SEROTYPES AND VIRULENCE PROFILES OF NON-O157 SHIGA TOXIN PRODUCING *E. COLI* ISOLATED FROM BEEF, CHICKEN MEAT AND ITS PRODUCTS

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ABSTRACT

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This study was conducted on 300 samples (150 beef and 150 chicken meat) collected from Menofia, Cairo and El-Kalyobia governorates for detection of STEC. STEC were isolated from beef and chicken meat on Trypticase Soya Broth and Sorbitol MacConkey agar supplemented with cefixime and tellurite supplements and were biochemically identified. Further identifications were performed including Vero cells cytotoxicity assay and PCR technique for specific VT1/VT2 and eae genes. Vero cells cytotoxicity assay was performed on 130 suspected colonies obtained from 300 samples collected from raw meat and meat products (150) and raw chicken and products (150) revealed that 56 of *E. coli* isolates were STEC. By PCR, 56 (100%) of the 56 strains were confirmed to be STEC. In comparison to Vero cells cytotoxicity, the sensitivity of PCR were 100%. The most common serogroups of STEC in samples were O111, O26, O103, O119, O128, O86, O45, O146, O119 and O121. *E.coli* O111, O26, O103, O91, O86 and O119 that proved to have *Stx1* and *Stx2* genes. *E.coli* O128 and O121 had *only Stx1*, while *E.coli* O146 had *only Stx2*.Concerning the *eae* gene responsible for the attaching and effacing lesions, *E. coli* O111 and O26 isolates proved to possess such gene. In conclusion raw beef, raw chicken and products constitute an important reservoir of STEC infection to man and it was declared that PCR technique is the most rapid, sensitive and efficient approach for detection of STEC in beef and chicken products.

Key words: *STEC; Serovars; Genotypes; meat and products*

INTRODUCTION

Pathogenic *E. coli* have been broadly classified into two major categories; the diarrheagenic *E. coli* and the extraintestinal pathogenic *E. coli*. Among the diarrheagenic *E. coli*, there are currently six categories including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusively adherent *E. coli* (DAEC) and enterohemorrhagic *E. coli* (EHEC)/Shiga toxin-producing *E. coli* (STEC) Xiaodong. (2010).

Shiga toxin–producing *E. coli* (STEC), also known as verotoxin-producing *E. coli* (VTEC) or enterohaemorrhagic *E. coli* (EHEC), have been known as a group of highly pathogenic *E.coli* strains producing one or more Shiga toxins (Monaghan *et al., 2011*). The term verocytoxin producing *E. coli* was derived from observation of strains producing a toxin with a profound and irreversible cytopathic effect on Vero cells "African green monkey kidney" (Konowalchuk *et al.,* 1977).

STEC represent a hazardous public health problem worldwide causing various human gastrointestinal tract diseases, including watery or bloody diarrhea and might develop a life-threatening diseases, such as hemorrhagic colitis (HC), Thrombotic Thrombocytopenic Purpura (TTP) and Haemolytic Uraemic Syndrome (HUS). The later is characterized by thrombocytopenia, microangiopathic haemolytic anaemia and acute renal failure (Pennington, *2010*).

STEC strains produce two powerful phage-encoded cytotoxins causing tissue damage in humans and animals, called Shiga toxins or verotoxins (Stx1/VT1 and Stx2/VT2), which are the common feature and main virulence factors of STEC and are directly correlated with human pathogenicity (Lindgren *et al., 1993*). Stx2 is the most powerful toxin, and the toxin producing strains are usually associated with more severe infections (Muniesa *et al., 2004 and* Gyles,

2007). In addition, some STEC strains can tightly attach and form attaching and effacing lesions to intestinal epithelial cells through an adhesin called intimin, which is encoded by the *eae* gene.

The aim of the present study was to determine the occurrence, serovars and virulence gene profile of STEC isolated from raw beef, beef products, raw chicken and chicken products samples collected at the retail level in Egypt.

MATERIALS and METHODS

Isolation of STEC from meat and chicken meat samples:
This study included 300 random locally raw beef (50), 100 produced beef product samples (raw kofta, beef burger, fresh sausage and beef luncheon), raw chicken (50) and 100 produced chicken product samples (chicken burger, chicken sausage and chicken luncheon) were collected from different super markets at Menofia, Cairo and El-Kalyobia governorates, Egypt in clean sterile containers and transported with a minimum of delay to the laboratory.

