The Antimicrobial Susceptibility and Detection of Virulence Genes of *Escherichia coli* O157:H7 Isolated from Leafy Green Vegetables

S. E. Haramain and S. O. Yagoub

ABSTRACT

Vegetables can be vehicles for transmission of *E. coli* O157:H7 to humans, therefore, this study carried out in order to investigate the presence of Enterohemorrhagic *E. coli* in ten different types of leafy green vegetables, determined their susceptibility to thirteen antibiotics and detected the presence of some virulence genes.

Method: Five-hundred samples of green leafy vegetables namely (Lettuce, Spanish, Rocket, Parsley, Mallow, Coriander, Portulaca, Lettuce, Dill, Basil and Chard) were examined for presence of *E. coli* O157:H7, by using standard microbiological tests (CHROMagar™ O157:H7), further detection of *E. coli* O157:H7 was done by Multiplex PCR (mPCR) for the detection of virulence genes (stx1, stx2, intimin and hlyA) These genes are causative factors of settlement, adhesion, and attack of STEC bacteria to gastrointestinal mucosa.

Results: *E. coli* O157:H7 was isolated from eight (80%) out of ten types of green leafy vegetable as 12 (2.40%) in which the highest percentage of isolation was shown in Dill and Chards samples as (4.2%), Coriander and Mallow showed percentage of isolation as (3.33% and 3.03%) respectively, Parsley, Portulaca and Lettuce showed percentages of isolation as 2.43%, 1.92%, respectively, the least percentage of isolation was shown in Rocket (1.7%), No *E. coli* O157:H7, was detected in Spinach and Basil. Makkah collected samples showed isolation of 7 isolates out of 12 (58.33%). All isolates were resistant to Methicillin (5µg), Metronidazole (5 µg) and Ampicillin (10 µg). Stx2 (110 bp), Stx1 (349 bp), hly A (165 bp) genes were detected. All isolates showed negative results for presence of intimin gene (890 bp). This study concluded that there is a high risk for occurrence of *E. coli* O157:H7 outbreaks due to consumption of the green leafy vegetables sold in Jeddah Central Market.

Keywords: *E. coli* O157:H7, Green leafy vegetables, Virulence genes (stx1, stx2, intimin and hlyA), Antibiotic susceptibility.

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I. INTRODUCTION

Green leafy vegetables play a major role in the worldwide diet probably due to its health benefits and low cost availability [1]. Vegetables are known to be rich in vitamins, iron, calcium, proteins, fats and minerals, dietary fibers and other nutrients including flavonoids, carotenoids and phenolic compounds that may lower the risk of cancer, heart disease and other illnesses [2]-[4].

Fresh vegetables are normally carried non-pathogenic epiphytic microorganisms, however, during growth, harvest, transportation, and further handling the product can be contaminated with pathogens from animal and human sources [5], [6]. As most of these products are eaten without further processing, their microbial content may represent a risk factor for the consumer's health [6]. An increasing number of disease outbreaks in humans have been associated with consumption of contaminated vegetables [7]. Enterohemorrhagic *Escherichia coli* (EHEC) is the most important recently emerged group of food borne

pathogens [8], [7]. It can cause severe gastrointestinal disease, including fatal infections, and is being detected more frequently worldwide. Transmission occurs through consumption of undercooked meat, unpasteurized dairy products and vegetables or water contaminated with feces of carriers [9], [10]. Although, several investigations have been carried out on the laboratory to diagnose these organisms in recent years, this group remains the most difficult to detect. EHEC strains not only produce potent cytotoxins (verotoxins) but have also acquired the ability to adhere to the intestinal mucosa in an intimate fashion [11]. A common characteristic of all EHEC strains is the production of an EHEC-specific plasmid mediated hemolysin encoded by the hlyA gene [12] and at least one Shiga-like toxin (encoded by stx1 or stx2) [13]. *E. coli* O157 infection causes watery diarrhea, vomiting and abdominal cramping, bloody diarrhoea (haemorrhagic colitis), non-bloody diarrhea and occasionally resulting in life-threatening systemic complications including hemolytic uremic syndrome [14].

