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PREVALENCE AND CHARACTERIZATION OF SHIGA-TOXIN O157:H7 AND NON-O157:H7 ENTEROHEMORRHAGIC *ESCHERICHIA COLI* ISOLATED FROM DIFFERENT SOURCES

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ABSTRACT

Shiga-toxin–producing Escherichia coli (STEC) is recognized as an important foodborne pathogen responsible for sporadic cases to serious outbreaks worldwide. The morbidity and mortality associated with several recent outbreaks due to STEC have highlighted the threat this organism poses to public health. This study was conducted to identify, characterize the virulence traits and antibiogram of enterohemorrhagic E. coli (EHEC) from different sources. A total of 384 samples from human, animal and environmental sources were collected from different locations in Ismailia city, Egypt. E. coli isolates (n = 283) were identified by conventional microbiology culture, and phenotypically characterized with biochemical and motility tests. Multiplex PCR (mPCR) was applied for the detection of virulence genes (stx1, stx2, eaeA, and EHEC hlyA). From the overall prevalence of E. coli isolates, 31 % (89/283) were isolated from stools of people with diarrhea; 17.3 % (49/283) were from stools of sheep, cattle and chicken with diarrhea; 16.5 % (47/283) were from urine of peple with a urinary tract infection; 17.3 % (49/283) were from fresh water; 6.4 % (18/283) from seafood; 6.02 % (17/283) from processed meat products; 3.9 % (11/283) from dairy products; and 1.1 % (3/283) from poultry products (liver). The antibiotic sensitivity pattern showed that the isolates carried a multidrug resistance (MDR) phenotype to at least four antibiotics from different classes: erythromycin (E), gentamicin (CN), cefazolin (CZ), thiampinicol (TP), vancomycin (VA), ciprofloxacin (CIP), and ampicillin (AM). Shiga toxin was identified in ten suspected EHEC by mPCR. Serotyping of these 10 E. coli isolates demonstrated the circulation of five serotypes (O157, O158, O114, O125 and O26) (10/283 [4%]): three isolates from people (serotype O157, O158), four isolates from animals (serotype O114, O26), two isolates from meat products (serotype O125, O158), and an isolate from fresh water (serotype O114). This study identified STEC O157 from human cases with diarrhea, and demonstrated that meat and water were contaminated with more than one serotype of non-O157 STEC strains. This is a cause of concern owing to their potential to cause human infections.

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Keywords: enterhemorrhagic, Shiga toxin, *Escherichia coli,* O157, non-O157, diarrhea, food

Abbreviations: CR: Congo red; *eaeA*: intimin gene; EHEC: enterohemorrhagic *E. coli*; EHL: enterohemolysisn; *hlyA*: hemolysin gene; HUS: hemolytic uremic syndrome; MDR: multi-drug resistance; MDRI: multi-drug resistance index; mPCR: multiplex PCR; STEC: Shiga-toxin–producing *E. coli*; *stx1*or *stx2*: Shiga-like toxin genes; UTI: urinary tract infection

Introduction

Enterohemorrhagic *Escherihia coli* (EHEC) is the most important recently emerged group of foodborne pathogens (17, 24). 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 (20), primarily because Shiga-toxin– producing *E. coli* (STEC) are found as part of the normal intestinal flora of animals (23). Person-to-person transmission has also been documented (36, 49). Although, several investigations have been carried out on the laboratory diagnosis of these organisms in recent years, this group still 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 (7, 24, 30, 47). A common characteristic of all EHEC strains is the production of an EHEC-specific plasmid-mediated hemolysin encoded by the *hlyA* gene (52) and at least one Shiga-like toxin (encoded by *stx1*or *stx2*) (47). Many produce intimin, a 97 kDa attachment- and-effacement protein that is encoded by the *eaeA* gene (42).

The present study aimed at investigating the prevalence of STEC strains from different human and environmental

sources in one of the governorates in Egypt. The virulence characteristics and antimicrobial susceptibility were also studied.

Materials and Methods

Sample collection

A total number of 384 samples were collected from different sources in Ismailia City, Egypt. Sampling included various types of food, water and clinical samples represented by stool and urine to survey the prevalence and distribution of EHEC in the collected samples.

