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RESEARCH ARTICLE

COMPARATIVE PATHOGENICITY, TOXICITY AND PULSE TYPES OF O157 AND NON -O157 *Escherichia coli*

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ABSTRACT

Shiga toxin producing *Escherichia coli* (STEC) are recognized as an important foodborne pathogen, responsible for sporadic cases to serious outbreaks worldwide (Wani *et al*., 2003). The morbidity and mortality associated with several recent outbreaks due to STEC have highlighted the threat this organism poses to global public health. The present study describes the molecular characterization of STEC expressing five different serotypes (O157, O158, O114, O125 and O26) isolated from different sources in Egypt and investigates their clonal relationship. The present study investigated the ability of each Shiga toxin producing strains with different genetic backgrounds to induce disease *in vivo* using a rat model. STEC strains were identified and characterized by PCR and DNA sequencing analysis; clonality was determined by comparing pulsetypes generated during pulsed field gel electrophoresis. Ten STEC isolates (three from human stool, four from animal stool, two from meat products and one from untreated water) were positive for a combination of *stx* genes; three were positive for both *stx1* and *stx2*, the remaining were only positive for *stx1*. Two of the STEC isolates contained *eae,* whereas one carried the enterohemorrhagic *E. coli* (EHEC) hemolysin gene, *hlyA*. All STEC isolates exhibited *in vivo* toxic effects after inoculation of STEC bacterial broth or their respective purified toxin to experimental rats. Subtyping of the ten STEC isolates by pulsed-field gel electrophoresis (PFGE) revealed three distinct restriction patterns. Sixty percent (6/10) of the isolates shared the same PFGE (mrp1) pattern, representing the most common profile; and 30% (3/10) shared a second common (mrp II) pattern. Only one strain (10%) showed a distinct and unique mrp III PFGE profile. *In vivo* challenge experiments with O157 and non O157 STEC induced disease in rats, including pronounced epithelial lesions and severe vascular damage. This study identified STEC O157 from human cases with diarrhea, and demonstrated that meats and untreated water available in Egypt were contaminated with diverse non-O157 STEC strains. This finding is of concern due to the potential of these organisms to cause human disease.

INTRODUCTION

Shiga toxin-producing enterohemorrahgic Escherichia coli (STEC) strains of serogroup O157:H7 and non O157:H7 STEC serogroups cause hemorrhagic colitis, which is may be followed by hemolytic-uremic syndrome (HUS) and/or acute encephalopathy (Paton and Paton 2004 and Wani *et al.,* 2003). Shiga toxin, encoded by either the stx1 or stx2 genes, plays a critical role in the pathogenesis of diarrhea caused by *E. coli* O157:H7 (Sheng *et al.,* 2004). However, the pathogenesis of these diseases is not well understood. Based on experimental findings (Fujii *et al.,* 1993), a possible mechanism for the development of these diseases following an O157:H7 infection has been proposed: shiga toxin hematogenously disseminates from the gut to the kidney or the brain (Eijikita *et al.,* 2000) resulting in tissue damage. *E. coli* (STEC) or Verotoxigenic Vero toxin producing *E. coli*) is usually acquired by consuming contaminated food or water, although person-to person transmission has not been ruled out (Gal-Mor and Finlay 2006). Most individuals infected with EHEC recover from the infection without further complications. However, 8–10% of patients, primarily children and the elderly, may go on to develop complications such as HUS, characterized by acute renal failure, thrombocytopenia and hemolytic anemia (Tarr *et al.,* 2005).Although EHEC is not invasive and is restricted to the lumen of the gut (Acheson *et al.,* 1996) in some circumstances Stx produced within the intestinal tract is able to cross the epithelial barrier and enter the blood stream. Stx targets the endothelium of susceptible tissues, resulting in intestinal as well as systemic dysfunction (Ochoa and Cleary 2003). Despite progress made during recent years regarding the involvement of inflammatory response in HUS pathogenesis, relatively little is known about EHEC-induced local changes in the intestinal tract and its association with systemic disease. In order to define these changes adequately, an animal model of EHEC oral infection is needed. In rabbits, EHEC induces gastrointestinal symptoms similar to humans (Ritchie *et al.,* 2003), however the absence of renal injury by Stx and the paucity of genetic and immunological resources are important limitations for the use of this animal model. The ability of STEC to cause serious disease in humans is related to the production of one or more Shiga-like toxins (Stx1, Stx2, or variants), which inhibits protein synthesis of host cells, leading to cell death (Djordjevic *et al.,* 2003). Epidemiological investigation of STEC is complicated by the ubiquitous nature and lack of heterogeneity between clonal strains. The discriminatory capacity of many methods used to subtype isolates of *E. coli* causing enterohemorrhagic disease is insufficient to resolve differences between clonal members. Therefore phenotypic and genotypic methods that are highly discriminatory are particularly useful in this context, maximizing the likelihood of detecting the often minimal amount of variation between strains (Putnam *et al.,* 2004). These

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typing methods are of great use to identify the sources and routes of transmission of organisms and make intervention strategies.

MATERIALS AND METHODS

STEC strains

Ten serologically confirmed STEC isolates (Sahar *et al* in press 2013) isolated from different human/environmental sources are subjects of the current study. The ten isolates belonged to five different serogroups (O157 (n=2), O158 (n=3), O114 (n=2), O125 (n=1) and O26 (n=2).

Antimicrobial susceptibility testing of STEC isolates

Antimicrobial susceptibility was determined using the Kirby-Bauer disk diffusion technique with Mueller Hinton agar medium, as described by the Swedish Reference Group for Antibiotics (CLSI, 2010). Antibiotics (Oxoid, KS.USA) used are listed in Table 1.