25 g of each beef product was added into 225 ml of Tryptic Soy Broth and incubated overnight at 37 ^{o}C. Subculture was done from Tryptic Soya broth on Sorbitol MacConkey Agar (SMAC) with cefixime and tellurite to obtain the suspected colonies of the concerned bacteria. The obtained colonies were prepared for VCA to detect STEC. Positive samples were confirmed to be STEC by PCR reaction to determine the type of *Stx* and serotyping. (Konowalchuk *et al.,* 1977).

Vero cell assay of the suspected *E. coli* strains
The cytotoxicity of the suspected *E. coli* isolates for vero cells was determined by using tissue culture supernatant and thereby detecting only high level of production of these cytotoxins based on Konowalchuk *et al.* (1977).

This test was carried out in 96 well tissue culture plates. 90µL of sterile physiological saline was added to each of the test wells, while 50µL of the physiological saline was added to the negative control wells. 60 µL of the bacterial lysates was added to each well. 50µL of RPMI medium containing 10% calf serum, 2mM L-glutamin, 100 U penicillin/ml and 100 µg streptomycin /ml were added to each one of the test wells. A suspension of vero cells was prepared and 50 µL of this suspension was seeded in each well of the test wells. 50 µL of 1% SDS solution was added to each of the positive control wells. The plates were incubated at 37^{o}C in 5% CO2 atmosphere, observed daily by using inverted microscope for detection of cell lysis and vacuolation.

Serotyping *E. coli* isolates
The isolates were serologically identified according to Kok *et al.* (1996) by using rapid diagnostic *E.coli* antisera sets (DENKA SEIKEN Co., Japan) for detection of the Shiga toxin-producing *Escherichia coli* serovars.

Detection of *Stx1, Stx2* and *eae* genes of STEC isolated from samples using Multiplex PCR:
The multiplex PCR was performed as described by Paton and Paton, 1998 at the laboratory of infectious diseases and Internal medicine, faculty of Veterinary Medicine, University of Sadat City, Egypt.

Genomic DNA extraction: Chromosomal DNA was isolated from STEC isolates using
Gene JET Genomic DNA Purification Kit (Fermentas)

DNA amplification for Multiplex PCR reaction.
20 ng of chromosomal DNA was used per reaction, where amplifications were performed in 25ul of buffer solution containing 3uM of oligonucleotides, 200uM of each deoxynucleoside triphosphate, 3.5 mM $MgCl_2$ and 2.5U of DNA Taq polymerase. Mixtures were overlaid with mineral oil and amplification was performed in PCR thermal cycler. Samples were subjected to 35 PCR cycles, each consisting of 1 min of denaturation at95°C; 2 min of annealing at 65°C for the first 10 cycles, decrementing to 60°C by cycle 15; and 1.5 min of elongation at 72°C, incrementing to 2.5 min from cycles 25 to 35. Amplified DNA fragments were resolved by gel electrophoresis (Sambrook *et al.,* 1989) using 2 % (w/v) agarose. Gels were stained with 0.5 mg of ethidium bromide per ml for 15 min, and documented with a UVP documentation system.

Table 1: Primer sequence of shiga toxin producing *E.coli*.

Gene	Primer sequence	Predicted size	Reference
Stx1	5'- ATAAATCGCCATTCGTTGACTAC -3' 5'- AGAACGCCCACTGAGATCATC - 3'	180 bp	Paton and Paton (1998)
Stx2	5'- GGCACTGTCTGAAACTGCTCC -3' 5'- TCGCCAGTTATCTGACATTCTG -3'	255 bp	Paton and Paton (1998)
eae	5 ' GCATCACAAGCGTACGTTCC 3 ' 5' CCACCTGCAGCAACAAGAGG 3'	384 bp	Paton and Paton (1998)

RESULTS

Table 2: Comparison of the results of cultivation on SMAC medium with VCA and multiplex polymerase chain reaction (PCR) for detection of STEC in raw beef, beef products, raw chicken and chicken products.

Samples	No. of examined samples	No. of +ve colonies on SMA medium	No. of samples +ve VCA.	No. of samples tested by PCR and were +ve VCA.
Raw beef	**50**	29	15 (51.72 %)	15 (100 %)
Beef products	**100**	44	18 (40.90%)	18 (100 %)
Raw chicken	**50**	22	10 (45.45%)	10 (100 %)
Chicken products	**100**	35	13 (37.14%)	13 (100 %)
Total	**300**	130	56 (42.08%)	56 (100 %)

Table 3: Incidence of Shiga toxin producing *E. coli* (STEC) serovars isolated from examined meat and its products samples.

