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Detection of Six *E. coli* O157 Virulence Genes in Water Samples Using Multiplex PCR

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ESCHERICHIA COLI O157 strains have emerged as important human enteric pathogens. *E. coli* O157 strains may be transmitted in a variety of ways, including drinking water, recreational water and wastewater. One hundred and seventy five water samples were collected from different water sources from June 2010 to July 2011 and examined for classical bacterial indicators (total bacterial counts at 37^{o}C and 22^{o}C, total coliforms, fecal coliforms and fecal streptococci) and *E. coli* O157. Total coliforms (TC), fecal coliforms (FC) and fecal streptococci (FS) among the collected water samples were 83, 76 and 76 out of 175 (MPN- index/100 mL) with incidence ratio of 47%, 43% and 43%, respectively. *Escherichia coli* O157 was detected in water samples using HiCrome EC O157:H7 agar and multiplex PCR targeting six virulence genes {*stx1* (Shiga toxin 1 gene), *stx2* (Shiga toxin 2 gene), *eae* (intimin gene), *hlyA* (hemolysin gene), *rfbE* (O157 antigen gene), and *fliC* (flagellar antigen gene)}. The sensitivity test showed that the multiplex PCR amplified genes with a minimum of 100 CFU of *E. coli* O157. Conventional method using HiCrome media indicated that 57 out of 175 examined water samples (32%) contained *E. coli* O157. The multiplex PCR indicated that, 60 water samples were positive for at least one of the six targeted virulence genes. The most prevalent virulence genes in *E. coli* O157 isolates were Shiga toxin 2 gene (*stx2*) (98%), intimin gene (*eae*) (98%) and O157 antigen gene (*rfbE*) (98%) followed by Shiga toxin 1 gene (*stx1*) (84%) then flagellar antigen gene (*flic*) (66%) while Hemolysin gene (*hlyA)* (0%) was not detected in any *E. coli* O157 isolates.

Keywords: *E. coli* O157, Virulence gene, Multiplex PCR, Water.

Fecal contamination is responsible for the presence of pathogens in natural environment, among which *E. coli*, enteric bacteria, can survive for long periods in the intestinal tracts of warm-blooded animals and also in other environments as faeces (Wang *et al.,* 1996), surface water (Akashi *et al.,* 1994), wastewater (Vernozy-Rozand *et al.,* 2002) and in biofilm (Castonguay *et al.,* 2006). Coliforms (including *E. coli*) are used as bacterial indicators of fecal contamination of food and water; it can even be representative of the global presence of pathogenic bacteria (Edberg *et al.,* 2000).

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Escherichia coli O157:H7 causes a wide spectrum of human diseases, including bloody and non-bloody diarrhea, hemorrhagic colitis, occasional kidney failure, hemolytic uremic syndrome (HUS) and death at times (Shelton & Karns, 2001).

Infection with *E. coli* O157:H7 can be caused by the ingestion of meat (Willshaw *et al.,* 1994), and uncooked fruits and vegetables (Pebody *et al.,* 1999)**.** An outbreak of *E. coli* O157:H7 infections through drinking water was first reported in the USA in 1989 (Swerdlow *et al.,* 1992) since then *E. coli* O157 contamination of drinking and recreational water has emerged as important cause of human disease (Chalmers *et al.,* 2000). The presence of *E. coli* O157 in drinking water offered to livestock contributes also to the prevalence of infection in animals and may lead to the contamination of meat products and the environment (Elder *et al.,* 2000).

Conventional pathogen detection methods depending on coliform assays are well known for their technical limitations. So, the introduction of PCR technique has allowed for various new approaches in water-borne pathogen research because of its high sensitivity, specificity, and speed as well as the culture independent assay capacity (Lee *et al.,* 2006).

Many detection methods of *E. coli* O157 have been employed to rapidly detect low levels of these pathogens in food, beverages and water. Techniques used include traditional enrichment and plating methods with selective media such as Sorbitol MacConkey agar and Rainbow agar (Manafi & Kremsmaier, 2001 and Meng *et al.,* 2001). *Escherichia coli* O157 is particularly difficult to confirm from enrichment cultures due to the problem of high background levels of competing microorganisms including other serotypes of *E. coli*, so a variety of immunological methods have been developed for the detection and enumeration of *E. coli* O157 whole bacteria (Park & Durst, 1999).