Table 1. Antibiogram, Serotypes and origin of STEC strains by virulence genes

Isolate: origin	Virulence genes	Serotype	Resistance Profile	Non- Resistance Profile
EC255: Urine (Female)	*stx 1/ stx2hlyA*	O157	Am 10, E 15, P10 SPT, F300, TE30, TP 30, VA 30 DA 2, RA 5 IPM 20	CIP 5, NOR 10
EC94: Child stool	*Stx 1*	O157	Am 10, P 10, SPT, AMC 30, IPM 20, CN 120, CZ 30 ,VA 30	F 300, TE 30, CIP 5, NOR 10- TP 30 DA 2,
EC306: Meat (Kofta)	*-Stx 1/ Stx2*	O158	Am 10, E 15, P 10, TE 30, RA 5, DA 2, AMC 30, IPM 20, CN 120, NOR 10, CZ 30, TP 30, VA 30	CIP 5
EC294: Calf stool	*Stx 1*	O158	Am10, E15, P10, SPT, F 300, TE 30, CIP 5 , TP 30	RA 5, DA 2 , AMC 30, CN 120, NOR 10, CZ 30
EC158: Urine (Male)	*-Stx 1*	O158	Am 10, E 15, P 10, TE 30, RA 5, DA 2, AMC 30, CN 120, CIP 5, NOR 10, CZ 30, TP 30, VA 30	SPT, F 300, IPM 20 RA 5
EC322: Sheep (stool)	*-Stx 1/ Stx2*	O114	E 15, SPT, F 300, TE 30, IPM 20, CN 120, CIP 5, NOR10, TP 30, VA 30	Am 10, RA 5, DA 2, AMC 30
EC357: Raw water	*-Stx 1-eae*	O114	Am10, E15, SPT, F 300, TE 30, RA 5, DA 2, CN 120, TP 30, VA 30	CIP 5, NOR 10
EC150: Meat (Sausage)	*Stx 1*	O125	Am 10, E 15, P 10, TE 30, RA 5, DA 2, CN 120, NOR 10, CZ 30, TP 30,VA 30	IPM 20, SPT, F300
EC0111: Chicken (stool)	*-Stx 1-eae*	O26	Am10, E15, P10, SPT , F300 TE 30, RA 5, DA 2, AMC 30, CIP 5	IPM 20, TP 30, VA 30
EC291: Cattle (stool)	*-Stx 1- eae*	O26	Am 10, SPT, CN 120, VA 30	E 15, F 300, RA 5 DA 2 , AMC 30, IPM 20, NOR 10, CZ 30, TP 30

The investigated antibiotics in the current study were as follows: Amoxicillin/Clavulanic Acid AMC 30, Ampicillin AM 10, Cefazolin CZ 30, Ciprofloxacin CIP 5, Clindamycin DA, Erythromycin E 15 , Gentamicin CN 120, Imipenem IPM 20, Nitrofurantion F 300, Nitrofloxacin NOR 10, Penicillin P 10, Rifampin RA 5 , Spectinomycin SPT 100, Tetracycline TE 30, Thiampinicol TP 30 and Vancomycin VA 30

Break points for sensitivity (S), intermediate (I) and resistance (R) to any given antibiotic was determined by measuring the easily visible and clear zone of each antimicrobial agents and results were interpreted according to the guidelines set by the Clinical and Laboratory Standards Institute (CLSI, 2010).

PFGE

Macrorestriction analysis of DNA resolved by pulsed-field gel electrophoresis was performed following a standardized protocol established for PulseNet USA, US Centers for Disease Control and Prevention (CDC 2010 and Ribot *et al.,* 2006) for Escherichia coli O157:H7, Salmonella, and Shigella rods. Briefly, bacterial suspensions were prepared by harvesting bacterial colonies directly from overnight growth of isolates on Trypticase Soy Agar; organsims were diluted to a concentration of 109 colony forming units per /mL (=1:1.3Nephelometric turbidity units (NTU). Bacterial cells were embedded in 1% SeaKem Gold: agarose (Cambrex Bio Sciences Rockland, Inc, Charles city, IA, USA) in TE buffer (10 mM Tris:1 mM EDTA, pH 8.0). The bacteria in each of two plugs from each isolate were lysed in a 50-ml tube containing 5 ml Cell Lysis Buffer (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% Sarcosyl) in the presence of 25 µl Proteinase K stock solution (20 mg/ml; Sigma, St. Louis, MO, USA). In a 54ºC shaker incubator for a minimum of 12 h, with constant and vigorous agitation (150-175 rpm). DNA embedded in agarose was washed 3x with water then with 1x TE buffer. For restriction digestion, a 2.0 -2.5 mm agarose slice containing DNA representing one isolate or the Salmonella, enterica serovar. Braenderup H9812 standard (Bio-Rad Laboratories) was incubated with 40 units of XbaI, a restriction enzyme, at 37ºC for a minimum of 16 h. Restricted plugs were loaded into a 1% agarose gel and fragments of DNA were were separated by PFGE using a CHEF DR III electrophoresis apparatus (Bio-Rad, Richmond, CA, USA). The initial switch time was 2.2 s and the final switch timewas 54.6 s, at 6 V, with an included angle of 120° for 18 h. Gels were stained with ethidium bromide (Bio-Rad Laboratories) and images were documented using a Gel Doc 1000 system.

Virulence determinace

This work was conducted in the Anatomy and Histology Unit, Faculty of Medicine and Surgery Suez Canal University. All ten STEC isolates with different serogroups and genetic characteristics were tested for their pathogenicity and toxicity using male rats according to the method described by (Gyles, 1992). Animals: 104 healthy male rats (68-80 gm) were purchased from (Animal House- Abu-Rawash region (Giza, Egypt, serving all Universities and National Academy of Science institute in Egypt and housed in micro isolator cages. Rats were maintained at an ambient temperature of 23 ± 2ºC and provided with food and water. Research was conducted in compliance with the U. S. Animal Welfare Act and other U. S. federal regulations relating

to animals and experiments involving animals and all research adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council.

