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PREVALENCE AND MOLECULAR CHARACTERIZATION OF SHIGA TOXIN PRODUCING *E. COLI* ISOLATED FROM SOME LOCALLY PRODUCED BEEF PRODUCTS

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Abstract: -

Shiga toxin-producing *Escherichia coli* (STEC) may cause severe gastrointestinal and systematic diseases in humans that result from consumption of meat products. STEC were isolated from some beef products 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. It was obvious from the obtained resuts that the incidance of shiga-toxin producing *E. coli* were 22%, 12%, 10%, 16% and 2% in minced meat, beef burger, beef sausage, beef kofta and beef luncheon, respectively. The serovars of shiga-toxin producing *E. coli* isolated from the examined meat 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 beef beef products constitute an important reservoir of STEC infection to man and it was decleared that PCR technique is the most rapid, sensitive and efficient approach for detection of STEC in beef products.

Keywords: STEC; Serovars; Genotypes; beef products

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INTRODUCTION

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***). STEC represent a hazardous public health problem worldwide causing various human gastrointestinal tract diseases,including watery or bloody diarrhoea, and might develop a lifethreatening disease, such as haemorrhagic colitis"HC", thrombotic thrombocytopenic purpura "TTP" and haemolytic uraemic syndrome "HUS"(***Pennington., 2010***).

Shiga toxin-producing *Escherichia coli* (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 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 beef products samples collected at the retail level in Egypt.

Material and methods

Isolation of STEC from meat product samples:

This study included 250 random locally produced beef product samples (50 each of minced beef, raw kofta, beef burger, fresh sausage and beef 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 ºC. 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 37ºC 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 beef product 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 GeneJET 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.

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 (1): Incidence of Shiga toxin producing *E. coli* (STEC) in the examined beef product samples using Vero-Cytotoxicty assay and Multiplex PCR.

Beef products	No. of examined samples	No. of positive STEC samples	
		VCA	Multiplex PCR
Minced meat	50	14 (28%)	11 (22%)
Beef burger	50	7 (14%)	6 (12%)
Beef sausage	50	7 (14%)	5 (10%)
Beef kofta	50	9 (18%)	8 (16%)
Beef luncheon	50	1 (2%)	1 (2%)

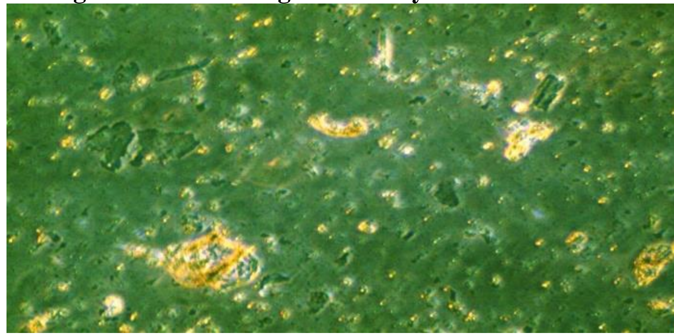
Table (2): Incidence of Shiga toxin producing *E. coli* (STEC) serovars isolated from examined beef product samples.

E. coli Serovars	Meat products				
	Minced meat	Beef burger	Beef sausage	Beef kofta	Beef luncheon
O111	3	1	1	2	--
O26	1	1	--	1	--
O103	1	1	1	--	--
O91	--	--	--	1	--
O119	1	--	1	--	--
O128	2	--	--	2	1
O86	1	1	1	1	--
O146	1	1	--	1	--
O45	--	1	1	--	--
O121	1	--	--	--	--
Total	11	7	7	9	1

Table (3): Occurrence of some virulence genes in serovars of Shiga toxin-producing *E.coli* (STEC) isolated examined meat product samples.

Serovars	No. of ex. isolates	Stx1 alone		Stx2 alone		Stx1&Stx2		Eae	
		NO.	%	NO.	%	No.	%	No.	%
O111	7	6	85.7	7	100	6	85.7	5	71.4
O26	3	2	66.6	3	100	2	66.6	2	66.6
O103	3	1	33.3	2	66.6	2	66.6	0.0	0.0
O91	1	1	100	1	100	1	100	0.0	0.0
O119	2	1	50	2	100	1	50	0.0	0.0
O128	5	5	100	0.0	0.0	0.0	0.0	0.0	0.0
O86	4	0.0	0.0	4	100	0.0	0.0	0.0	0.0
O146	3	0.0	0.0	3	100	0.0	0.0	0.0	0.0
O45	2	2	100	0.0	0.0	0.0	0.0	0.0	0.0
O121	1	0.0	0.0	1	0.0	0.0	0.0	0.0	0.0

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.