A number of PCR based assays have been developed for detection of *E. coli* O157 in food, water and feces. Some of these assays have targeted only Shiga toxin genes (*stx*) (Witham *et al.,* 1996). Gannon *et al.* (1992) developed a duplex PCR procedure to detect Shiga toxin 1 (*stx1*) and Shiga toxin 2 (*stx2*) genes. Moreover, several multiplex PCR procedures have been developed to detect different combinations of the major virulence genes. Fagan *et al.* (1999) included intimin gene (*eae*) and hemolysin gene (*hlyA*) to form a four gene PCR reaction to detect *eae, hlyA, stx1* and *stx2* genes**.** Fratamico *et al.* (2000) developed five gene multiplex PCR procedures to detect different combinations of *fliC, stx1, stx2, eae, hlyA* and *rfbE*. Two separate multiplex PCR procedures, described by Gannon *et al.* (1997) and Fagan *et al.* (1999) were routinely used**,** to identify the five genes, *eae, stx1, stx2, hlyA* and *fliC.* Bai *et al.* (2010) developed a multiplex PCR procedure that can detect six virulence genes (*fliC, stx1, stx2, eae, rfbE* and *hlyA*) of *E. coli* O157. So this study was aimed to detect *E. coli* O157 using both multiplex PCR and HiCrome EC O157:H7 agar from water samples. In addition to, characterize *E. coli* O157 using multiplex PCR targeting six virulence genes (*fliC, stx1, stx2, eae, rfbE* and *hlyA*) to determine the most frequent virulence gene in Egyptian aquatic environment.

Materials and Methods

Sampling sites

One hundred and seventy five water samples were collected from different water sources in Egypt having a wide range of microbial load during the period June 2010 to July 2011, the samples included: 50 surface water samples from 10 sites 2 km along the River Nile (Rossita Branch) (around mixing point with El-Rahawy Drain), 20 water samples from El-Rahawy Drain along 5 km, ten raw wastewater samples from El-Kasr El- Aini Hospital, 40 untreated groundwater samples (New Valley Governorate), 40 treated groundwater samples (Kalubyia Governorate) and fifteen sea water samples from Mediterranean Sea at Marsa Matroha Governorate. Depending on water source, a quantity of 1 L- 20 L volume water samples were collected in sterile sampling bottles, then transferred to laboratory at National Research Center (NRC) within 1-8 hr in ice box.

Microbiological examination

Detection and enumeration of classical bacterial indicators; total bacterial counts (TBC), TC, FC and FS were carried out according to American Public Health Association (APHA, 2005) using pour plate technique for TVBC and multiple tube fermentation technique (MTF) for TC, FC and FS. The detection of *E. coli* O157 was carried out using HiCrome EC O157:H7 selective agar base (HiMedia, India) plates supplemented with novobiocin and potassium tellurite (HiMedia, India). Typical colonies of *E. coli* O157 isolates were confirmed by subculturing on HiCrome MacConky Sorbitol agar base (HiMedia, India) supplemented with tellurite and cefixime (HiMedia, India) and incubated at 37^{o}C for 24 hr. Indole and oxidase tests (APHA, 2005) were also performed to confirm the *E. coli* O157 isolates**.**

PCR sensitivity test on E. coli O157:H7

The PCR sensitivity test was carried out using *E. coli* O157:H7 (ATCC 35150) obtained from *VACSERA Co*., Egypt as a positive control. The positive control was centrifuged at 3000 rpm for 15- 20 min. The pellets were resuspended in phosphate buffered saline (PBS), two portions of 10-fold serial dilutions (from 10^{-1}- 10^{-9}) were prepared. Each portion was tested using multiplex PCR and another portion counted using standard plate count agar.

PCR specificity test on E. coli O157:H7

The primer specificity of multiplex PCR was carried out using *E. coli* O157:H7 (ATCC 35150) as positive control. While negative controls included *E. coli* (ATCC 25922), *Enterobacter cloacae, Klebsiella pneumonia, Proteus mirabilis, Listeria monocytogenes* (ATCC 25152), *Salmonella enterica* serovar Typhimurium **(**ATCC 14028), *Pseudomonas aeurgniosa, Staphylococcus aureus* and *E. coli* all negative controls were obtained from Bacteriology Lab., Water Pollution Research Department, National Research Center, Egypt. Both postive and negative controls were incubated at 37^{o}C for 18- 24 hr in tryptic soya broth (TSB), then centrifuged at 3000 rpm for 15- 20 min; washed twice by sterile PBS pellets resuspended in 1mL of PBS, DNA extracted and multiplex PCR carried out according to the procedure described below.

DNA extraction of E. coli O157 isolates

The DNA extraction of *E. coli* O157 isolates was carried out according to Bai *et al.* (2010), One colony of each isolate was suspended in one mL of sterile distilled water and boiled for 10 min then preserved in ice for 5 min. After centrifugation at 12000 rpm for 10 min, 300- 500 µL of supernatant transferred to eppendorf, 5 µL of supernatant were used as template in multiplex PCR reaction.

Preparation of water samples for PCR

Water samples were filtered with nitro-cellulose membrane (0.2 µm pore size and 47 mm in diameter (Whattman Co.)). The membrane filter was transferred to 10 mL TSB with 10% glycerol plate. Plates were incubated overnight at room temperature with gently shaking. DNA extractions were carried out according to the methods described by Kapperud *et al*. (1993) and Waage *et al.* (1999).