Bacterial inocula

For each STEC isolate three flasks (250 ml capacity), each containing 50 ml Trypticase Soya broth supplemented with 0.6% yeast extract, were prepared. Flasks were inoculated with 5 ml of each *E. coli* isolate, and incubated at 37°C in a shaking incubator (180 rpm) for three days. Cultures were serially diluted to determine the concentration and the sub lethal dose (SLD) (Raife *et al.,* 2006). Extraction and purification of exotoxins: After three days incubation time, the medium of each isolate was centrifuged at 15000 rpm for 20 min and cell free medium containing crude exotoxins was prepared by filtration through 0.22µm membrane filter glass fiber filter paper, (Whatman, Germany). Sterile, crude toxin preparations were precipitated using cold acetone for 18 h. An equal amount of acetone (1:1 volume) as that of the broth was used for the precipitation. After precipitation the mixture was centrifuged at 16000 rpm min at 4ºC. The filtrate was discarded and the residue was dissolved in 5 ml of 25 mM phosphate buffer at pH 7.0 to obtain the purified toxins which were stored at 4ºC until biological assessment (Hesham *et al.,* 2010).

Intraperitoneal STEC animal studies

Rats used in these experiments were grouped into two categories: the first group (I) was challenged with the isolates grown in broth cultures and the second group (II) was challenged with the corresponding purified toxin mixture from each isolate. Both groups were injected through an intraperitoneal (i.p.) route with respect to animal body mass. Test doses were determined to be 0.2 µl/gm for each tested fluid from each toxin and whole bacterial each separately) (Siegler and Oakes 2005.). Each isolate was tested separately using four rats housed in separate cages. Four additional rats per set were unexposed to any challenge and used as a negative control. Rats were examined daily for any physical or clinical changes through the duration of the study; this included changes in fur texture, skin consistency, eyes, mucus membranes, orifices, and clinical signs of respiratory behavior changes. Results were recorded after 24 and 48 h for diarrhea and after 5 days for mortality (Hesham *et al.,* 2010).

Acute toxicity was determined and evaluated as described elsewhere. Animals showing signs of morbidity were sacrificed and organs were processed for further histopathological studies (Hosler *et al.,* 2003).

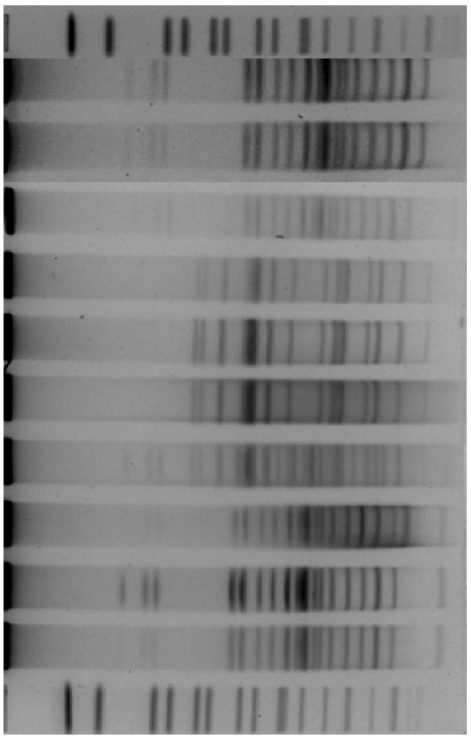
Histopathological studies

Histopathological sections of tissue samples from liver, kidney, and the gastrointestinal tract of rats challenged with the ten STEC isolates and their corresponding toxins were investigated. These organs were removed from sacrificed rats and prepared for histopathological examination according to (Lillic and Fullmen, 1976). Rat tissue from the organs were collected, excised, and fixed in 10% neutral buffered formalin. Ground tissues were passed through ascending grades of alcohol in the following step tissues while they were embedded in paraffin wax, then sectioned blocks were persued into sections each of thickness 4-5mm this section is not fluid and should be reworded to be more clear. Sections from each experimental animal were loaded on to clean glass slides, stained with haematoxylin and eosin, and examined microscopically.

RESULTS

PFGE and antibiogram

The 10 STEC strains belonged to different serotypes and with varied virulence genes (Stx1-Stx2, eae, and hlyA) were subjected to finger printing by PFGE after digestion of their genomic DNA with Xba1 restriction enzyme. A total of 3 macrorestriction patterns (MRPs) were identified among the 10 strains (the patterns are very similar; the PFGE needs to be analyzed via a dendrogram). PFGE MRPs were recorded as mrp I, II, and III. Sixty percent (6/10) of the total examined strains shared the same (mrp1) PFGE pattern which represents the most homogenous profile; and 30% (3/10) shared the second common (mrp II) pattern, while one strain (10%) showed a distinct mrp III PFGE profile. PFGE with mrp I included six STEC strains belong to four serotypes (O157 (n=2), O158 (n= 2), O114 (n=1), O26 (n=1), PFGE with mrp II included three strains belong to O26 (n=1), O114 (n=1) and O158 (n=1). While PFGE with mrp III included one strain with unique serotype O125 (n=1) (Figure: 1). It was found that STEC isolates with different antibiogram pattern and



Strain	Source	Serotype	MRPs	*Stx1*	*Stx2*	*eae*	*hly A*
Salmonella ser. Braenderup H9812 standard							
EC94	Child Stool	O157	I	+	–	–	–
EC158	Urine (Male)	O158	I	+	–	–	–
EC291	Cattle (stool)	O26	I	+	–	+	–
EC357	Raw water	O114	II	+	–	+	–
EC294	Calf (stool)	O158	II	+	–	–	–
EC0111	Chicken (stool)	O26	II	+	–	+	–
EC150	Meat (sausage)	O125	III	+	–	–	–
EC322	Sheep (stool)	O114	I	+	+	–	–
EC306	Meat (Kofta)	O158	I	+	+	–	–
EC255	Urine (Female)	O157	I	+	+	–	+
Salmonella ser. Braenderup H9812 standard							

Fig. 1. Macro- Restriction patterns MRPs of Xba1-digested genomic DNA STEC from Egypt background

with different virulence characteristics displayed the same PFGE mrp. In addition, STEC strains belonging to different serotypes and isolated from human and animal source share the same PFGE profile.