E.coli Serovars	Raw beef	Beef products	Raw chicken	Chicken products
O111	4	5	3	4
O26	3	3	1	2
O103	1	1	1	--
O91	--	2	--	2
O119	1	--	1	--
O128	2	2	1	2
O86	2	1	1	1
O146	1	1	--	1
O45	--	1	2	--
O121	1	2	--	2

Table 4: Occurrence of some virulence genes in serovars of Shiga toxin-producing *E. coli* (STEC) isolated from raw beef and beef products.

Serovars	No. of examined isolates	Stx1 alone		Stx2 alone		Stx1&Stx2		eae	
		NO.	%	NO.	%	No.	%	No.	%
O111	8	0.0	0.0	4	50	4	50	6	75
O26	4	4	100	4	100	4	100	0.0	0.0
O103	3	0.0	0.0	3	100	0.0	0.0	0.0	0.0
Other STEC	18	8	44.4	10	55.5	0.0	0.0	0.0	0.0
Total	33	12	36.6	21	63.6	8	24.3	6	18.2

Table 5: Occurrence of some virulence genes in serovars of Shiga toxin-producing *E. coli* (STEC) from raw chicken and chicken products.

Serovars	No. of examined isolates	Stx1 alone		Stx2 alone		Stx1&Stx2		eae	
		NO.	%	NO.	%	No.	%	No.	%
O111	6	6	100	6	100	6	100	3	50
O26	4	4	100	2	50	0.0	0.0	0.0	0.0
Other	13	4	30.7	9	69.2	0.0	0.0	0.0	0.0
Total	23	14	60.9	17	73.9	6	26.1	3	13.0

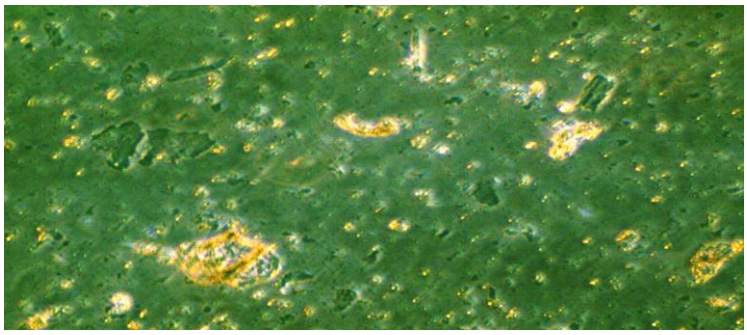

Photo 1: Cytotoxic effect of Shiga toxin containing bacterial lysate of STEC on Vero cells.

The Cytopathic effects of Shiga toxin containing bacterial lysate of STEC were observed after incubation with culture filtrates there was a change from spindle-shaped cells characteristic of normal Vero cells to round and shriveled cells, and these changes were followed by gradual destruction of the monolayer.

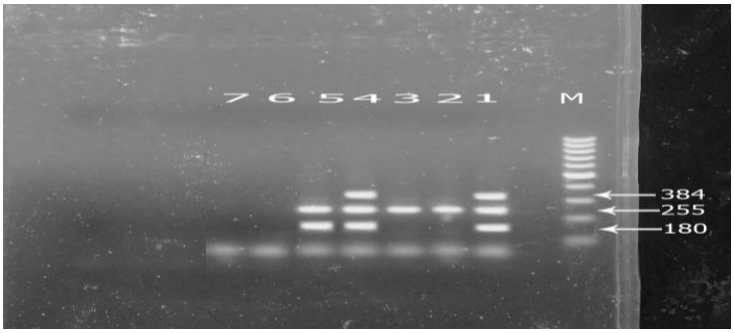

Figure 2: Agarose gel shows six positive strains of EHEC for shiga toxin 1 and shiga toxin 2 and eae genes. 180 bp, 255bp, 384 bp respectively isolated from beef.
Lane (M): MW marker = 100 bp DNA ladder (Promega).
Lane (1): Positive Control *(E. coli O157H7* provided by Animal Health research Institute, Egypt).
Lane 2- O_{86} has stx2 genes
Lane 3- O_{121} has stx2 genes
Lane 4- O_{111} has the 3 genes stx1, stx2 and eae genes
Lane 5- O_{103} harbor stx1 and stx2genes
Lane 6- Negative Control.