Most of the *E. coli* O157:H7 outbreaks during (1996 to 2008) were associated with leafy green vegetables, where *E. coli* O157:H7 and *Salmonella* spp, counted 72% of the pathogens involved [11]. Many studies reported outbreaks of *E. coli* O157: H7 due to consumption of green vegetables such as Romaine lettuce [15] white radish sprouts, fresh spinach, lettuce, cantaloupe melon, apple juice, tomatoes and radish sprouts [14], lettuce [16], bagged baby spinach and bagged shredded lettuce [17], The Center for Disease Control and Prevention (CDC) estimates that *E. coli*O157:H7 causes 73,480 illnesses, 2168 hospitalizations and 61 death per year in the USA alone [18]. Critical points of pre-harvest contamination with pathogens in the vegetable production chain are the result of untreated animal manure as well as irrigation with contaminated water or runoff water from nearby areas with animal fecal deposits. Thus, it has been speculated as to what extent human disease outbreaks are associated with the application of animal manure as fertilizer, which is a common practice in organic vegetable production, because applications of mineral nitrogen fertilizers are prohibited. This study was carried out in order to investigate the presence of Enterohemorrhagic *E. coli* O157:H7 (EHEC) in different kinds of leafy vegetables.

II. MATERIALS AND METHODS

A. Samples Collection

Five-hundred leafy green vegetable samples were collected from Jeddah Central Market of Vegetables and Fruits, Jeddah district, western area- kingdom of Saudi Arabia. Samples were obtained covering ten types of leafy green vegetables (Lettuce, Spanish, Rocket Parsley, Mallow, Coriander, Portulaca, Lettuce, Dill, Basil and Chard). All samples were collected freshly in the morning and transported to the laboratory in sterile separate polyethylene bags (20 cm × 35cm).

B. Samples Preparation

Damaged and diseased particles were removed from leafy green vegetable samples. Chopping board, knife, vegetable trays, were wiped with 70% ethanol. Each sample were cut into small pieces (1 cm³) under aseptic conditions and then twenty-five grams sub-samples of each leafy green vegetable sample were weighed and transferred into sterile Stomacher bags (tempo bags, Bio Merieux SA), 225 ml of sterile buffered peptone water (BPW) were added automatically by Delumat S (AES CHEMUNEX, Bio Merieux SA) devise (dilution system), All samples were blended in Stomacher 400 (AES CHEMUNEX, Bio Merieux SA) for 2 min.

C. Isolation and Identification of *E. Coli* O157:H7

Primary isolation occurs on modified soy broth by blending 25 grams of the examined samples in 225 ml of modified tryptic soy broth mTSB modified using stomacher at medium speed for one minute and incubated aerobically at 37 °C for 24 h according to [19], [20]. 100μ were cultured on CHROM™ agar *E. coli*. O157: H7 (CHROM TM agar CE75006-Barais - France) and incubated at 37 °C

for 24 hours; *E. coli* O157:H7 had been grown producing pink to mauve colonies.

D. Multiplex PCR for Detection *Intmin*, *Stx1*, *Stx2* and *HylA* Genes in *E. coli* O157:H7

The PCR were carried out to confirm the presence of *intmin*, *stx1*, *stx2* and *hylA* genes in *E. coli* O157:H7 isolates, the amplification was carried out by using primers targeting *intmin*, shiga toxin type, shiga toxin type2 and *hylA* genes. PCR assay included 25 μl final reaction mixture which consisted of 5 μl master mix (intron biotechnology, south Korea) which consisted (taq polymerase, reaction buffer and DNTPs) and 2 μl of each 10 P moll forward and reverse for amplification *Intmin*, shiga toxin type1, shiga toxin type 2 and *hylA* gene (Table I) 5 μl from DNA template and 13 Ml from (Ddh2o) to complete the volume to 25 ml final reaction mixture and then incubated in Thermal Cycler. The cycling program was performed with an initial denaturation for 5 min at 94 °C then 35 cycles of denaturation for one min at 94 °C, annealing for 1min at 55 °C and extension for 1 min at 72 °C and final extension for 5 min at 72 °C. 1% agarose gel electrophoresis was prepared with 1.5 μl from ethidium bromide, 5 ml from amplified product were electrophoreses and visualized under UV Tran illuminator.

