence of diarrhea that

suggesting *efa1/lifA*-positive strains may be true diarrhea pathogens. Pervious study in Nairobi, Kenya, aims to detection some virulence factors of (DEC) by Multiplex and conventional PCR assays, and demonstrated (DEC) secreted protein C (EspC) 12.2% (8/66) and (4/66) of fyuA gene, 19.8% (13/66) expressed *efa/lifA* gene [25]. The results are compatible with previous study in University of Baghdad, Iraq; include, isolated 32 isolates of *E. coli* recovered from 350 fecal samples, and showed high percentages of isolates gave positive results with stx1primer equal to target product size (614bp) [26]. Other study in Iran refers to *stx1-eae*, are only observed in *E. coli* O157:H7 that cause human infections in South of Iran, within the human disease-associated strains, those producing Shiga toxin type 2 (Stx2) appear to be more commonly responsible for serious complications such as HUS than those producing only Shiga toxin type 1 (Stx1), this result explanation why don't appear stx2 in our result, because not recorded any patient Suffers from HUS [27]. Our study focusing on detection *ler* gene because the expression of LEE genes is regulated by the transcription factor Ler, which is encoded by the *ler* gene, *ler* is the first gene in the LEE1 operon, as well as a few studies about *ler* gene give stimulation for designed a special primer for gene. In Iraq, there was no recorded studiesabout molecular detection of *ler* gene in *E.coli* O157:H7. Current study compatible with some studies in worldwide that include detection *ler* gene in EHEC and study the expression of this gene, such as study in 2014, refers to the role of iron in LEE (locus for enterocyte effacement) virulence gene expression in EHEC, the expression of LEE genes was greatly reduced in fur mutants irrespective of the iron concentration. The expression of the *ler*gene was affected at a post-transcription step by fur mutation. Further analysis showed that the loss of fur affected the translation of the *ler* gene by increasing the intracellular concentration of free iron, and the transcription of the antisense strand was necessary for regulation, the results indicate that LEE gene expression is closely linked to the control of intracellular free iron homeostasis [28]. The importance for molecular detection of Ler, because it essential for the expression of multiple LEE-located genes in both EPEC and EHEC, including those encoding the type III secretion pathway, the secreted Esp proteins, Tir, and intimin. Ler is therefore central to the process of attaching and effacing (AE) lesion formation [29].

4. Conclusions

All Enterohemorrhagic Escherichia coli (EHEC) serotype O157:H7 were present in diarrheal stool samples of children under two years old, and several serotype O157:H7 were multidrug resistant, all (EHEC) serotype O157:H7 isolates have regulatory gene (Ler).

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