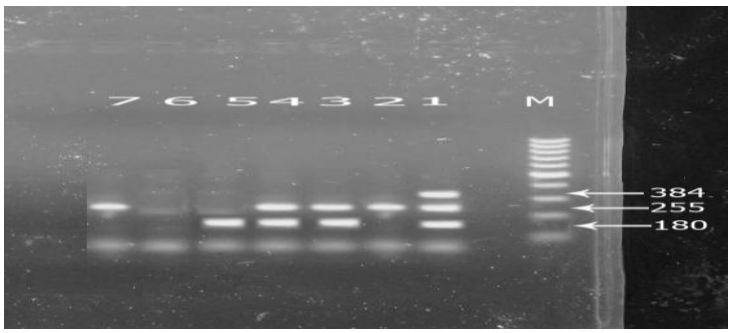

Figure 3 Agarose gel shows six positive strains of EHEC for shiga toxin 1 and shiga toxin 2 and eae genes. 180 bp, 255bp, 384 bp respectively isolated from chicken.
Lane (M): MW marker = 100 bp DNA ladder (Promega).
Lane (1): Positive Control *(E. coli O157H7* provided by Animal Health research Institute, Egypt).
Lane 2- O_{146} harbor stx2 genes
Lane 3- O_{91} harbor stx1 and stx2genes
Lane 4- O_{103} harbor stx1 and stx2genes
Lane 5- O_{45} has stx1 genes
Lane 6- negative control.
Lane 7- O_{86} has stx2 genes

DISCUSSION

Shiga toxin-producing *E. coli* (STEC) is a serious public health concern worldwide. This pathogen causes diarrhea, hemorrhagic colitis and hemolytic-uremic syndrome. Shiga toxin produced by STEC has been considered a prime virulence factor. Shiga toxins are classified into two groups, *Stx1* and *Stx2*, on the basis of immunological properties. Though O157:H7 is the most predominant serovars isolated from sporadic cases and outbreaks, more than 100 serovars of non-O157 STEC have been isolated from animals and humans (Abd –EL-All, 2005). Since most of the food poisonings due to STEC are related to the consumption of beef or beef products, cattle have been considered a major reservoir of STEC. However, other vehicles, such as contaminated water, vegetables, and fruits, have been increasingly recognized as an infection source of STEC (Shima *et al.,* 2006).

The results recorded in Table (2) showed that from 300 meat samples collected from raw beef (50), 100 produced beef product samples, raw chicken (50) and 100 produced chicken product samples were collected from different super markets at Menofia, Cairo and El-Kalyobia governorate, 130 samples yielded positive culture SMAC-CT. Further identifications of the isolated colonies were performed by Vero cells cytotoxicity assay which revealed that 56 of *E. coli* isolates (42.08%) were verotoxin producing *E. coli*. The results obtained in this study agreed with Ramotar *et al.* (1995) who reported that SMAC was positive for only 30 % of verocytotoxin-positive samples. In comparison to Vero cells cytotoxicity, the sensitivity of PCR were 100 %. PCR test was compared with Vero cytotoxicity assay for a number of reasons. Firstly, the profound sensitivity of Vero cells to *Stx* which was first observed by Konowalchuk *et al.* (1977), Secondly, the cytotoxicity for this cell line remains the "gold standard" for confirmation of putative STX-producing isolates (Byomi, 1995). In comparison of PCR and Vero cells cytotoxicity 56 out of 56 (100 %) positive cases by PCR were also positive by Vero cells cytotoxicity. The usefulness of PCR requires no emphasis as a means for detection of shiga toxins encoding genes from the DNA material extracted from meat and products. Interestingly, the results obtained in this study agreed with that of Ramotar *et al.* (1995) who evaluated a method for rapid detection of verotoxin-producing *E. coli* in stool samples by PCR and detected 34 of 36 (94%) of samples that were positive by colony blot and free verotoxin (FVT) that was performed by using vero cell monolayers. Similarly, Zaki and El-Adrosy (2007) reported that PCR is sensitive and fast method for detection of STEC.