PFGE MRP I

All six isolates with mrp I were shiga toxin producers. Three isolates (O157:EC255 – O158:EC306 – O114:EC322) were stx1 and stx2 producers, while the other three isolates (O158:EC158 – O26:EC291– O157:EC94) were producers of stx1 only. Intimin eae genes were absent in all isolate belonging to this pattern, except for one (O26:EC291). Only one isolate (O157:EC255) produced enterohaemolysin hlyA. Isolates with mrpI included STEC from different origins: two isolates were isolated from two patients with urinary tract infections (UTI); one isolate was collected from the stool of child with diarrhea, two isolates each obtained from the stool of a sheep, and cattle with diarrhea, and one strain isolated from raw meat (kofta) Fig (1). The antibiogram for *E. coli* isolates carrying different virulence genes is demonstrated in Table 1. All isolates with PFGE mrp I demonstrated multi-drug resistant (MDR) to at least four antibiotics belong to different classes: erythromycin, gentamicin, cefazolin, thiampinicol, vancomycin, ciprofloxacin, and ampicillin. Isolates belonging to serotype O157 were sensitive to ciprofloxacin (CIP) and norfloxacin (NOR). However, 30% of isolates expressing non-O157 serotypes were resistant to CIP, and 90% were MDR to at least three antibiotics. Three isolates were resistant to spectinomycin (O114:EC322 – O157:EC94 – O157:EC255), three isolates (O114:EC 322–O158:EC306–O158:EC158) were resistant to nitrofloxacin, four isolates (O157:EC255 – O114:EC322 –O158:EC 306–O158:EC158) were resistant to tetracycline, three isolates (O157:EC255 – O158: EC306 – O158:EC158) were resistant to rifampin, three isolates (O157:EC255–O158:EC306–O158:EC158) were resistant to clindamycin, four isolates were resistant to amoxicillin/clalvulanic acid (O158:EC306 – O158:EC158 –O157:EC94) and three isolates (O157:EC255– O158:EC306– O157:EC94) were resistant to imipenem. It is of particular interest to identify resistance to imipenim (IPM) in two non-O157 strains isolated each from urine and stool of two children with gastroenteritis.

PFGE MRP II

This pattern included three strains characterized as shiga toxins 1 producer. Two out of the three strains (O26:EC0111– O114:EC357) were proved to produce Intimin eae gene, while the third strain O158:EC294 was negative for eae gene. hlya gene was absent in all strains belonged to this pattern. PFGE MRP II included STEC isolated from Chicken, Calf and untreatedwater. All strains of this PFGE pattern showed resistance to Ampicillin, Erythromycin, Penicillin, spectinomycin, Nitrofurantion, Tetracycline. While each strain of this pattern had its own characteristic antibiotics profile for other antibiotics groups; O158:EC294 was resistant to Ciprofloxacin, Thiampinicol, and was sensitive to Amoxicillin/ Calvulanic Acid, Gentamicin, Nitrofloxacin, Cefazolin. O26:EC0111 strain was resistant to Ciprofloxacin and was sensitive to Nitrofurantion, Imipenem, Thiampinicol and Vancomycin. O114:EC357 strain was resistant to Rifampin, Clindamycin, Gentamicin, Thiampinicol and Vancomycin, while was sensitive to Ciprofloxacin Nitrofloxacin. All strains belonged to this pattern were typed as non O157

PFGE MRP III

Only one STEC strain included in this pattern: O125:EC150 was positive to Stx 1, and negative for both Intimin eae and. hlyA genes. The strain was isolated from meat product and was resistant to all antibiotics except Spectinomycin 100 Nitrofurantion 300 and Imipenem 20.

Pathogenicity

Clinical signs of CNS (Central Nervous system) were the most striking clinical observation in rats inoculated with STEC. As shown in Tables (2, 3) both bacterial fluids and purified toxins gave comparable pre-mortem CNS signs such as decreased appetite, abdominal cramps, back legs paralyzation, convulsion and comma. These signs were recorded within 2-4 hours after ip injection. While rats challenged with purified toxins demonstrated more severe signs (paralyzation and rapid morbidity) when compared to those challenged with the bacterial broth. All of the previous clinical findings were observed in 100% of the injected animals, while gastrointestinal signs (GTI) such as diarrhea was observed in 66% of the rats injected by bacterial broth as opposed to 42% injected with the purified toxins. Mortality within 24 hours Tables (2, 3) was recorded in all investigated groups. Microscopic lesions: Histological lesions were frequently found in intestinal, kidney and liver tissues of animals inoculated by STEC, eae producing *E. coli* and hly producing EHEC, while control rats had no morphological lesions in any of organs sampled. The findings for specific anatomical sites Table (4, 5, and 6) were as below:

Intestine (Colon)

Histologic signs were evident in the intestine of all rats inoculated with all pathogenic strains, such lesion were more common in caecum and colon than in ileum. Within the inoculated groups, the degree and intensity of the lesions were differed from animal to another and with the duration of the exposure. Intestinal lesions were seen as early as 24 hour PI (post injection) but were more pronounced in rats necropsied at later time after injection. Table (4): the clearest findings were the shift of goblet cells, irregular distribution of goblet cells in mid crypts and replacement by immature cells with large nuclei in addition to extensive dilatation, infiltration of lamina proportion with occasional eosinophils, intraepithelial cells and Neutrophils. Dilatation of some goblet cells in mid-crypt areas and accumulation of mucus and bulging mucus droplets toward the lumen, crypts contained intensively stained immature cells were obviously observed in strains produce the two type of toxins Stx1 and stx2 with a serotype O157 and O114 and some other serotype (O26) which produce only stx1.