Photo (2) Agarose gel shows five positive strains of EHEC for shiga toxin 1 and shiga toxin 2 and eae genes. 180 bp, 255bp, 384 bp respectively.
Lane (M): MW marker = 100 bp DNA ladder (Promega).
Lane 1- O_{111} has the 3 genes stx1, stx2 and eae genes
Lane 2- O_{86} has stx2 genes
Lane 3- O_{121} has stx2 genes
Lane 4- O_{26} has the 3 genes stx1, stx2 and eae genes
Lane 5- O_{103} harbor stx1 and stx2genes
Lane 6- negative control

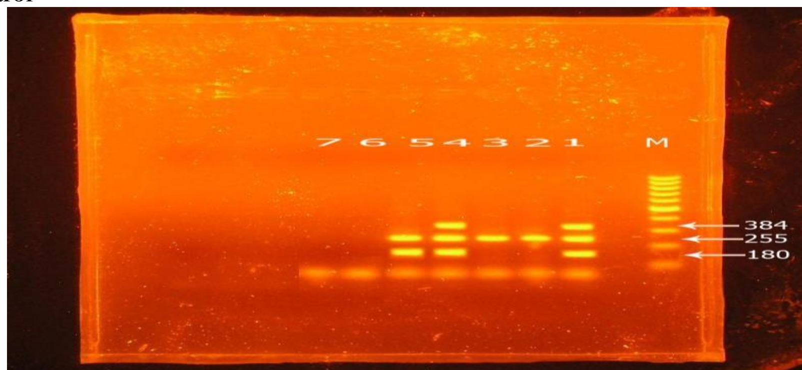


Photo (3) Agarose gel shows six positive strains of EHEC for shiga toxin 1 and shiga toxin 2 and eae genes. 180 bp, 255bp, 384 bp respectively.
Lane (M): MW marker = 100 bp DNA ladder (Promega).
Lane 1- O_{111} has the 3 genes stx1, stx2 and eae genes
Lane 2- O_{146} harbor stx2 genes
Lane 3- O_{91} 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

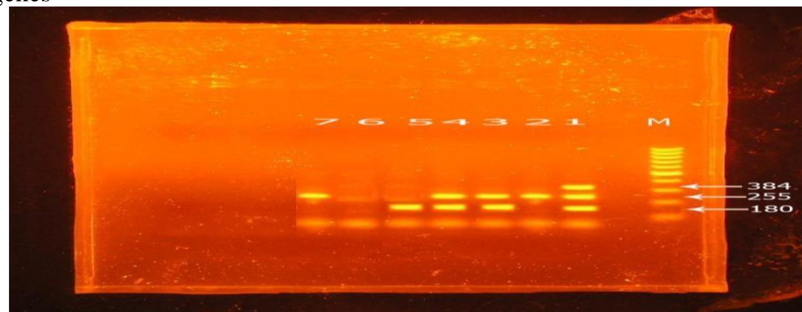
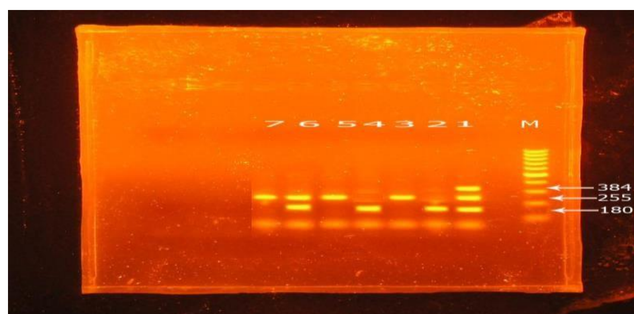


Photo (4) Agarose gel shows seven positive strains of EHEC for shiga toxin 1 and shiga toxin 2 and eae genes. 180 bp, 255bp, 384 bp respectively.
Lane (M): MW marker = 100 bp DNA ladder (Promega).
Lane 1- O_{111} has the 3 genes stx1, stx2 and eae genes
Lane 2- O_{128} harbor stx1 genes
Lane 3- O_{146} harbor stx2 genes