The Cytotoxic effect of shiga toxin cotaining bacterial lysate on vero cells was illustrated in photo (1). In the present study vero cytotoxicity assays was used as screening test for STEC. The test was done only on samples that gave characteristic colonies on sorbitol monitol agar plates. Detection of STEC was done on basis of positive VCA. The positive samples were confirmed by serotyping using polyvalent and monovalent "O" *Escherchia coli* antisera. Further confirmation was done by using multiplex PCR reaction to determine the type of *Stx*.

Paton and Paton, (1998) stated that Vero cytotoxicity assay has played an important role in establishing a diagnosis of STEC infection, particularly where subsequent isolation of the causative organism has proven to be a difficult task. When testing such crude samples, the sensitivity is influenced by the abundance of STEC, the total amount and potency of the STX produced by the organism concerned, and the degree to which the particular STX is released from the bacterial cells. PCR provide rapid and valuable diagnostic method while, detection of *Stx* by tissue culture cytotoxicity is labor-intensive, time-consuming, and cumbersome. Not all microbiology laboratories perform tissue culture work with Vero cell monolayers available on demand. Moreover, rapid diagnosis is important, and the results of cytotoxicity testing are generally not available befor 48 to 72 hrs. (Paton and Paton, 1998). The current results agree, to some extent, with those recorded by Hussein & Bollinger (2005) and Hussein (2007) as they found non O157 STEC to be more prevalent in beef products than *E. coli* O157. The prevalence rates of non O157 STEC ranged from 2.4 to 30.0% in ground beef, from 17.0 to 49.2% in sausage. Testing other beef products revealed prevalence rates of 19.0% (Zhao *et al.,* 2001) and 62.5% (Samadpour *et al.,* 1994).

However, (Smith and Scotland, 1988.) pointed out that the two examined samples were positive VCA and were confirmed to be non-STEC, Since the presence of cytoxicity in a crude filtrate could be due to other bacterial products or toxins, positive samples should always be confirmed and typed by testing for neutralization of cytotoxicity by specific preferably monoclonal antibodies to *Stx1* or S*tx2*. Moreover, Abd-El-Latif (2003) detected two STEC which were positive for VCA while only one of them was positive to PCR.

Table (3) revealed that the serological identification of shiga-toxin producing *E.coli* isolated from the examined raw beef samples were O111, O26, O103, O119, O128, O86, O146 and O121, from beef products samples, the isolated serovars O111, O26, O91, O103 ,O86,O121,O128 O146 and O45. But the isolated serovars from raw chicken were O111, O26, O103, O119, O128, O86 and O45, while those from chicken products were O111, O26, O91, O128, O86, O121 and O146.

Fantelli and Stephan (2001) detected EHEC or STEC in 2.3% of minced meat samples, while Abd-El-Latif (2003) isolated EHEC from minced meat, burger and sausage in 16% of the samples.

Shiga toxin producing *E. coli* (STEC) organism of different serovars have been isolated from human and from apparently healthy domestic animals .Many of these isolates were typical STEC belonging to serovars O26, O111 and O157 (Karamali, 1989). Also, verotoxin producing *E. coli* (VTEC) non O157 serovars O26, O103, O111 are among the most important emergency food borne pathogen groups particularly O26 which able to cause large spectrum of illness in human as hemorrhagic colitis (HC) to hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Dambrosio *et al.,* 2007).

Enterohaemorragic *Escherichia coli* (EHEC) constitutes a subset of STEC serovars including *E.coli* O157 and non - O157 serogroups like O26, O111, O103, and O145. STEC may be transmitted from animal reservoirs to human not only via ingestion of contaminated food or water but also by contact with STEC-positive animal or with their environment (Alfredo *et al.,* 2005).

Enterohaemorrhagic *E.coli* (EHEC) produces two types of illness, haemorrhagic colitis and hemolytic uraemic syndrome (HUS). Haemorrhagic colitis results from colonic mucosal oedema, errosion and haemorrhage. The incubation period is 3 to 4 days. The symptoms start by sudden pain followed by watery diarrhea, nausea and vomition in the early stages of illness and abdominal distension with severe pain after the onset, disease progress over 2 days to bloody diarrhea. Haemorrhagic colitis was primarily foodbrone and was associated most frequently with *E. coli* as recorded by Riley (1987), Bhong *et al.* (2008), Lee *et al.* (2009) and Xiaodong (2010).