Kidney

The severity and intensity of signs of the examined tissues varied according to the type of inoculate since animal injected with strains O157:EC255 and O114:EC322 with two types of shiga toxins displayed a more pronounced necrosis as compared to the control group. (Table: 5, Figure: 3). Animals inoculated by purified toxins demonstrated more severe and marked lesions. Tubules: suffered from epithelial vacuolization and necrosis, hyaline casts and interstitial edema. Vascular damage: was recorded as fibrin thrombi, focal hyaline deposits, intimal thickening in addition to diffuse hyaline deposits and fibrin thrombi.

Liver

Histological signs were restricted to liver steatosis and ballooning with portal and lobular inflammation in the majority of sampled organs (Table 6).

DISCUSSION

Enterohemorrhagic Escherichia coli (EHEC) O157 and non O157 infections are considered a public health problem in both developed and developing countries because of their increasing incidence and the severity of clinical presentation. In Egypt there is no sufficient surveillance data about outbreaks or infections induced by STEC although of the high incidence of diarrheic outbreaks among preschool children. Data Collected during the last ten years indicated that Epidemiological studies in Egypt were restricted to livestock particularly dairy and beef cattle as a natural reservoirs of these organisms (Hassanain and Zaabal 2004). Previous Egyptian Studies carried out on cattle by products, poultry and diarrheic individuals

Table 2. Pre-mortem signs recorded from experimental rats inoculated with live STEC isolates

Isolate Source	Serotypes	Diarrhea	Decreased appetite	Abdominal cramps	Back legs paralyzation	Convulsions and coma
		24hrs	24hrs	2-4 hrs	2-4hrs	Mortality 1- 7 days
EC255: Urine (Female)	O157 Stx 1/ stx2 +hly (A)	+	+	+	+	3\4
EC94:Child (stool)	O157 Stx 1	+	+	+	+	2\4
EC306 : Meat (Kofta)	O158 Stx 1/ stx2	−	+	+	+	3\4
EC294:Calf (stool)	O158 Stx 1	+	+	+	+	1\4
EC158:Urine (male)	O158 Stx 1	+	+	+	+	3\4
EC322 : Sheep (stool)	O114 Stx 1/ stx2	−	+	+	+	3\4
EC357: Raw water	O114 Stx 1+ eae	+	+	+	+	3\4
EC150:Meat (Sausage)	O125 Stx 1	−	+	+	+	2\4
ECO111:Chicken (stool)	O26 Stx 1 + eae	−	+	+	+	3\4
EC291 : Cattle (stool)	O26 Stx 1+ eae	+	+	+	+	3\4

Table 3. Pre-mortem signs recorder from experimental rats inoculated with purified toxin from STEC isolates

Isolate Source	*Serotypes*	*Diarrhea*	*Decreased appetite*	*Abdominal cramps*	*Back legs paralyzation*	*Convulsions and coma*
		24 hrs	24hrs	2-4 hrs	2-4hrs	Mortality 1- 3 days
EC255: Urine (Female)	*O157 Stx 1/ stx2 +hly(A)*	+	+	+	+	4\4
EC94:Child (stool)	*O157 Stx 1*	+	+	+	+	4\4
EC306 : Meat (Kofta)	*O158 Stx 1/ stx2*	−	+	+	+	4\4
EC294:Calf (stool)	*O158 Stx 1*	−	+	+	+	4\4
EC158:Urine (male)	*O158 Stx 1*	−	+	+	+	4\4
EC322 : Sheep (stool)	*O114 Stx 1/ stx2*	−	+	+	+	4\4
EC357: Raw water	*O114 Stx 1+ eae*	−	+	+	+	4\4
EC150:Meat (Sausage)	*O125 Stx 1*	−	+	+	+	4\4
ECO111:Chicken (stool)	*O26 Stx 1 + eae*	−	+	+	+	4\4
EC291 : Cattle (stool)	*O26 Stx 1+ eae*	+	+	+	+	4\4

+: positive result — : negative result

Table 4. Post-mortem results s for the examined Colon for whole bacteria (B.B.) and purified toxin (P.T.)

Isolate Source	Serotypes	Shift of goblet cells		Infiltration of lamia proporia		Eosinophillic		Necrosis	
		B.B	P.T	B.B	P.T	B.B	P.T	B.B	P.T
EC255: Urine (Female)	O157 Stx 1/ stx2 +hly A	+++	+++	+++	+++	+++	+++	+++	+++
EC94:Child (stool)	O157 Stx 1	+++	+++	+++	+++	+++	+++	+++	+++
EC306 : Meat (Kofta)	O158 Stx 1/ stx2	+++	+++	+++	+++	+++	+++	+++	+++
EC294:Calf (stool)	O158 Stx 1	++	+++	+++	+++	+++	+++	+++	+++
EC158:Urine (male)	O158 Stx 1	+++	+++	+++	+++	+++	+++	+++	+++
EC322 : Sheep (stool)	O114 Stx 1/ stx2	+++	+++	+++	+++	+++	+++	+++	+++
EC357: Raw water	O114 Stx 1+ eae	+	+++	+++	+++	+++	+++	+++	+++
EC150:Meat (Sausage)	O125 Stx 1	++	+++	+++	+++	+++	+++	+++	+++
ECO111:Chicken (stool)	O26 Stx 1 + eae	+	+++	+++	+++	+++	+++	+++	+++
EC291 : Cattle (stool)	O26 Stx 1+ eae	++	+++	+++	+++	+++	+++	+++	+++

Table 5. Post-mortem results s for the examined Kidney for whole bacteria (B.B.) and purified toxin (P.T.)