TABLE I: DETAILS OF PRIMERS USED FOR PCR

Specificity Amplicon	Oligonucleotides sequence (5' – 3')
<i>intmin</i>	F.GTGGCGAATACTGGCGAGACT
	R.CCCCATTCTTTTCACCGTCG
Shiga toxin type1	F.CAACACTGGATGATCTCA
	R.CCCCCTCAACTGCTAATA
shiga toxin type2	F.ATCAGTCGTCACACTGGT
	R.CTGTCTGCTGTCACAGTGACAAA
<i>hylA</i> genes	F.ACGATGTGGTTTATTCTGGA
	R- CTTACCGTGACCATACATAT

E. Antibiotic Sensitivity Test

Antimicrobial susceptibility of the isolated *E. coli* O157:H7 was carried out by disc diffusion method on Mueller-Hinton agar using commercial antibiotics (μg/disc) [21]. Results were recorded by measuring the inhibition zones and scored as susceptible or resistant according to the recommendations of the Clinical and Laboratory Standards. Antibiotics used in this study were Methicillin (5 μg), Chloramphenicol (30 μg), Gentamicin (10 μg), Amoxicillin (25 μg), Streptomycin (10 μg), Amikacin (30 μg), Cephalexin (30 μg), Metronidazole (5μg), Tetracycline (30 μg), Ampicillin (10 μg), Doxycycline (30 μg), Ciprofloxacin (5 μg) and Norfloxacin (10 μg).

III. RESULTS

A total of 12 (2.40%) of *E. coli* O157:H7 was isolated from 500 samples of leafy vegetables in which the highest percentage of the isolation was shown with Dill and Chards as both of them showed isolation of *E. coli* O157:H7 as (4.2%) of the examined samples, Coriander (3.33%), Mallow (3.03%), Parsley (2.43%), both Portulaca and Lettuce showed positive isolation of (1.9%) of the collected samples while the lowest percentage (1.7%) was isolated

from Rocket, no *E. coli* O157:H7 was detected in Spinach and Basil as shown in (Table II). According to the region of the collection the highest percentage of the isolation of *E. coli* O157:H7 was shown in samples collected from Makkah area where seven out of 12 were shown positive isolation of *E. coli* O157:H7 (58.33%) as shown in Table III.

A. Multiplex PCR Detection for Genes in *E. coli* O157:H7 Isolates

Ten virulence genes were detected from the seven *E. coli* O157:H7 isolates as followed, Stx2 (110 bp) gene was detected in three isolates of *E. coli* O157:H7 (42.85%), Stx1 (349 bp) gene was detected in four isolates of *E. coli* O157:H7 (57.14%), hly A gene (165 bp) was detected in three isolates of *E. coli* O157:H7 (42.85%) as shown in Table III. Three isolates of *E. coli* O157:H7 (42.85%) out of seven positive isolates showed positive detection for the two genes namely (stx1/ stx2, stx2/hlyA and stx1/hlyA). Four isolates of *E. coli* O157:H7 (57.14%) out of seven positive isolate showed positive detection for the only one gene (stx1). All isolates showed negative results for intimin gene (890 bp), as shown in Table IV and Fig. 2.

B. Antibiotics Susceptibility of Isolated *E. coli* O157:H7

This study showed that all isolates were resistance to, Ampicillin (10 µg), Metronidazole (5 µg) and Methicillin (5 µg), followed by Amoxicillin 25 µg (72.7%), Tetracycline, 30 µg (36.4%), Cephalexin, 30 µg (27.3%), Doxycycline, 30 µg (27.3%), Ciprofloxacin, 5 µg (18.2%) and Streptomycin 10 µg (18.2%) the resistance pattern of the isolates were shown in Table V and Fig. 4. The same table and figure showed that all the isolates (100%) were sensitive to (Amikacin 30 µg and chloramphenicol 30 µg) followed by Gentamicin 10 µg (90.9%), Ciprofloxacin 5 µg (81.8%), Doxycycline 30 µg (72.7%), Norfloxacin 10 µg (72.7%), Streptomycin 10 µg (63.6%), Tetracycline 30 µg (45.5%).