Table [4&5] illustrates STEC isolated from meat product samples have virulence genes. The use of Multiplex PCR with specific primers for *Stx1, Stx2* and *eae* genes revealed the presence or absence of these genes in the tested isolates. The obtained results showed that the isolates *E. coli* O111, O26, O103, O91, O86 and O119 had *Stx1* and *Stx2* genes while, *E.coli* O128 and O121 had only *Stx1*. *E.coli* O146 had only *Stx2*.Concerning the *eae* gene responsible for the attaching and effacing lesions, *E. coli* O111 and O26 isolates possessed this gene.

According to, Hornitzky *et al.* (2002); Jenkenis *et al.* (2002) and Bollinger (2004) stated that serotypes O111, O26, O103, O128, O121, O91, O86 and O119 are Shiga toxin-producing *E. coli* (STEC). All of the STEC isolates produced 1, 2, 3 or 4 virulence factors (i.e. *Stx1, Stx2, Stx1&stx2* or *eae*) and were lethal to Vero (African green monkey cells). Therefore, the potential public health risk of these isolates should not be ignored.

In Egypt, many studies have been reported the prevalence of E.coli O157 in meat or milk products (Sayed *et al.,* 2001; Mohammed, 2002, and Abd-Ell-All, 2005) while, few studies have reported the prevalence of non-O157 (Byomi *et al.,* 2001 and Abd-El-All, 2005).

Bettleheim (2000) reported that STEC serovars other than O157H7, such as O111, O103, O26, and O145 are emerging human pathogens predominantly in Europe, Australia, and South America.

On conclusion, the raw beef and Chicken meat and its products were contaminated with non-O157 shiga toxin producing *E.coli*. By using the PCR assay on the basis of Stx1 and Stx2 genes is a more practical and reliable method for molecular epidemiological studies of STEC strains because of its ability to determine, meat and products should be considered a major reservoir of STEC.

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تصنيف وتوصيف العترات الحقلية المصرية للميكروب القولونى المفرز لتوكسين شيجا فى اللحوم والدواجن ومنتجاتها

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اجريت هذه الدراسة علي عدد ٣٠٠ عينة شملت اللحوم ومنتجاتها (١٥٠ عينة) والدواجن ومنتجاتها (١٥٠ عينة) تم تجميعها من محافظة المنوفية والقاهرة. تم فحص العينات معمليا لعزل الميكروب القولوني المفرز لتوكسين شيجا أولا بطريقة العزل علي الوسط المخصص (SMAC-Media) والذى أسفر عن وجو١٣٠عينة ايجابية للميكروب القولوني تم تأكيدها باستخدام الطرق البيوكيميائية. كذلك تم اختبار العينات الايجابية باستخدام vero cell assay (VCA) لتحديد العترات المفرزة لتوكسين شيجا. بعد ذلك تم تصنيف العينات الايجابية لاختبار VCA سيرولوجيا وكذلك تم تأكيدها باستخدام تفاعل البلمرة المتسلسل المتعدد (Multiplex PCR) لتحديد نوع الجين المسئول عن افراز سموم شيجا. هذا وقد أسفر فحص العينات عن تواجد ٥٦ عينة ايجابية لاختبارVCA تم تأكيدهم جميعا بالاختبارات السيرولوجية وكذلك باستخدام تفاعل البلمرة المتسلسل المتعدد (Multiplex PCR) بنسبة ١٠٠% مما يؤكد كفاءة وسرعة اختبار تفاعل البلمرة المتسلسل في تشخيص الميكروب القولونى المفرز لتوكسين شيجا. كما أسفر استخدام تفاعل البلمرة المتسلسل المتعدد (Multiplex PCR) بواسطة بادئات لجينات شيجا توكسين١ *Stx1* وشيجا توكسين٢ *Stx2* و *eae* عن وجود او غياب هذه الجينات في العينات المعزولة. وكانت أكثر المعزولات من اللحوم والدواجن هي O111, O26, O103, O119, O128, O86, O45, O146, O119 and O121. *E.coli* O111, O26, O103, O91, O86 and O119. بالاضافة الى ما سبق لوحظ ارتفاع معدل الاصابة في الاشخاص والحيوانات المصابة بالاسهال عن الاشخاص والحيوانات السليمة ظاهريا مما يؤكد علي دور الميكوب القولوني المفرز لتوكسين شيجا في حدوث الاسهال في الانسان.