Isolate Source	Serotypes	Tubular Epithelial vacuolization		Necrosis		Vascular damage		Interstitial edema	
		B.B	P.T	B.B	P.T	B.B	P.T	B.B	P.T
EC255: Urine (Female)	O157 Stx 1/ stx2 +hly (A)	+++	+++	+++	+++	+++	+++	+++	+++
EC94:Child (stool)	O157 Stx 1	++	+++	+++	+++	+ +	+++	+++	+++
EC306 : Meat (Kofta)	O158 Stx 1/ stx2	+++	+++	+++	+++	+++	+++	+++	+++
EC294:Calf (stool)	O158 Stx 1	+	+++	+++	+++	+++	+++	+ +	++
EC158:Urine (male)	O158 Stx 1	+	+++	+++	+++	++	+++	+ +	+++
EC322 : Sheep **(stool)**	O114 Stx 1/ stx2	+++	+++	+++	+++	+++	+++	+++	+++
EC357: Raw water	O114 Stx 1+ eae	+++	+++	+++	+++	+++	+++	+++	+++
EC150:Meat (Sausage)	O125 Stx 1	+	+ +	+ +	+++	+ +	+++	+	+++
ECO111:Chicken (stool)	O26 Stx 1 + eae	+++	+++	+++	+++	+++	+++	+++	+++
EC291 : Cattle (stool)	O26 Stx 1+ eae	+++	+++	+++	+++	+++	+++	+++	+++

+: mild lesion ++ moderate lesion +++ Sever lesion ++++ very sever (acute)

Table 6. Post-mortem results s for the examined Liver for whole bacteria (B.B.) and purified toxin (P.T.)

Isolate Source	Serotypes	Steatosis		Ballooning		Portal inflammation		lobular inflammation	
		B.B	P.T	B.B	P.T	B.B	P.T	B.B	P.T
EC255: Urine (Female)	O157 Stx 1/ stx2 +hly (A)	+++	+++	+++	+++	+++	+++	+++	+++
EC94:Child (stool)	O157 Stx 1	+	++	+++	+++	+++	+++	+++	+++
EC306 : Meat (Kofta)	O158 Stx 1/ stx2	+++	+++	+++	+++	+++	+++	+++	+++
EC294:Calf (stool)	O158 Stx 1	++	++	+	+++	+++	+++	+++	+++
EC158:Urine (male)	O158 Stx 1	++	++	++	++	++	+++	+	+++
EC322 : Sheep (stool)	O114 Stx 1/ stx2	+++	+++	+++	+++	+++	+++	+++	+++
EC357: Raw water	O114 Stx 1+ eae	+++	+++	+++	+++	+++	+++	+++	+++
EC150:Meat (Sausage)	O125 Stx 1	++	+++	+	+	+	++	++	+++
ECO111:Chicken (stool)	O26 Stx 1 + eae	+ +	+++	+++	+++	+++	+++	+++	+++
EC291 : Cattle (stool)	O26 Stx 1+ eae	+++	+++	+++	+ +	+ +	+ +	+ +	+++

+: mild lesion ++ moderate lesion +++ Sever lesion ++++ very sever (acute)

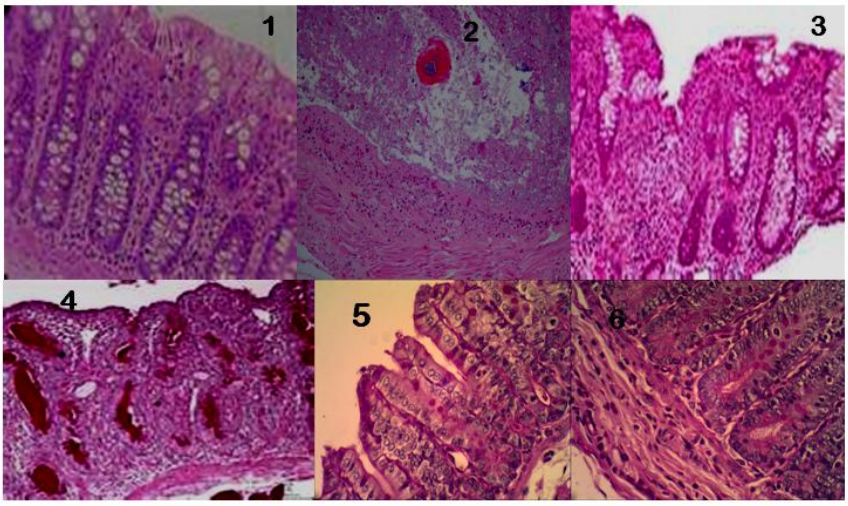


Fig. 2. Six histological cases of haematoxylin-eosin stained sections of examined colon from rats necropised 24 hours after injection. (1, 2, 3, 4, 5. 6): (1): control (no injection) showing normal goblet cell, (2.3): A case of colon necrosis with clear lesions due to I.P by *STEC O157 strain*, (4): colonic eosinophils. Neutrophils by I.P of *O125 strains* (5,6): represented upnormality and shifting of the goblet cells of the colonic epithelial tissues and replacement by immature cells with large nuclei. Do you have a positive control, a known STEC isolate? Do you have a negative control? These are important for comparative analyses

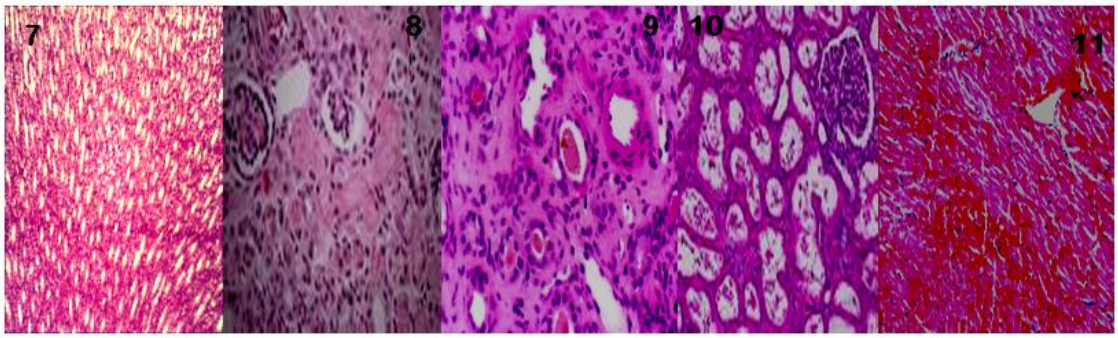


Fig. 3. Four histological cases haematoxylin-eosin stained sections of examined kidneys from rats necropised 24 hours after injection (7, 8, 9,10,11): (7): control (no injection) (8) :Showing Acute kidney tubular necrosis ○resulted from I.P of *O157 and O26 strain*, (9): Showing vacuolization in kidney tubules from I.P of O114 *STEC* strain (10): representing