TABLE II: NUMBER AND PERCENTAGES OF *E. COLI* O157:H7 ISOLATED FROM LEAFY GREEN VEGETABLES SAMPLES USING CHROMAGARTM MEDIA

Samples	NO of sample collected	NO and % of isolated <i>E. coli</i> O157:H7
Spinach	33	00(00.00%)
Rocket	60	01(01.70%)
Parsley	82	02(02.43%)
Mallow	66	02(03.03%)
Coriander	60	02(03.33%)
Portulaca	52	01(01.92%)
Lettuce	52	01(01.92%)
Dill	24	01(04.20%)
Chards	48	02(04.20%)
Basil	23	00(00.00%)
	500	12(02.40%)

TABLE III: NUMBER AND PERCENTAGE OF THE ISOLATION OF *E. COLI* O157:H7 FROM SOME REGIONS IN KSA

Regions	NO. and % of <i>E. coli</i> O157 isolates
Madena	04(33.33%)
Makkah	07(58.33%)
Al-Bahah	01(08.34%)
Total	12

TABLE IV: CHARACTERIZATION OF THE IDENTIFIED *E. COLI* O157:H7 BY MULTIPLEX PCR FROM LEAFY GREEN VEGETABLES

<i>E. coli</i> O157:H7	stx1	stx2	hlyA	stx1 and stx2	stx1/2 and hlyA	stx1 and hlyA	Intimin gene
No of the presence of gene	3	3	1	0	1	0	4
No of absence of gene	9	9	11	12	11	12	8

TABLE V: ANTIBIOTICS SUSCEPTIBILITY OF *E. COLI* O157 ISOLATED FROM LEAFY VEGETABLES OF JEDDAH CENTRAL MARKET

Antimicrobial agent	Conc. mcg/disc	Sensitive%	Intermediate %	Resistant %
Methicillin	5 µg	0(00.0%)	0(00.0%)	11 (100%)
Chloramphenicol	30 µg	11(100%)	0(00.0%)	0(00.0%)
Gentamycin	10 µg	10(90.9%)	1(09.1%)	0(00.0%)
Amoxicillin	25 µg	02(18.2%)	1(09.1%)	8(72.7%)
Streptomycin	10 µg	07(63.6%)	2(18.2%)	2(18.2%)
Amikacin	30 µg	11(100%)	0(00.0%)	0 (00.0%)
Cephalexin	30 µg	01(09.1%)	7 (63.6%)	3(27.3%)
Metronidazole	5 µg	00(00.0%)	0(00.0%)	11 (100%)
Tetracycline	30 µg	05(45.5%)	2(18.2%)	4(36.4%)
Ampicillin	10 µg	00(00.0%)	0(00.0%)	11 (100%)
Doxycycline	30 µg	08(72.7%)	0(00.0%)	3(27.3%)
Ciprofloxacin	5 µg	09 (81.8%)	0(00.0%)	2(18.2%)
Norfloxacin	10 µg	08(72.7%)	3(27.3%)	0(00.0%)

Fig. 1. Multiplex PCR for detection intimin gene, shiga toxin type1 gene, shiga toxin type2 gene and hlyA gene in *E. coli* O157:H7 isolates.

Showed lane (M) marker (100 bp), lane (1) mixed positive control for intimin gene, shiga toxin type1, shiga toxin type 2 and hlyA gene, lane (2) and (3) positive sample for hlyA gene (165 bp), lane (4) positive sample for shiga toxin type1 (110 bp), lane (5) positive sample for shiga toxin type1, shiga toxin type 2(110 bp) and (349 bp), lane (6) positive sample for shiga toxin type2 lane (7) positive sample for hlyA gene and lane (8) negative control.

Fig. 2. Presence and absence of virulence genes in *E. coli* O157:H7 isolates from leafy vegetables.

Fig. 3. Antibiotics susceptibility of *E. coli* O157:H7 isolated from leafy vegetables towards antibiotics.