Selection and synthesis of primers

Detection of *E. coli* O157 by multiplex PCR was carried out according to many authors (Table 1) using primers targeting six virulence genes of *E. coli* O157 as follow, *stx1* (Shiga toxin 1 gene), *stx2* (Shiga toxin 2 gene), *eae* (intimin gene), *hlyA* (hemolysin gene), *rfbE* (O157 antigen gene) and *fliC* (flagellar antigen gene) in one microtube. The primers of this study were synthesized by Bio-Basic Inc., Canada.

PCR condition optimization

Multiplex PCR conditions were carried out according to Bai *et al.* (2010); however no DNA amplification were carried out to obtain PCR products, so different conditions were used to reach an optimal condition. Multiplex PCR in this study was used in two ways; first one to confirm and characterize the suspected colonies of *E. coli* O157, another way directly detect *E. coli* O157 in water samples. All PCR reactions were performed in TC-S thermal cycler (BOECO, Germany). Multiplex PCR was first tested individually on *E. coli* O157 (ATCC 35150). Factors tested include; primer concentration over a range of 0.1- 0.5 µM., the dNTPs concentration over a range of 200- 400 µM., annealing temperatures over a range 50- 65^{o}C, Taq DNA polymerase concentration over a range 2- 4 units and PCR cycle number over a range 25- 35 cycles. After a series of tests, the following optimal conditions for the six gene multiplex PCR procedure were established as: reaction volume of 50 µL consisting of 5 µL of DNA template and 45 µL master mix (BioFlux) from 5 µL of 10X PCR buffer (containing 7.5 mM of $MgCl_2$, 50 mM of KCl, 20 mM of Tris–HCl (pH 8.4)), 0.5 µL from each primer (mixture of equal amount of the 100 mM primer stocks), 250 µM of dNTPs, and 4 units of Taq DNA polymerase (Bio-Rad, CA). The PCR program used after optimization was: 94°C denaturation for 3 min, 35 cycles of 94°C denaturation for 30 s, 60°C annealing for 30 s, 72°C extension for 75 s, and a final step of 72°C extension for 5 min. The amplified DNA was separated on 2% agarose gel and stained with 0.5 µg/mL of ethidium bromide with Ladder ΦX174 DNA/HaeIII digest (TOYOBO, Japan). The DNA bands were visualized and documented with a GelDoc UVP Fluorescent Imaging System (UVP, UK).

TABLE 1. Primers sequence and six target virulence genes for detection of *E. coli* O157 by multiplex PCR

Primers name	Target gene	Primer sequence (5′ to 3′)	Tm °C	product size	Reference
fliC-F	flagellar antigen	AGC TGC AAC GGT AAG TGA TTT	56.0	949 bp	Wang *et al.* (2000)
fliC-R		GGC AGC AAG CGG GTT GGT C	64.0		
Stx1-F	Shiga toxin 1	TGT CGC ATA GTG GAA CCT CA	57.8	655 bp	Bai *et al.* (2010)
Stx1-R		TGC GCA CTG AGA AGA AGA GA	57.8		
Stx2-F	Shiga toxin 2	CCA TGA CAA CGG ACA GCA GTT	59.9	477 bp	Fagan *et al.* (1999); Bai *et al.* (2010)
Stx2-R		TGT CGC CAG TTA TCT GAC ATT C	58.2		
eae-F	intimin	CAT TAT GGA ACG GCA GAG GT	57.8	375 bp	Bai *et al.* (2010)
eae-R		ACG GAT ATC GAA GCC ATT TG	55.7		
rfbE-F	O157 antigen	CAG GTG AAG GTG GAA TGG TTG TC	61.5	296 bp	Bertrand and Roig (2007)
rfbE-R		TTA GAA TTG AGA CCA TCC AAT AAG	55.1		
hly4-F	hemolysin	GCG AGC TAA GCA GCT TGA AT	57.8	199 bp	Bai *et al.* (2010)
hly4-R		TGC GCA CTG AGA AGA AGA GA	57.8		

Where: R- Reverse, F- Forward

Results

Sensitivity and specificity test

The sensitivity of multiplex PCR was assessed using serial dilution of *E. coli* O157:H7 (ATCC 35150) suspended in PBS (Table 2). The detection limit of *E. coli* O157 (showing clear PCR product) by multiplex PCR was 100 CFU. Dilutions from stock to 10^{-4} showed four PCR positive products namely Shiga toxin 1 gene (*stx*1), Shiga toxin 2 gene (*stx*2), intimin gene (*eae*) and O157 antigen gene (*rfbE*), respectively. Further dilutions from 0^{-5} to 10^{-7} revealed the disappearance of Shiga toxin 1 gene (*stx*1) only, whereas the last two dilutions (10^{-8} and 10^{-9}) lacked all PCR products (Fig. 1).