Food. A total of 99 food samples were obtained randomly in plastic bags from different local markets. The food products included meat products, poultry products, seafood and dairy products. Twenty-five grams of each sample were combined with 225 mL of modified tryptic soy broth mTSB and incubated with agitation (120 rpm) for 24 h at 37 °C as described (18, 22). After 24 h enrichment, aliquots of 100 µL were plated onto Eosin Methylene Blue (EMB) to presumptively identify isolates as Gram-negative enteric bacteria and *E. coli* (green-metallic colonies), and onto Sorbitol MacConkey agar (SMAC) to test for sorbitol non-fermenting bacteria (colorless colonies). After 18 h to 24 h at 37 °C, characteristic colonies from EMB agar (green-metallic colonies) and SMAC agar were transferred onto Tryptic Soy agar (Difco) for the biochemical identification.

Water. Thirty-one water samples were collected from Ismailia freshwater canal and examined according to the standard methods described by APHA (4). Typical green metallic colonies were introduced into SMAC agar and incubated at 37 °C for 24 h (4).

Stool. Fecal specimens were collected following standard methods described elsewhere (11, 12). Stool specimens were collected at the early stages of enteric illness from patients with diarrhea to ensure that pathogens are usually abundant in the stool and before antibiotic therapy has been started. A fresh stool sample, or a rectal swab from a fresh specimen was collected with a sterile rectal swab and inoculated into Tryptic soy broth transport medium (11). Upon arrival to the lab, samples were streaked onto sorbitol MacConkey agar (Difco, USA) culture plates supplemented with 0.05 mg/L cefixime in order to examine the sorbitol fermentation activity. After 24 h at 37°C, plates were examined and individual *E. coli*-like colonies with a pink/pale color were selected and subjected to further identification using biochemical standard procedures (18).

Urine. Specimens (n = 76, 24 from male patients and 52 from female patients) were collected from the midstream urine (MSU) of symptomatic cases with an urinary tract infection (UTI). Within few hours after collection, all urine samples were examined following standard procedures (32). Urine specimens were diluted with 5 % saline solution, by treating 10 µL of urine with 190 µL of saline solution. Then, 100 µL from each diluted sample was directly used to inoculate EMB culture agar plates. Using a sterile loop, grown bacterial colonies were restreaked onto Cefixime Sorbitol MacConkey agar plates, and incubated for 24 h at 37 °C.

Phenotype characterization

Identification of the isolates. Purified suspected *E. coli*-like colonies (n = 283) were identified by examining the morphology of growing colonies on both EMB agar, and McConkey Agar plates. Gram-staining properties were determined following the procedure described by Merchant and Packer (45), and. *E. coli*-like colonies were subjected to different biochemical tests, including sugar fermentation test, Indole production test, Methyl-Red and Voges–Proskauer (IMIVC) tests, following the standard methods described in Cowan (18).

Antibiotic susceptibility test. The total number of *E. coli* isolates (n = 283) were tested for their susceptibility to 16 antimicrobial agents: augmentin, ampicillin, cefazolin, ciprofloxacin, clindamycin, erythromycin, gentamicin, imipenem, nitrofurantion, nitrofloxacin, penicillin, rifampin, spectinomycin, tetracycline, thiampinicol, vancomycin. The test was performed on Mueller–Hinton Agar and the Kirby–Bauer disk diffusion technique was used (33). The antibiogram of each *E. coli* colony was determined based on the breakpoints of the inhibition zone diameters for individual antibiotic agents and as recommended by the disk manufacturer. The results were interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (16) for antimicrobial susceptibility testing (16). Resistance to more than four antibiotics was taken as MDR. MDRI of individual isolates was calculated by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics to which the isolate was exposed (13):

$$MDRI\,(\%) = \frac{\text{Number of antibiotics resisted}}{\text{Total number of antibiotics used}} \times 100$$

Isolates with MDRI values of over 0.2 % or 20 % were considered highly resistant.

Congo red (CR) binding assay. This test was used to test the individual *E. coli* isolates for their binding activity with the Congo red dye as a marker indicative for intestinal invasion (6, 39, 48). Individual *E. coli* colonies were cultured on Congo red medium and incubated at 37 ºC for 24 h. Culture plates were then transferred at room temperature for additional 24 h incubation. Growth of red colonies indicates Congo-red–positive (CR+) *E. coli* results. Different intensities in the dye uptake were scored as (+), (++) and (+++), while appearance of white colonies (*E. coli* which did not uptake the dye) indicates Congo-red–negative (CR−) *E. coli*.

Enterohemolysin (EHL) production. Detection of hemolysin *E. coli* producers was performed according to the method described in Beutin et al. (8) and Blanco et al. (9). Briefly, individual *E. coli* colonies were grown at 37 ºC for 24 h on tryptose blood agar (Difco) supplemented with 10 mmol/L $CaCl_2$ and 5 % washed defibrinated sheep blood in phosphate

saline. Positive beta-hemolytic reaction was indicated by a completely clear zone under and around the colonies.