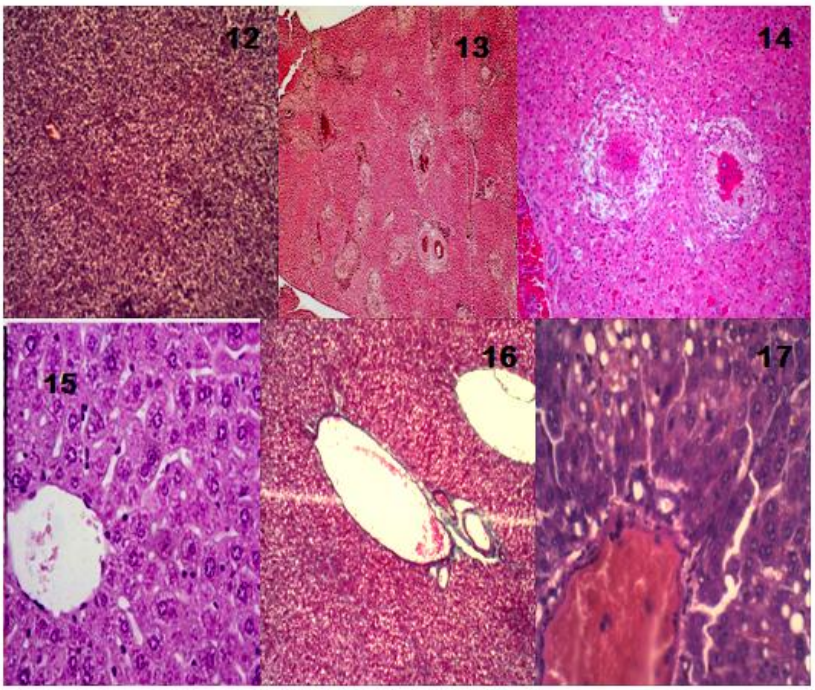


Fig. 4. Six histological cases haematoxylin eosin, mathon stained sections of examined liver from rats necropised 48 hours after injection. (12, 13, 14, 15, 16, 17) (12): control (no injection), (13): Portal inflammation caused by O157 strain, (14): liver necrosis caused by STEC O157 strain, (13,14) represent clear liver steatosis caused by O114 and O125, (17) damaged tissue caused by O157 strain

(Iman *et al.,* 2010) had illustrated the prevalence's of variant pathogenic *E. coli* pathovar that do not belong to STEC serogroup This study is the first study from Egypt that characterizes at the molecular level strains of shiga-toxin *E.coli* producers isolated from different sources using the PFGE Diversity observed among the strains was explored to further assess their ability to cause disease in vivo. The present study demonstrates that non-O157 STEC comprises 60% of the STEC strains causing infections and were identified from a diverse and separate source, either from human stool and urine specimens collected from diarrheic patient, or from food products such as unprocessed meat products, or from environmental sample (water).

PFGE macrorestriction pattern of XbaI digested DNA from STEC strains was applied to determine the genetic clonal relatedness among the identified STEC strains. A total of 3 macrorestriction PFGE patterns were detected among the non-O157 STEC strains with one pattern shared with the O157 group. The present study concluded that STEC strains with undistinguishable mrp PFGE pattern demonstrated a remarkable variation in their genetic characteristics, serotypes, source as well as their phenotypic resistance profile; a conclusion which corroborate with that reported in (Dean-Nystrom *et al.,*1998) who used PFGE to study clonal diversity among strains with different genetic background and isolated from a cohort of 48 newborn calves, although 97% of isolates belonging to just only two different PFGE patterns, yet, a clear phenotypic and genotypic diversity was noticed among the tested isolates. In the current study, six out of the 10 investigated strains including O157 and non-O157 serotypes shared the same mrp (type I). Sixteen types of antibiotics were tested to examine their resistance profile, and their antibiogram indicated that all 6 STEC strains developed a multi- drug resistance (MDR) pattern to at least four classes of antibiotics including Amoxicillin/Clavulanic Acid 30, Penicillin 10. Spectinomycin Erythromycin 15, Gentamicin 120, Cefazolin 30, Thiampinicol 30, Vancomycin 30, Ciprofloxacin 10. Our study indicated that MDR patterns were more pronounced in STEC isolated from clinical samples such as UTI (O157:EC255,O158:EC158), as well as from meat product (O158:EC306) where resistance to 13 types of antibiotics were noted among these strains, a finding alarm to a serious impact in limiting the selection of treatment drug. This finding corroborated with the study reported in (Mubita *et al.,* 2008), who reported that both clinical and environmental strains displayed MDR phenotype to most of the previously mentioned antibiotics. Many authors documented that the use of antibiotics is strongly associated with the prevalence of antimicrobial resistance in *E. coli* isolates in food-producing animals (Kang *et al.,* 2005). Similar finding has been reported in other *E. coli* pathovar in many other studies from Egypt (Putnam *et al.,* 2004, Shaheen *et al.,* 2004) and different parts of the world (Hoge *et al.,*1998, Okeke *et al.,*2000, Shapiro *et al.,* 2001, Turner *et al.,* 1988) There is an increasing isolation rate of MDR