The specificity of multiplex PCR was carried out using both positive and negative controls. PCR products of positive control (Lane 2) showed bands of Shiga toxin 2 gene (477 bp), intimin gene (375 bp) and O157 antigen gene (296 bp). On the other hand, the negative controls strains showed no PCR products for any of the negative controls (Fig. 2).

TABLE 2. *E. coli* O157:H7 cell number limits for PCR detection .

Dilution	Average CFU/mL	PCR
Stock	TNTC	+
10^{-1}	TNTC	+
10^{-2}	$8.9x10^4$	+
10^{-3}	$2.1x10^4$	+
10^{-4}	$6.3x10^3$	+
10^{-5}	$2.1x10^3$	+
10^{-6}	$2.1x10^2$	+
10^{-7}	$1.0x10^2$	+
10^{-8}	25	-
10^{-9}	Zero	-

TNTC: Too Numerous To Count

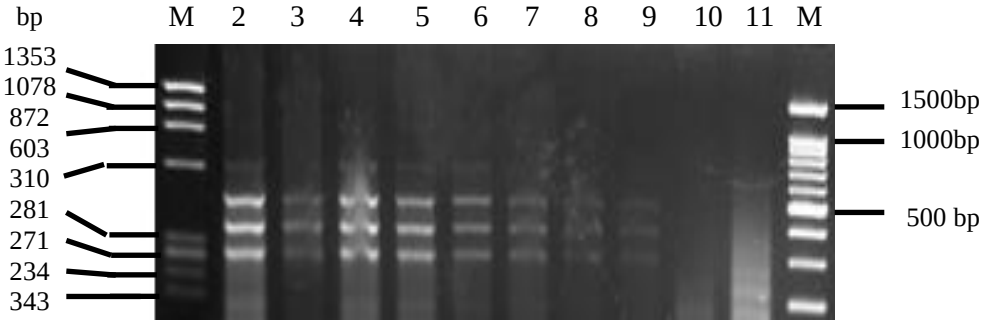

Fig. 1. Sensitivity test of multiplex PCR, Lane M: ØX 174 ladder, Lane 2: Stock, Lanes 3- 11: serial dilution from 10^{-1}- 10^{-9}, lane M: 100 bp DNA Ladder RTU.

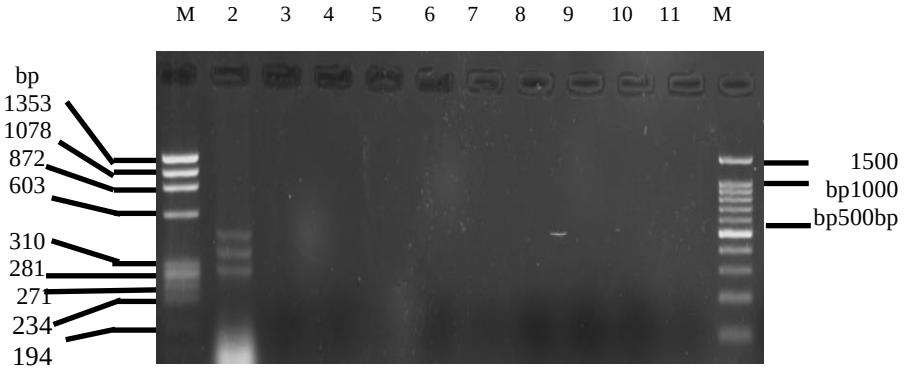

Fig. 2. Specificity test of multiplex PCR.

Lane M: ØX174 ladder, Lane 2: positive control *E. coli* O157 (ATCC 35150), Lane 3: *E. coli* (ATCC 25922), Lane 4: *Enterobacter cloacae*, Lane 5: *Klebsiella pneumonia*, Lane 6: *Proteus mirasbilis*, Lane 7: *Listeria monocytogenes* (ATCC 25152), Lane 8: *Salmonella* Typhimurium **(**ATCC 14028), Lane 9: *Pseudomonas aeurgniosa*, Lane 10: *Staphylococcus aureus*, Lane 11: *E. coli*, Lane M: 100 bp DNA Ladder RTU.

Detection of bacterial indicators and E. coli O157 in different water samples

Results in Table 3 show the range and average counts of bacterial indicators; total viable bacterial counts at 37^{o}C and 22^{o}C, total coliforms, fecal coliforms and fecal streptococci (MPN-index/100 mL) and the range and average counts of the selected pathogenic *E. coli* O157 (CFU/100 mL) using HiCrome EC O157 medium. The counts varied according to water source, the highest counts were found in El-Rahawy Drain for bacterial indicators and *E. coli* O157 followed by River Nile, then hospital wastewater. Both TC and FC were detected in untreated ground water but FS and *E. coli* O157 were not detected. Whereas the treated ground water and sea water showed absence of TC, FC, FS and *E. coli* O157. *E. coli* O157 was detected in 57 out of 175 examined water samples (32%) using HiCrome EC O157 medium while the multiplex PCR indicated that, 60 out of 175 examined water samples (34%) were positive for at least one of the six targeted virulence genes (Table 4).