Detection of virulence genes

DNA template preparation

Boiling method. DNA lysate was prepared by the boiling method as described by Fragan et al. (24). A 1.0 mL aliquot of L.B culture of each *E. coli* isolate was centrifuged in a 1.5 mL microcentrifuge tube at 7000 rpm for 10 min and the supernatant was discarded. The cell pellet was resuspended in 400 µL of sterile de-ionized water and boiled for 15 min to 20 min, and the insoluble material was removed by centrifugation for 5 min. The supernatant was collected and stored at -20 °C until further testing.

Genomic DNA. Total purified genomic bacterial DNA was extracted from overnight cultures of *E. coli* isolates on Trypticase Soya Agar TSA, with a genomic DNA mini kit (QiAGen, QIAamp, USA) according to the manufacturer's instructions.

PCR assay for virulence genes. The DNA templates of 283 samples were subjected to mPCR for screening of virulence genes *stx1* and *stx2*, *eae A* and *hyl A* genes, with specific primers as described elsewhere (**Table 1**). Positive controls included DNA lysate prepared from clinical specimens positive for *Shigella dysentery* (WS15443); and *eae* and *hlyA*, which were kindly provided by US Naval Medical Research Unit #3 (NAMRU-3) Laboratories, Cairo, Egypt. The production of enterohemolysin was assayed on 75 *E. coli* isolates which had demonstrated a beta-hemolytic phenotype. The invasion capability was tested only on shiga-toxin–producing strains by *eae A* specific primers.

PCR cycle conditions. PCRs for detection of *stx1*, *stx2*, *eae* and *hlyA* genes were performed with commercially manufactured oligonucleotide primers. Primers and the predicted lengths of PCR amplification products are listed in **Table 1**. These primers were chosen because they amplify conserved regions of the target genes and allow single-step identification of amplified DNA fragments that are visualized as stained bands after migration on agarose gel electrophoresis. PCRs were performed in 25 µL reaction mixture containing 2 µL of template DNA, 5 µL of 5x PCR buffer, 4 µL of a 2.5 mmol/L mixture of deoxynucleoside triphosphates, 2 µL of 25 mmol/L $MgCl_2$, 0.25 µL of 5 U Ampli Taq Gold DNA polymerase per microliter, and 2 µL of a 20 µmol/L solution of each primer. The thermo-cycling conditions were done in a Gene Amp PCR system 9700 (AB Applied Biosystems).

Gel electrophoresis. PCR products (5 µL) were evaluated in 2 % agarose gel at 120 mV for 30 min. A molecular marker (1 kb DNA Ladder; Gibco/BRL) was run concurrently. The DNA bands were visualized and photographed under UV light after the gel was stained with ethidium bromide.

Serotyping analysis

Serotyping for somatic "O" antigen of *E. coli* cultures propagated in an 18 h nutrient broth culture at 37 °C was determined commercially in the Clinical Microbiology Reference Unit, Central Health Laboratories (Cairo, Egypt). Slide agglutination test (39) was used, with 8 diagnostic polyvalent and 43 corresponding monovalent anti-coli sera provided by Denka Seiken (Japan). The strains which failed to give agglutination results with any of the available antisera were recorded as non-typeable strains.

Results and Discussion

STEC cause cases of diarrhea, bloody diarrhea and hemorrhagic colitis. STEC infection also causes hemolytic-uremic syndrome (HUS), a life-threatening condition characterized by hemolytic anemia, thrombocytopenia, and renal failure (56). Transmission of STEC occurs through contaminated foods, such as ground beef, through contaminated water and by person-to-person spread (52, 55). In some countries, O157:H7 (O157) is the most common serotype of STEC (26) and is the serotype most often associated with HUS (57). Approximately 150 non-O157 STEC serotypes also cause diarrheal disease (44, 50). Some studies suggest that non-O157 STEC infection causes milder disease than O157 infection (10). Most of the medical significance reports on O157 STEC are published in developed countries, and only a handful of reports are available in developing countries, with nearly no data available on non-O157 STEC from countries in North Africa and the Middle East. The aim of the present study was, first, to identify the frequencies of O157 and non O157 STEC among different human, animal and environmental sources; and, second, to characterize in more details the genetic background and virulence traits of suspected STEC isolates.