Lane 4- O_{45} harbor stx1 genes
Lane 5- O_{86} has stx2 genes
Lane 6- O_{119} harbor stx1 and stx2genes
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 serovare 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 occurrence of Shiga toxin-producing *E. coli* (STEC) in beef products as examined by Vero Cytotoxicity Assay (VCA) and Multiplex Polymerase Chain Reaction was illustrated in table (1).
The obtained results revealed that 28%, 14%, 14%, 18% and 2% of minced meat, beef burger, beef sausage, beef kofta and beef luncheon respectively were contaminated with *shiga toxin producig E. coli* using Vero-Cytotoxicty assay method assay.

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*.

The profound sensitivity of Vero cells to *Stx* was first observed by **Konowalchuk et al. (1977**), and cytotoxicity for this cell line remains the "gold standard" for confirmation of positive STX-producing isolates **(Byomi, 1991).**

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.

Table (1) revealed that shiga-toxin producing *E. coli* also can be detected by Mutiplex PCR in minced meat, beef burger, beef sausage, beef kofta and beef luncheon by the following ratio 22%, 12%, 10%, 16% and 2% respectively. 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% (**Samadpouret 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 *Stx2***.** Moreover, **Abd-El-Latif (2003)** detected two STEC which were positive for VCA while only one of them was positive to PCR. Table (2) revealed that the serological identification of shiga-toxin producing *E.coli* isolated from the examined minced meat samples were O111, O26, O103 , O119 ,O128 ,O86, O146 and O121, from beef burger samples, the isolated serovars O111, O26 , O103 ,O86, O146 and O45. But the isolated serovars from beef sausage were O111, O103 , O119 O86 and O45, while those from beef kofta were O111, O26 , O91 ,O128 ,O86, and O146. finally the only one serovars isolated from beef luncheon was O119.

Fantelli and Stephan (2001) detected EHEC or STECin 2.3% of minced meat samples, while **Saleh (2001)** 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 [3] illustrates STEC isolated from meat product samples have virulence genes.The use of Multiplex PCR with specific primers for *Stx1, Stx2 ,eae* and ehly 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) ; Bollinger (2004) and Hassanain and Ahmed (2008**) 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.

Photo (2) agarose gel shows five strains of EHEC positive for shiga toxin 1 and shiga toxin 2 and eae genes. At size180 bp, 255bp, 384 bp. respectively. Lane 1 and 4 include O111 and O26 which contain the three genes stx1, stx2 and eae so it contains 3 bands. Lane 2 and 3 contain PCR products of O86 and O121 it carries stx2 genes so we can detect 1 band appear in the agarose while lane 5 include O103, it carries stx1 and stx2 genes so we can detect 2 bands.

Photo(3) agarose gel shows six strains of EHEC positive for shiga toxin 1 and shiga toxin 2 and eae genes. At size 180 bp, 255bp, 384 bp. respectively. Lane 1include O111 which contain the three genes stx1,stx2 and eae so it contains 3 bands. Lane 2 and 7 contain PCR products of O146 and O86, it carry stx2 genes so we can detect 1 bands appear in the agarose. Lane 3 and 4 contain PCR products of O91 and O103 , it carry stx1 and stx2 genes so we can detect 2 bands.

Photo(4) agarose gel shows seven strains of EHEC positive for shiga toxin 1 and shiga toxin 2 and eae genes. At size 180 bp, 255bp, 384 bp. respectively. Lane 1include O111 which contain the three genes stx1,stx2 and eae so it contains 3 bands. Lane 2 and 4 contain PCR products of O128 and O45, it carry stx1 genes so we can detect 1 bands appear in the agarose. Lane 3, 5 and 7 contain PCR products of O146 and O86, it carry stx2 genes so we can detect 1 bands appear in the agarose. Lane 6 contain PCR products of O119, it carry stx1 and stx2 genes so we can detect 2 bands.

In Egypt, many studies have been reported the prevalence of *E.coli* O157 in meat or milk products **(Sayed et al.,2001; Mohammed, 2002, , Abd-Ell-All, 2005 and Hassan and ELMalt, 2008)** 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.

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