In River Nile water; the prevalence of TC, FC, FS and *E. coli* O157 were 50 (100%), 45(90%), 46 (92%) and 32 (64%), respectively. From the multiplex PCR results, it was found that, *E. coli* O157 was positive in 35 (70%), the presence of six virulence genes (*flic, stx1, stx2, eae, rfbE* and *hly*) was 0 (0%), 10 (20%), 25 (50%), 23 (46%), 15 (30%) and 0 (0%), respectively (Table 4).

In case of El- Rahawy drain samples, the prevalence of TC, FC, FS and *E. coli* O157 was 20 out of 20 (100%). The multiplex PCR results confirmed such prevalence of *E. coli* O157 was detected in 20 out of 20 water samples with prevalence percentage 100% and the presence of six virulence genes (*flic, stx1, stx2, eae, rfbE* and *hly*) was 1 (5%), 8 (40%), 17 (85%), 19 (95%), 15 (75%) and 1 (5%), respectively (Table 4).

TABLE 3. Range and average values of bacterial indicators and *E. coli* O157 from different water sources.

Water source	No. of samples	Total bacterial counts (CFU/ml)		MPN-index/100 ml			(CFU/100 ml) *E. coli* O157
		37°C	22°C	Total coliforms	Fecal coliforms	Fecal streptococci	
River Nile	50	30- $8.4x10^5$ $1.7x10^5$	70- $9.6x10^5$ $1.4x10^5$	30- $4.6x10^5$ $5.8x10^4$	30- $4.6x10^5$ $3.3x10^3$	30- $1.1x10^6$ $4.0x10^4$	4.0- $7.3x10^3$ $3.1x10^2$
El-Rahawy Drain	20	$1.1x10^4$-$4.4x10^6$ $8.5x10^5$	$1.2x10^4$-$8.0x10^6$ $1.6x10^6$	$3.0x10^3$-$1.1x10^6$ $3.0x10^5$	$3.0x10^3$-$1.1x10^6$ $1.8x10^5$	$7.0x10^3$-$1.5x10^6$ $3.3x10^5$	14- $9.6x10^3$ $2.5x10^3$
Hospital wastewater	10	$4.0x10^3$-$2.0x10^5$ $6.4x10^4$	$1.6x10^3$-$3.0x10^5$ $5.9x10^4$	$1.5x10^3$-$4.8x10^4$ $1.5x10^4$	$1.4x10^2$-$4.8x10^4$ $8.6x10^3$	30- $2.1x10^3$ $6.2x10^2$	98- $1.7x10^3$ $2.6x10^2$
Untreated ground water	40	1.0- $1.1x10^3$ 152	2.0- $1.2x10^3$ 180	1.0- 3.0 0.125	1.0 0.025	ND	ND
Treated ground water	40	1.0- 22 7	2.0- 28 10	ND	ND	ND	ND
Sea water	15	$1.0x10^2$-$5.2x10^3$ $9.2x10^2$	$1.0x10^2$- $5.6x10^3$ $1.4x10^3$	ND	ND	ND	ND

ND: Not Detected

TABLE 4. Number and percentage of positive samples for TC, FC, FS and *E.coli* O157 by culture method and multiplex PCR from water samples.

Water source	No. of samples	TC	FC	FS	*E. coli* O157 by culture	*E. coli* O157						
						Direct multiplex PCR	Multiplex PCR					
							fliC	*stx1*	*stx2*	*eae*	*rfbE*	*hly*
River Nile	50	50 (100%)	45 (90%)	46 (92%)	32 (64%)	35 (70%)	0 (0%)	10 (20%)	25 (50%)	23 (46%)	15 (30%)	0 (0%)
El-Rahawy Drain	20	20 (100%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)	1 (5%)	8 (40%)	17 (85%)	19 (95%)	15 (75%)	1 (5%)
Hospital wastewater	10	10 (100%)	10 (100%)	10 (100%)	5 (50%)	5 (50%)	0 (0%)	0 (0%)	5 (50%)	5 (50%)	5 (50%)	0 (0%)
Untreated ground water	40	3 (7.5%)	1 (2.5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Treated ground water	40	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Sea water	15	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total samples	175	83 (47%)	76 (43%)	76 (43%)	57 (32%)	60 (34%)	1 (0.5%)	18 (10%)	47 (26%)	47 (26%)	32 (18%)	1 (0.5%)
E. coli O157 isolates	45				42 (93%)	44 (98%)	30 (66%)	38 (84%)	44 (98%)	44 (98%)	44 (98%)	0 (0%)

Percentage was calculated according to each type of examined water samples.