A total of 283 *E. coli* isolates were recovered from 384 samples collected from different sources in Ismailia City, Egypt, one of the largest governorates located in the North East of Egypt, on the west bank of the Suez Canal. The prevalence of *E. coli* isolates among the different sources is demonstrated in **Table 2**. The prevailing part of the *E. coli* isolates were obtained from human stools (31.43 %), animal stools (17.3 %), human urine (16.5 %), fresh water (8.0%), and processed meat products (6.1 %), while a lower percent of *E. coli* isolates was from dairy products (3.9 %), and poultry products (1.1 %). Seventy percent (n = 194) of the 283 *E. coli* isolates clearly demonstrated an invasive phenotype on CR agar, while 26 % (n = 75) showed a beta-hemolytic pattern on Blood agar. An invasive pattern was recorded in considerably high ratios: 86 % of the isolates from diarrheic animals, 83 % of those from seafood isolates, 82 % of the isolates from meat products, 77 % of the ones from UTI patients, and 73 % of the isolates from diarrheic patients. The beta-hemolytic phenotype was widespread among the isolates from various food products: poultry (66 %), processed meat (47 %), and seafood (33 %). Twenty-two percent (n = 61) of the isolates shared a common phenotype with respect to the presence of both an invasive pattern on CR and beta-hemolytic activity. Fourteen of these 61 isolates were from human stools, 16 from animal stools, 13 from UTI patients, 6 from fresh water, 10

TABLE 1

Primer sequences of examined virulence genes and predicted lengths of PCR amplification products

Cycle conditions	Product length (bp)	Oligonucleotide sequence (5'–3')	Target Gene
96 °C 5 min, 95 °C 1 min, 55 °C 1 min, 72 °C 1 min for 30 cycles, 72 °C 7 min	349	F-CAACACTGGATGATCTCAG R-CCCCCTCAACTGCTAATA	*stx1*
96 °C 5 min, 95 °C 1 min, 55 °C 1 min, 72 °C 1 min for 30 cycles, 72 °C 7 min	110	F-ATCAGTCGTCACTCACTGGT R-CTGCTGCTGTCACAGTGACAAA	*stx2*
96 °C 5 min, 95 °C 1 min, 48 °C 1 min, 72 °C 1 min for 30 cycles, 72 °C 7 min	165	F-ACGATGTGGTTTATTCTGGA R-CTTCACGTGACCATACATAT	*hlyA*
96 °C 5 min, 95 °C 1 min, 55 °C 1 min, 72 °C 1 min for 30 cycles, 72 °C 7 min	890	F-GTGGCGAATACTGGCGAGACT R-CCCCATTCTTTTTCACCGTCG	*eaeA*

Stx1: Shiga toxin 1: *Stx2*: Shiga toxin 2; *hly*: enterohemolysin; *eae*: intimin

TABLE 2

Prevalence of *E coli* isolates by source, hemolytic activity, Congo red indicator, and virulence gene

Source	Sample type	Number of samples (n = 384)	Isolated *E. coli* (n = 283)	Prevalence of *E. coli* isolate, %	Beta hemolytic activity (n = 75)	Congo red indicator (n = 194)	Virulence genes *stx*1	*stx*1/2	*stx*1+eae	*stx*1/2+*hly*	*hly*A
Fresh water	Fresh canals	31	49	17.3	7 (14.2 %)	19 (39 %)	1				
Human stools	Male	37	38	13.41	5 (13 %)	27 (71 %)					
	Female	27	20	7.1	8 (40 %)	19 (95 %)					
	Children	43	31	10.92	7 (22.5 %)	19 (61.2 %)	1				1
Human urine	Male	24	8	2.82	5 (62.5 %)	8 (100 %)					
	Female	52	39	13.7	9 (23 %)	28 (71 %)	1			1	
Animal stools	Cattle	33	14	4.9	5 (35.7 %)	14 (100 %)	1				
	Calves	20	5	1.78	2 (40 %)	3 (60 %)	1				
	Sheep	9	20	7.1	5 (25 %)	15 (75 %)	1				
	Chickens	9	10	3.55	4 (40 %)	10 (100 %)	1		1		
Meat products	Minced	11	3	1.1	3 (100 %)	2 (66.6 %)					
	Steaks	3	6	2.12	1 (16.6 %)	6 (100 %)					
	Sausage	8	2	0.7	2 (100 %)	1(50 %)	1				
	Kofta	6	2	0.7	1 (50 %)	2 (100 %)	1				
	Burger	8	0	0	0	0					
	Luncheon	8	4	1.4	1 (25 %)	3 (75 %)					
	Liver	4	0	0	0	0					
Poultry products	Livers	6	3	1.1	2 (66 %)	2 (66.6 %)					
	Lamps	8	0	0	0	0					
Seafood	Oysters	9	7	2.5	2 (28.5 %)	6 (85.7 %)					
	Calamari	7	4	1.4	2 (28.5 %)	4 (100 %)					
	Bivalves	7	7	2.5	2 (28.5 %)	5 (71.4 %)					
Dairy products	Raw milk	6	4	1.4	1 (25 %)	1 (25 %)					
	Yogurt	4	4	1.4	0	0					
	Cheese	4	3	1.1	1 (33 %)	0					