strains belonged to Enteropathogenic *E. coli* in Nigeria (Okeke *et al.,* 2000), Thailand (Hoge *et al.,* 1998), Kenya (Shapiro *et al.,* 2001) and Israel (Turner *et al.,*1988). Despite the MDR pattern reported in other studies involving traveler's diarrhea, yet susceptibility to ciprofloxacin was noted among the E.coli strains which makes it remain as te drug of choice for the treatment of (Dupont, 2006 and Ericsson, 2003). In the present study there is an increasing incidence of infections caused by non O157 STEC belonged to different serotypes (O25, O114, and O158) which demonstrated a strong association with severe disease upon in vivo challenge of experimental animals. Most studies investigate the systemic disease and the severity of clinical presentation caused by STEC O157, where approximately 10% of infected patients develop complications such as hemolytic uremic syndrome (HUS) characterized by acute renal failure, thrombocytopenia and hemolytic anemia (Djordjevic *et al.,*2003).

The precise sequence of events leading to HUS is still understood incompletely. Because of the lack of a reproducible small animal model for STEC infections, in vivo studies examining STEC–host early interactions are limited and insufficient. We have utilized rat as experimental model to test the abilities of the different STEC O157 and non-O157 serotypes (with different virulence genes: shiga toxins Stx1 and Stx2 in addition to Enteroheamolysin hly (A) and Intimin eae) to induce in vivo toxicity and systemic disease.and death. The data presented herein demonstrate that tested male rat show enhanced susceptibility upon exposure to *E. coli* O157 and non-O157 strains owning different virulence genes (shiga toxin 1and 2 in addition to Enteroheamolysin and Intimin), which has lead to systemic disease and death in a percentage of rats i.p injected with either toxin or whole cell bacterial extracts. The rapid lethality of the examined STEC strains was more likely due to STEC strains that are lacking intimin eae (non-invasive) gene. Stx1 is required to induce disease and causing death but eae was not necessary involved in such outcome (Evelyn *et al.,* 2003). The involvement of Shiga toxin in the development of HUS is suggested by the close association of the syndrome with Shiga toxin-producing strains of S. dysenteriae and enterohemorrhagic *E. coli* (Gunzer *et al.,* 2002). Shiga toxins are responsible for the vascular component of such HUS disease. In support of this concept is a report that Shiga-like toxin caused colonic hemorrhage when presented intravenously to rabbits (Teel *et al.,* 2002). A similar conclusion was drawn by a study that demonstrated that Shiga toxin was required by Shigella. Dysenteriae 1 for colonic vascular damage but was not necessary for S. dysenteriae 1colonization, invasion of colonic epithelium, or production of diarrhea in primates (Dean-Nystrom *et al.,* 1998). It is also known that human endothelial cells, including renal cells, express the glycolipid receptor for Shiga toxins and respond in a cytotoxic manner to these agents (Melton-Celsa *et al.,* 2002). Thus, it appears that hemorrhagic colitis may be required for the development of HUS in allowing entry of Shiga toxin (or Shiga-like toxin into the circulation) (Brando *et al.,* 2008).

Histopathological results of the examined animals in the current study indicated that Intimin is not required for the pathogenicity of non-O157 STEC, disease was evident in all groups of animals inoculated with STEC whether the isolate had intimin or not. This conclusion agrees with others (Evelyn *et al.,* 2003). Who compared the pathogenicity of intimin-negative non-O157:H7 Shiga toxin (Stx)-producing Escherichia coli (STEC) O91:H21 and O104:H21 strains with the pathogenicity of intimin-positive O157:H7 and O157:H_strains in neonatal pigs. Our results indicated that the combination between the two shiga toxins together enhanced the susceptibility of rats and increased the pronounced systemic lesions in three target organs especially in the examined kidneys. This observation was recorded in group of rats injected with strains carrying the two stx (1,2) genes (such as O157:EC255, O114:EC322 isolated from UTI and Sheep stool samples, respectively) where a higher degree of vascular damage and tubular necrosis was noticed as compared to those rats challenged with STEC strains carrying one shiga toxin gene (stx1); a conclusion which agrees with the hypothesis in (Melton-Celsa *et al.,*2002) on the direct correlation between the degree of vascular damage and the quantity of Shiga toxin or Shiga-like toxin produced by these pathogens which reflects the synergistic cytotoxicity between Shiga toxin 1 and 2. Another conclusion is that mucosal lesions were seen in the large intestines of all rats inoculated with either eae negative or eae STEC strains. These lesions occurred both in cells on the surface of the intestine and in crypts in the cecum and colon. A similar absence of goblet cells and discharge of mucus has been seen in STEC-inoculated ligated colonic loops in calves (Schmidt *et al.,* 2000). The presence of STEC-induced changes in porcine intestinal mucus suggests that porcine mucus, like human mucus and mouse mucus (Melton-Celsa *et al.,* 2002, Melton-Celsa *et al.,* 1996). Clear shifting in the goblet cells of the intestine and their replacement with immature cells with large nuclei suggests that shiga toxins may play a role in alternation of goblet cells expressing mechanism. No evident difference was observed between the in vivo damage caused by the O157 STEC and the non O157 STEC, the equivalent intestinal tissues damage caused by non-O157 strains and the O157 strains is a consequence of a balance of the virulence factors produced by the two types of STEC strains (Evelyn *et al.,* 2003). It was also evident that the severity of systemic damage and lesions formation were time dependant, more severe and obvious damage were recorded in later investigations than in the earlier stages of the experiment. An important conclusion is that all types of Styx 1 and Stx2 produced by the strains used in this study cause similar systemic disease and CNS signs in the examined rats. Premortem signs of CNS in all examined animals during the current study merits further investigation of shiga toxins on rats with a special concern to the brain as a vital organ.

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