In hospital wastewater samples, the presence of TC, FC and FS was detected in 10 out of 10 (100%) wastewater samples and *E. coli* O157 was positive in 5 out of 10 (50%) using both culture method and multiplex PCR with only three detected virulence genes (*stx2, eae* and *rfbE*) (Table 4).

Escherichia coli O157 was not detected in untreated, treated ground water and sea water either by the conventional cultivation methods or by PCR technique (Tables 3, 4). Generally, the occurrences of TC, FC and FS in the collected water samples were 83, 76 and 76 with incidence of 47%, 43% and 43%, respectively. *E. coli* O157 was detected in 57 (32%) and 60 (34%) by cultural methods and multiplex PCR, respectively. From the multiplex PCR results, the characterizations of six virulence genes (*flic, stx1, stx2, eae, rfbE* and *hly*) were: 1 (0.5%), 18 (10%), 47 (26%), 47 (26%), 32 (18%) and 1 (0.5%), respectively. The most dominant virulence gene in different water samples was the Shiga toxin 2 gene (*stx*2) and the intimin gene (*eae*) followed by O157 antigen (*rfbE*) gene, Shiga toxin 1 gene (*stx*1) then flagellar antigen (*flic*) gene and hemolysin (*hly*) gene (Table 4). Fourty- five random *E. coli* O157 isolates were confirmed as; indole positive, oxidase negative and non-sorbitol fermenting. The confirmed isolates were regarded as presumptively positive *E. coli* O157. Using biochemical and multiplex PCR, 42 (93%) and 44 (98%), respectively were further confirmed as *E. coli* O157 (Table 4 and Fig. 3). The characterization of 44 *E. coli* O157 isolates by multiplex PCR was: 30 (66%), 38 (84%), 44 (98%), 44 (98%), 44 (98%) and 0 (0%) positive for six virulence genes (*flic, stx1, stx2, eae, rfbE* and *hly*), respectively.

Sequence analyses of six PCR positive *E. coli* O157 showed that the most frequent strains were *E. coli* O157:H7 strains (acc.no.NZ DS 571135.1 and NZ KB 453139.1) with high homology.

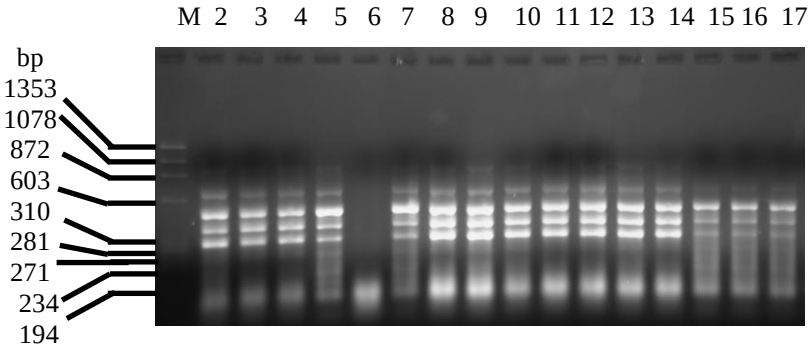

Fig. 3. Multiplex PCR of *E. coli* O157 isolates Lane M: ØX 174 ladder, Lane 2- 5: *E. coli* O157 isolates Lane 6: negative control *Listeria monocytogenes* (ATCC 25152), Lane 7- 17: *E. coli* O157 isolates.

Discussion

Waterborne diseases have been regarded as a major global health problem throughout history. Microbial contamination remains a critical risk factor in water in many parts of the world, municipal sewage become the conduits for the passage of pathogens into surface water (Irvine *el al.,* 1995), *Escherichia coli* O157 is waterborne pathogen that has emerged as a major cause of hemorrhagic colitis and is transmitted to humans either by food or water, it can cause HUS mainly by secretion of Shiga toxins encoded by the virulence genes *stx1* and/or *stx2* and others variants (Bidet *et al.,* 2005). The human infectious dose is very low, and ingestion of amounts as few as 10 cells is thought to be sufficient to cause illness (Chart, 2000).