TABLE 3

Antibiotic resistance pattern of *E. coli* (n = 283) isolates from different origins

Antimicrobial agent	Resistance pattern									
	N	%	Fresh water	Human stools	Animal stools	UTI	Meat	Poultry	Diary	Seafood
			49 (17.3 %)	89 (31 %)	49 (17.3 %)	47 (16.5 %)	17 (6.02 %)	3 (1.1 %)	11 (3.9 %)	18 (6.4 %)
Augmentin (AMC/30)	141	50	4	52	36	27	11	1	2	8
Ampicillin (AM/10)	211	75	39	55	47	36	15	3	6	10
Cefazolin (CZ/30)	156	55.1	21	49	33	32	9	0	1	11
Ciprofloxacin (CIP/5)	148	52.1	13	53	45	20	6	1	6	11
Clindamycin (DA/2)	161	57	25	51	45	24	9	1	0	6
Erythromycin (E/15)	161	60	27	43	45	30	13	1	7	3
Gentamicin (CN 120)	119	42	24	34	29	20	9	0	0	3
Imipenem (IPM/10)	82	29	14	23	31	7	6	0	0	1
Nitrofurantoin (F/300)	101	36	27	23	16	23	4	2	3	3
Nitrofloxacin (NOR/10)	118	42	23	45	33	9	4	2	0	2
Penicillin (P/10)	180	64	23	51	49	33	13	0	1	10
Rifampin (RA/5)	167	59	22	51	46	27	10	1	0	10
Spectinomycin (SPT/100)	113	40	31	25	31	16	6	0	0	4
Tetracycline (TE/30)	141	50	32	35	42	22	4	0	0	6
Thiamphenicol (TP/30)	153	54	31	40	38	28	9	0	0	7
Vancomycin (VA/30)	195	69	35	46	47	35	15	1	6	10

from meat products, 1 from poultry, and 1 from seafood. These percentages were near to those obtained by other authors (1, 31, 51) who found that a positive Congo red and hemolysin profile were widespread among *E. coli* strains isolated from diseased animals. In our study, most of the CR- and EHL-positive strains were observed in clinical samples isolated from patients with diarrhea and UTI, which indicates the potential of these strains to cause disease and corroborates with findings described elsewhere (29), emphasizing the role of hemolysin-producing *E. coli* in the increased pathogenic potential and the critical role these organisms play in causing extra-intestinal infection.

The antibiogram pattern of the 283 *E. coli* isolates shown in **Table 3** indicates that antibiotic resistance is common among most of the *E. coli* isolates. The results demonstrated that 75 % (n = 211) of the isolates were resistant to ampicillin, followed by 69 % (n = 195) resistant to vancomycin, 64 % (n = 180) to penicillin, and 60 % (n = 169) to erthromycin. The results showed that near 50 % of the tested isolates were augmentin resistant. Less common was the resistance to NOR (36 %) and IPM (28 %). The antibiograms of the *E. coli* strains carrying different virulence genes is given in **Table 4**. Most strains had a significantly high MDRI, demonstrating resistance to at least four antibiotics belonging to different classes: E, CN, CZ, TP, VA, CIP, and AM. Strains from the O157 serotype were sensitive to CIP and NOR, while 30 % of the non-O157 strains were found to be resistant to CIP, and 90 % showed MDR to at least three antibiotics.

The detection of EHEC poses a challenge for clinical microbiology laboratories. Key issues concerning their detection include the need to distinguish EHEC from commensal varieties of *E. coli* (5, 21). The currently accepted method for detection is direct plating on SMAC or other media such as cefixime-SMAC or SMAC supplemented with cefixime and tellurite (CT-SMAC) (51). Culture on SMAC is a simple method for screening for EHEC O157, but it cannot identify non-O157 serotypes. The multiplex PCR approach for detection of *stx1*, *stx2*, *eaeA* and EHEC *hlyA* genes, is advantageous for rapid detection of EHEC pathogenicity factors and is highly recommended. Our study demonstrates that non-O157 STEC strains compose a significant proportion (70 %) of all STEC strains detected. SMAC culture was selected for detection of STEC, and 10 STEC strains primarily appeared as typical *E. coli* colonies (pink-red), and could not be differentiated from other pathogenic or non-pathogenic (commensal) *E. coli* which grow on the same medium. We applied the PCR assay for Shiga- toxin detection and found the assay to be an excellent, sensitive, and specific tool for accurate detection of STEC related both to O157 and to non-O157 serotypes. Our data support the consideration of others for the mandated use of a Shiga-toxin assay for the identification of STEC (37).