Fig. 4. Resistance pattern of *E. coli* O157:H7 isolated from leafy green vegetables towards antibiotics.

IV. DISCUSSION

E. coli O157:H7 is the most studied strains among all other pathogenic strains of *E. coli* because it has been recognized as the leading causes of human food borne infections throughout the world with fatal complications such as hemolytic uremic syndrome (HUS) that ends in renal failure. This strain can be transmitted to humans by direct and indirect methods such as contaminated vegetables, fruits, and meat. Therefore, our study was carried out to detect the percentage of the isolation, molecular characterization of *E. coli* O157:H7, in leafy green vegetables samples collected from Jeddah central markets, Saudi Arabia and presence of virulence genes (*stx1*, *stx2*, *intimin*, *hlyA*). In the present, study 12 out of 500 (2.4%) *E. coli* O157:H7 was isolated from eight out of ten types of green leafy vegetable as (80%). These results were confirmed the finding of [22] who reported that Out of the 60 samples of vegetables, 10 samples (16.66%) were positive for *Escherichia coli* O157:H7, On the other hand [23], found that no *E. coli* O157 was detected in both raw unwashed vegetables and salads also [24] did not detect *E. coli* O157 among all isolated *E. coli* from vegetables similarly [25] found that the percentages of the presence of *E. coli* O157 ranged from (0% to 33%) in onions and cabbage. According to the current findings and results of the

previous studies carried out in the area, *E. coli* O157 is very rare.

In our study three types of virulence genes (*stx1*, *stx2* and *hlyA* genes) were detected from the seven *E. coli* O157:H7 isolates observed as followed, *Stx2* (110bp) gene was detected in three isolates of *E. coli* O157:H7 (42.85%), *Stx1* (349 bp) gene was detected in four isolates of *E. coli* O157:H7 (57.14%), *hlyA* gene (165 bp) was detected in three isolates of *E. coli* O157:H7 (42.85%) as shown in Table IV. [26] reported that eight samples were positive for presence of *E. coli* O157:H, three contained *stx1*, five contained *stx2*. On the other hand, three isolates of *E. coli* O157:H7 (42.85%) out of seven positive isolates showed positive detection for the two genes namely (*stx1*/ *stx2*, *stx2*/*hlyA* and *stx1*/*hlyA*), four isolates of *E. coli* O157:H7 (57.14%) showed positive detection for the only one gene (*stx1*). All *E. coli* O157:H7 isolates in this study showed negative results for intimin gene (890bp) and this is agreed with [26] who stated that by using a PCR primer conceived for the *eae* (*intimin*) gene detection reported that no positive result was obtained to presence of various *eae* genes among all *E. coli* O157:H7 strains encoding the intimin.

This study showed that all the isolates of *E. coli* O157 (100%) were sensitive to (Amikacin 30 µg and chloramphenicol 30 µg) followed by Gentamicin 10 µg (90.9%), Ciprofloxacin 5 µg (81.8%), Doxycycline 30 µg (72.7%), Norfloxacin 10 µg (72.7%), Streptomycin 10 µg (63.6%), Tetracycline 30 µg (45.5%) and amoxicillin 10 µg (36.4%). While all isolates were showed high rate of resistance to Ampicillin 10µg, Metronidazole 5 µg and Methicillin 5 µg, followed by Amoxicillin 25 µg (72.7%), Tetracycline, 30 µg (36.4%), Cephalexin, 30 µg (27.3%), Doxycycline, 30 µg (27.3%), Ciprofloxacin 5 µg (18.2%) and Streptomycin 10 µg (18.2%) this results agreed with [27] who found that isolated *E. coli* O157 showed resistance against Ampicillin (100%), gentamicin (90.47%), tetracycline (85.71%), and ciprofloxacin (71.42%) and [22] reported that all identified isolates of *E. coli* O157:H7 were resistant to more than five antibiotics and the high rate of resistance was observed with amoxicillin.

In this study it was clear that the isolated *E. coli* O157:H7 were resistance to more than three antibiotics and can considered as MDR organism, this could be due to environmental contamination with antibiotic residues in agriculture processes and the use of antibiotics as a results of poor monitoring by regulatory bodies.

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