The last decade has seen a significant increase in the development of PCR techniques for the rapid detection of water-borne human pathogens including *E. coli* O157 (Cupples *et al.,* 2010). PCR reactions are designed to either amplify a single product (Fincher *et al.*, 2009) or to use several primer pairs as part of a multiplex PCR (Campbell *et al.,* 2001). The advantage of multiplex PCR is the capacity to simultaneously detect a number of sequences from the target organism (Osek, 2003 and Duris *et al*., 2009); to detect several pathogenic species in a single sample (Kong *et al.,* 2002) or to detect critical virulence factors, *e.g*. *stx1* and *stx2*, which are involved in the production of the Shiga toxin, or *eae* which codes for intimin (Bopp *et al*., 2003; Osek, 2003; Garcia-Aljaro *et al.,* 2004 and Quilliam *et al.,* 2011). This study used multiplex PCR in comparison with HiCrome EC O157:H7 agar to detect *E. coli* O157 in different water samples. In addition to, characterize *E. coli* O157 using multiplex PCR through targeting six virulence genes (*fliC, stx1, stx2, eae, rfbE* and *hlyA*) in order to determine the most frequent virulence gene in Egyptian aquatic environment. With regard to the results of bacterial indicators, TC, FC and FS were detected in 83, 76 and 76 out of 175 water samples, respectively, these data revealed a high incidence of microbial load along El-Rahawy Drain and River Nile (Rossita Branch). A possible explanation that El- Rahawy Drain receives agriculture wastewater from surrounding fields in El-Rahawy village beside that, it receives large amount of treated wastewater from Zenin and Abou-Rawash wastewater treatment plants from Greater Cairo that are discharging directly into Rossita Branch. In Egypt, El- Jakee *et. al.* (2009) reported that, 90% of the collected water samples were positive for coliform group reached to > 1800 MPN- index/100 mL and the highest coliforms detection was recorded among samples collected from Baniswaf, Mansoria and Maruotia canals followed by drinking underground water (Gezera El Dahab, El Zomor and Kerdasa), River Nile, agriculture drain, untreated sewage water, treated sewage water and well samples with values of 24, 18, 16, 14, 8, 6 and 4%, respectively.

In the present study the detection limit of *E. coli* O157 was 100 CFU by multiplex PCR (Table 2). *E. coli* O157 was detected in 32% and 34% water

samples using cultural methods and multiplex PCR, respectively. From the results, it was found that *E. coli* O157 was detected in 57 and 60 out of 176 water samples using HiCrome EC O157 agar medium and multiplex PCR respectively; it means that the multiplex PCR was more sensitive than the cultivation methods in our study. *E. coli* O157:H7 strains (acc.no.NZDS 571135.1 and NZ KB 453139.1) were observed in the sequenced samples it means that *E. coli* O157:H7 was the most frequent in El- Rahawy drain water samples it may be due to receiving agriculture wastewater from surrounding fields in El-Rahawy village beside that, it receives large amount of treated wastewater and also presence of animal, cattles and rodents. The multiplex PCR, results, confirmed the characterization of six virulence genes (*flic, stx1, stx2, eae, rfbE* and *hly*) frequent in water samples were: 1 (0.5%), 18 (10%), 47 (26%), 47 (26%), 32 (18%) and 1 (0.5%), respectively. The most dominant virulence gene was the Shiga toxin 2 gene (*stx2*) and intimin gene (*eae*). Campbell (2001) reported that, a multiplex PCR, which was able to detect viable cells and to distinguish the serotype O157:H7 was used to confirm the presence of *E. coli* O157:H7 in soil and water. Moreover, the author reported that detection limits were 1 CFU mL^{-1} in drinking water and 2 CFU g^{-1} soil. Bai *et al.* (2010) used the same six primers (used in this study) to test 84 cattle fecal and 57 human clinical *E. coli* O157 strains for detecting the following six genes (*fliC, stx1, stx2, eae, rfbE* and *hlyA*). The 84 cattle strains differed only in *stx1* and *stx2* genes, and all possessed the other four genes. Among the cattle strains, 28% had *stx2*, 26% had *stx1*, and 28% had both *stx1* and *stx2*. Similarly all the 57 human strains (100%) possessed *fliC, eae, rfbE* and *hlyA* and differed in *stx1* and *stx2*. Of the 57 human strains, 38% had both *stx1* and *stx2*, 60% had *stx2* and only 2% had *stx1* alone. In the same study, both cattle and human strains were also tested with an O157-specific agglutination (*rfbE*) followed by the two separate multiplex PCR procedures on *fliC, stx1, stx2, eae* and *hlyA* genes. Bai *et al.* (2010) added that, sensitivity tests showed that the procedure amplified genes from a fecal sample spiked with a minimum of 104 CFU/g (10 cells/reaction) of *E. coli* O157. After a 6-h enrichment period of *E. coli* O157 spiked samples, a sensitivity level of 10 CFU/g was achieved.

In Egypt, El-Safey (2001) found specific shiga-like toxin (*stx1* and *stx2*), intimin (*eaeA*) and the enterohemorrhagic *E. coli* hemolysin (*hlyA*) genes in five *E. coli* O157:H7 strains isolated from Egyptian food. Whereas, In South Africa Müller *et al.* (2003) reported a prevalent rate of 20% *E. coli* O157:H7 from sewage and environmental sources.

In France, Bertrand & Roig (2007) used a specific and sensitive PCR assay based on the *rfbE* gene to detect low levels of *E. coli* O157 in wastewater. The set of primers used was designed to amplify an intragenic segment of the *rfbE* gene. The amplification assay detected 200 CFU of *E. coli* O157 in pure water. The prevalence of *E. coli* O157 in the effluents of 44 wastewater treatment plants was determined (7%).