Ten Shiga-toxin–producing strains were identified by mPCR screening of a total of 283 *E. coli* isolates. Three of

TABLE 4

Antibiogram, serotypes and origin of STEC strains by genetic, phenotypic characteristics (CR and EHL) and MDRI

Isolate origin	Positive genes	Serotype	Resistance profile	Non-resistance profile	Multi-Drug Resistance Index (MDRI) %	Heamolysis on Blood Agar	Invasion on Congo Red Agar
EC255: Urine (Female)	*stx 1 stx2 hlyA*	O157	Am, E, P, SPT, F, TE, TP,VA, DA, RA, IPM	CIP, NOR	68.75	β	+++
EC94: Stool (Child)	*stx1*	O157	Am, P, SPT, AMC, IPM, CN, CZ,VA	F, TE, CIP, NOR, TP, DA	50	β	+++
EC306: Meat (Kofta)	*stx1 stx2*	O158	Am, E, P, TE, RA, DA, AMC, IPM, CN, NOR, CZ, TP, VA	CIP	81.25	β	+++
EC294: Calf (Stool)	*Stx1*	O158	Am, E, P, SPT, F, TE, CIP, TP	RA, DA, AMC, CN, NOR, CZ	50	β	+++
EC158: Urine (Male)	*stx1*	O158	Am, E, P, TE, RA, DA, AMC, CN, CIP, NOR, CZ, TP, VA	SPT, F, IPM, RA	81.25	β	+++
EC322: Sheep (Stool)	*stx 1 stx2*	O114	E, SPT, F, TE, IPM, CN, CIP, NOR, TP, VA	Am, RA, D, AMC	56.25	β	+++
EC357: Fresh Water	*stx1 eae*A	O114	Am, E, SPT, F, TE, RA, DA, CN, TP, VA	CIP, NOR	62.5	β	+++
EC150: Meat (Sausage)	s*tx1*	O125	Am, E, P, TE, RA, DA, CN, NOR, CZ, TP, VA	IPM 20, SPT, F300	68.75	β	+++
EC0111: Chicken (Stool)	s*tx1 eaeA*	O26	Am, E, P, SPT, F, TE, RA, DA, AMC, CIP	IPM, TP, VA	62,5	β	+++
EC291: Cattle (Stool)	*stx1* eaeA	O26	Am, SPT, CN, VA	E, F, RA, DA, AMC, IPM, NOR, CZ, TP	25	β	+++

The investigated antibiotics in the present study were as follows: Augmentin (AMC 30), Ampicillin (AM 10), Cefazolin (CZ 30), Ciprofloxacin (CIP 5), Clindamycin (DA 30), Erythromycin (E 15), Gentamicin (CN 120), Imipenem (IPM 20), Nitrofurantoin (F 300), Nitrofloxacin (NOR 10), Penicillin (P 10), Rifampin (RA 5), Spectinomycin (SPT 100), Tetracycline (TE 30), Thiamphenicol (TP 30) and Vancomycin (VA 30).

the ten isolates (EC255:O157, EC306:O158, EC322:O114) were positive for both *stx1* and *stx2*. **Fig. 1** demonstrates the two PCR bands of *stx2* (110 bp) and *stx1* (349 bp). The other seven isolates (EC150:O125, EC0111:O26, EC294:O158, EC357:O114, EC291:O26, EC158:O158, EC94:O157) were positive for *stx1* (**Fig. 1**). The ten Shiga-toxin–producing strains were tested for the *eaeA* virulence gene as a marker indicative for intestinal invasion. Three of them were positive for the *eae* gene with a 890 bp band size (**Fig. 2**). In addition, PCR testing of the 75 beta-hemolytic *E. coli* isolates yielded two *hlyA* positive strains with a 165 bp band size (**Fig. 3**).

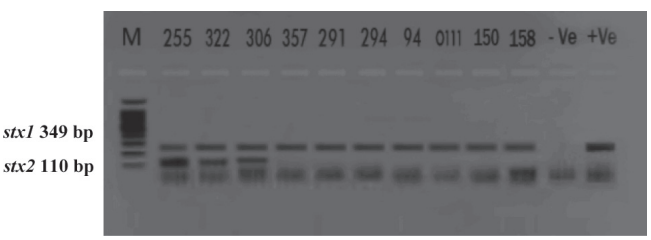

Fig. 1. Characterization of STEC strains by multiplex PCR showing positive pattern to both Shiga toxins (*stx*1 and *stx*2). Lane 1: DNA ladder (100 bp marker); Lane 2: 255 (UTI); Lane 3: 322 (sheep); Lane 4: 306 (kofta); Lane 5: 357 (water); Lane 6: 291 (cattle); Lane 7: 294 (calf); Lane 8: 94 (child); Lane 9: O111 (chicken); Lane 10: 150 (sausage); Lane 1: 158 (UTI); Lane 12: negative control; Lane 13: positive control (*Shigella dysentery*).

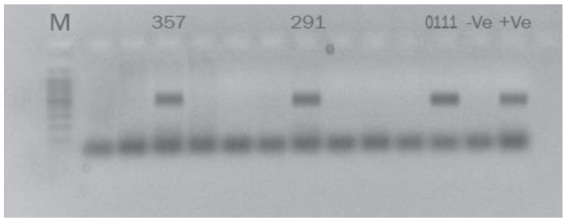

Fig. 2. Characterization of STEC strains by PCR showing positive pattern to *eaeA*. Lane 1: DNA ladder (100 bp marker); Lane 4: 357 (water), Lane 8: 291 (cattle); Lane 12: O111 (chicken); Lane 13: negative control; Lane 14: positive control (invasive *E. coli*).

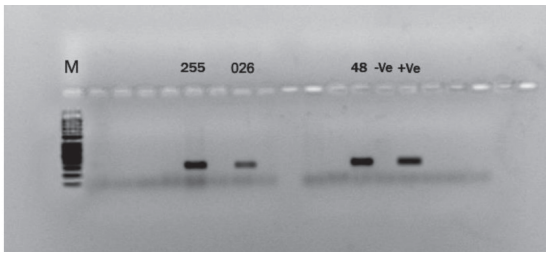

Fig. 3. Characterization of hemolysin producing *E. coli* strains by PCR showing positive pattern to *hlyA*. Lane 1: DNA ladder (100 bp marker); Lane 6: 255 (UTI); Lane 13: 48 (child); Lane 14: negative control; Lane 15: positive control.

When only a Shiga-toxin assay is used, it is important to subculture the positive samples for STEC detection and serotyping, in order to identify and limit potential outbreaks of STEC infection. We were able to identify molecular markers (*stx1*, *stx2* and *hlyA*) for EHEC in the ten STEC isolates, which represented 4 % of the total isolates. Three strains carried both *stx1* and *stx2*, seven strains carried *stx1* only, one strain carried *stx1*, *stx2* and *hlyA*, and three strains carried *stx1* and *eaeA*. It was of a particular concern to find that all the 10 STEC with O157 and non-O157 serotypes were positive for both CR and hemolysis tests on blood agar, indicating that both serotypes possess similar virulent phenotypic characteristics. Most studies of O157 and non-O157 STEC have included mixed populations of adults and children (10, 11). While outbreaks of non-O157 STEC have been reported in children (22), and in 38 % of STEC infections among a pediatric cohort (38), our results identified seven STEC strains that were isolated from clinical samples, of which four were isolated from stools of diarrheic animals and two (O157 and non-O157) were from adult patients with UTI and one (O157) was from the stool of a diarrheic child under 5 years of age. Data collected from multiple sites in the world showed that the highest incidence of both *E. coli* O157 infection and HUS occurred among 5-year-old children and that the median age of patients with HUS was 4 years (26). Nevertheless, the incidence of non-O157 STEC infection and the clinical squeal need to be more clearly defined (38). *E. coli* accounts for the majority of urinary tract infections in young adults and pregnant women (34). EHEC causing hemolytic colitis (HC) (35), a severe form of diarrhea, has been associated with hemolytic uremic syndrome (HUS), which may progress in some patients to renal failure (41). HUS is a life-threatening condition especially among children and elderly people (40). In Nigeria, Akinduti et al. (3) isolated EHEC serotype O157:H7 from urine samples of adult male and female patients suffering from severe UTI. The authors reported a very high prevalence (46.4 %) of *E. coli* 0157:H7 (n = 372, out of 1205) among the tested patients, demonstrating that renal diseases associated with severe UTI were majorly caused by *E. coli* O157:H7. The percent of STEC in our clinical samples from UTI cases is notably high, considering the small number of UTI cases (n = 47) included in the study. To the best of our knowledge, this is the first report from Egypt to identify non-O157 STEC from patients with UTI, which recommends further community and hospital-based surveillance studies to better estimate the prevalence of EHEC in this region and their impact on disease complexity. Christina et al. (15) surveyed 5110 children for STEC at the Children's Hospital Boston and demonstrated that 50 cases (0.9 %) had confirmed STEC infection; 33 were O157:H7 and 17 were non-O157:H7 belonging to different serotypes, such as O25:H11, O26:H11, O26:H28, O111: nonmotile, O118:H16, O126:H1, O130:H11, O145: nonmotile, and O146:H28. However, there were no significant differences in disease severity or laboratory manifestations of STEC infection between children with O157:H7 and those with non-O157 STEC. Our survey identified a comparable percent in the distribution of non-O157 serotypes as those reported by Christina et al. (15): we identified that four different non-O157 serotypes, which belonged to O158, O114, O125, and O26, were isolated from clinical samples, meat products and fresh water.