El- Jakee *et al.* (2009) mentioned that, from 14 *E. coli* strains isolated from different water sources in Egypt and characterized by monoplex PCR as follow 8 (57.1%) isolates carried *stx1* and 4 (28.6%) possessed *stx2* gene. Intimin (*eae*), *fliCh*7 and *hly*, virulence genes were detected in 3 (21.4%), whereas *hly* gene was found in 4 (28.6%) of the isolates. In Saudi Arabia, Abulreesh (2011) found that, 2.5% of 400 pigeon fecal samples were positive Shiga toxin- producing *E. coli*.

In conclusion, the cultivation on HiCrome media is considered inexpensive and reliable methods for the detection of *E. coli* O157 in concentrated water sample; samples with low counts (4 CFU/100mL) where easily detected. The multiplex PCR using selected primers targeting virulence genes of *E. coli* O157 offers more accurate and convenient methods for rapid detection of pathogenic *E. coli* and provides a clue for the degree of virulence. Although, the most frequent genes obtained in this study were Shiga toxin 2 gene (*stx*2), Intimin gene (*eae*) and O157 antigen gene (*rfbE*), It is recommended to optimize the PCR conditions to target as many as possible genes under investigation. At last, the type's numbers of the present genes, reflect the severity of the suspected infection.

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الكشف عن ستة جينات شديدة الاصابة فى *الايشريشيا كولاى* O157 فى عينات المياه باستخدام تفاعل البلمره المتعدد

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تعتبر بكتيريا *الايشريشيا كولاى* O157 من أهم مسببات الأمراض المعويه بالنسبة للإنسان. وتنتقل بكتيريا *الايشريشيا كولاى* O157 بطرق مختلفه وذلك من خلال مياه الشرب, والمياه المستخدمه فى الترفيه ومياه الصرف الصحى. لذلك فقد تم تجميع ١٧٥ عينه مياه من مصادر مختلفه خلال الفتره من يونيه ٢٠١٠ إلى يوليو ٢٠١١ لتعيين دلائل التلوث البكتيريه (العد الكلى البكتيرى عند درجتى حرارة ٢٢° م و ٣٧°م , مجموعة القولون الكليه, مجموعة القولون البرازيه ومجموعه السبحيه البرازيه) وأيضاً بكتيريا *الايشريشيا كولاى* O157. فقد اتضح أن مجموعة القولون الكليه, مجموعة القولون البرازيه والمجموعه السبحيه البرازيه قد تم الكشف عنهم فى ٨٣ و ٧٦ و ٧٦ عينه من مجموع الـ ١٧٥ عينه المياه التى تم تجميعها بنسب ٤٧٪ و ٤٣٪ و ٤٣٪ على التوالى. وقد تم أيضاً الكشف عن بكتيريا *الايشريشيا كولاى* O157 فى عينات المياه باستخدام طرق الزرع التقليديه على بيئه الـHiCrome EC O157:H7 agar و باستخدام الـ multiplex PCR للكشف عن مجموعة الجينات السته المسئوله عن شده الاصابه وهم: الجين المسئول عن افراز سموم الشيجا ١ ويسمى *(stx1)*, الجين المسئول عن افراز سموم الشيجا ٢ ويسمى (*stx2*) والجين المسئول عن التصاق بكتيريا *الايشريشيا كولاى* O157 و خلية العائل ويسمى (*eae*) و الهيمولسين جين و جين (O157 antigen gene) (*rfbE*)) و جين ((*flic*)) (flagellar antigen gene). ومن نتائج الـ multiplex PCR فقد وجد أن حساسية هذه الطريقه هى ١٠٠ مستعمره من بكتيريا *الايشريشيا كولاى* O157 للتفاعل الواحد. ويتضح أيضاً من نتائج الزرع التقليديه المستخدمه بيئة الـ HiCrome EC O157:H7 agar- أن ٥٧ عينه من ١٧٥ عينه تم عزل بكتيريا *الايشريشيا كولاى* O157 بنسبة ٣٢ ٪ بينما تم الكشف عن جين واحد من الست الموجوده بكتيريا *الايشريشيا كولاى* O157 على الأقل فى ٦٠ عينه من ١٧٥ عينه وذلك بإستخدام الـ multiplex PCR . ووجد أن اكثر الجينات تواجداً فى عزلات بكتيريا *الايشريشيا كولاى* O157 هى *stx2 بنسبة* (٩٨٪) و (eae) بنسبة (٩٨٪) و (rfbE) بنسبة (٩٨٪) و (stx1) بنسبة (٨٤٪) و(flic) بنسبة (٦٦٪) بينما لم يستدل على الهيمولسين جين بنسبة (٠٪)