We additionally demonstrated the *in vivo* comparable toxicity of Shiga toxin derived from both O157 and non-O157serotypes. Serotyping of the 10 STEC strains demonstrated the circulation of five serotypes (O157, O158, O114, O125 and O26) (**Table 4**): three isolates from people (serotype O157, O158), four isolates from animals (serotype O114, O26), two isolates from meat products (serotype O125, O158) and an isolate from fresh water (serotype O114). Three STEC strains, belonging to three serotypes, positive for both *stx1* and *stx2* were isolated from a urine sample of a female patient with UTI (serotype O157), a stool from an infected sheep (serotype O114) and from a meat product (serotype O158).

STEC are associated with a broad spectrum of human illnesses throughout the world, ranging from mild diarrhea to HC, HUS and TTP. Complications arising from antibiotic treatment of STEC-related human infections have also been reported (19). Our studies on the antibiotic susceptibility pattern for the 10 STEC strains indicated that all Shiga-toxin–producing strains (O157 and non-O157) showed resistance to commonly used antibiotics such as ampicillin, vancomycin, penicillin, erythromycin, tetracycline, augmentin, cefazolin, and ciprofloxacin. In addition, a considerably high MDRI phenotype was identified in both serotypes. It is of concern to find that 88 % (7/8) of the non-O157 strains showed resistance to ampicillin, of which six were MDR with E/T and 38 % were resistant to ciprofloxacin. The two O157 strains were resistant

to ampicillin and showed MDR to more than four antibiotics. The MDRI phenotype of both the O157 and non-O157 strains signifies that STEC are highly resistant to available antibiotics, limiting the selection of treatment choice. MDR among *E. coli* isolates has been subject of concern worldwide (14, 43).

Ground beef and other bovine products have often been implicated as sources of STEC (27), along with other food products (2, 53, 54). Occasional outbreaks of STEC have also been associated with public drinking water (55) and swimming in contaminated water (25, 28, 46). Our investigation also demonstrated that of three non-O157 STEC strains, two (EC306:O158, EC150:O125) were isolated from processed meat products and one (EC357:O114) from the main fresh water canal of Ismailia. It is not clear whether the contamination of meat and water was of animal or human origin but conceivably, a combination of the above origins could explain the presence of STEC strains found in such isolates.

We are in the process of analyzing PFGE results of all STEC strains to compare the clonality and define the relatedness of the strains. In this study, most of the STEC strains were shown to possess a combination of two or more of the virulence factors (CR indicator, hemolysin production as well as *stx1*, *stx2*, *hlyA* and *eaeA*).

Conclusions

The presence of multiple virulence factors increases the virulence of microorganisms, since such factors function synergistically to overcome and defeat normal host defenses. Thus, strains with more virulence factors are potentially considered as virulent pathogens. Our findings may imply that the isolates from the examined sources could be considered as potentially pathogenic. It is of interest that STEC serotype O104 was not identified from the environmental sources tested in the present study, as fenugreek imported from Egypt was blamed as the most likely source of the 2011 STEC epidemic in Germany that fatally affected 49 people. One of the limitations of our investigation, however, is that we could not identify (H) flagellar antigen for the isolated STEC, since it was commercially unavailable at the time of our study. Additionally, this is considered as a lab-based study and with the small number of disease cases involved and the lack of clinical data, we could not determine an association between disease severity and a specific STEC serotype.

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