A COHORT STUDY ON SHIGA TOXIN PRODUCING *E. COLI* O157:H7 ISOLATED FROM SOME MEAT PRODUCTS IN ASSIUT GOVERNORATE AS A CAUSE OF BLOODY DIARRHEA IN CHILDREN

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ABSTRACT

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The present study was conducted to investigate the presence of *E. coli* especially *E. coli* O157:H7 and to detect the presence of the stx1 and stx2 genes in isolates derived from a total of 80 samples including 20 samples each of frozen beef burgers, frozen sausages, beef burger sandwiches and sausage sandwiches. The samples were randomly collected from retail supermarkets and restaurants in Assiut, Egypt. In addition, 20 stool cultures collected from hospitalized children admitted in Assiut Pediatric University Hospital with history of diarrhea or fever. *E. coli* was detected in 9 (45%), 6 (30%), 1 (5%), 1 (5%) and 12 (60%) of frozen beef burgers, frozen sausages, beef burger sandwiches, sausage sandwiches and children stool samples, respectively. *E. coli* O157 was detected in eleven of the 100 (11%) samples tested (two from frozen beef burgers, three from frozen sausages, one from each of beef burger sandwiches and sausage sandwiches and four from children stool samples). Whereas H7 gene was not detected in all *E. coli* O157 positive samples, but, the genes stx1 and stx2 were detected in two *E. coli* O157 isolates obtained from two frozen sausage samples. The public health significance of this pathogen and consumer's safety were discussed.

Key words: *Escherichia coli, E. coli O157:H7, shiga toxin, beef burgers, sausages, and children stools*

INTRODUCTION

The microbiological safety of meat products is an important public health concern. Numerous epidemiological reports have identified pathogenic *E. coli*, particularly *E. coli* O157:H7, as major cause of disease outbreaks associated with contaminated meat (Olsvik *et al*., 1991; Meng and Doyle, 1998).

The strains of enterohemorrhagic *E. coli* (EHEC) are a subset of the Shiga-toxin-producing *E. coli* (STEC) strains that cause disease in humans and pose a threat to public health worldwide (Griffin, 1995). The serogroup O157 and O157:H7, in particular, have caused a number of human infections, through the consumption of foods of animal origin, particularly those originating from cattle, e.g., minced/comminuted beef (Williams *et al*., 2005). The pathogenicity of *E. coli* O157 and O157:H7 including STEC is associated with several virulence factors. The main factor contributing to their pathogenicity is their capacity to produce two potent phage-encoded cytotoxins called Shiga-toxins (namely, Stx1 and Stx2). Shiga-toxins cause disease, such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), through cytopathic effects on the vascular endothelial cells of the kidneys, intestines, the central nervous system, and other organs. In addition to the production of toxins, another virulence-associated factor expressed by STEC is a protein called intimin, which is encoded by the eae gene and is responsible for the intimate attachment of the STEC to the intestinal epithelial cells. This further causes attaching and effacing lesions in the intestinal mucosa (Garrido *et al*., 2006).

In 1982, *E. coli* O157 was first identified as a food-borne pathogen found in contaminated hamburger (Riley and Remis, 1983). Cattle and other ruminants have been established as the major natural reservoir for *E. coli* O157 (Rassmussen *et al.,* 1993) and thus play a significant role in the epidemiology of human infections (Griffin and Tauxe, 1991). Many environmental and food-borne sources have caused *E.*

coli O157 infections in humans through either consumption of food that is bovine in origin (Slutsker *et al*., 1988).

In 2007, the incidence of O157 and non- O157 STEC infections in the United States was 1.19 and 0.59 per 100,000 people, respectively (Centers for Disease Control and Prevention, 2009). The incidence of STEC infections in the European Union in 2006 and 2007 was 1.1 and 0.6 per 100,000 respectively (European Food Safety Authority (EFSA, 2009). About 70% of cases with human STEC infection in the U.S. and 50% in the E.U. were attributed to STEC O157 (EFSA, 2009). Besides the O157 serotype, in 2005 about 400 serotypes of STEC were known to be associated with illness in humans as agents of diarrhea, HC and HUS (Scheutz and Strockbine, 2005). In the light of the foregoing, this study therefore aimed at determining the presence of both *E. coli* and *E. coli* O157:H7 in raw and ready-to- eat (RET) meat products and children stool in Assuit Governorate, and to detect the harborage of stx1 and stx2 genes using the polymerase chain reaction (PCR).

MATERIAL and METHODS

Collection of samples:
The food samples examined were obtained from different supermarkets and shops selling ready-to-eat meat in Assiut Governorate. The samples were grouped in three categories. The first category consisted of 40 frozen meat product samples (20 samples each of beef burgers and sausages). The second category consisted of 40 thermally processed sandwiches (20 samples each of beef burgers and sausages). The third category consisted of 20 stool samples collected from diarrheic children from different clinical laboratories and hospitals in Assiut Governorate. Samples were transferred directly without delay to the laboratory in an ice box for bacteriological examination.

Preparation of samples:
At the laboratory, fresh samples were processed upon delivery. The frozen samples were thawed by overnight refrigeration; each sample was aseptically and carefully freed from its casing and mixed thoroughly in sterile mortar.

Bacterial isolation (De Boor and Heuvelink, 2000):

Selective enrichment:
For enrichment, Ten grams of each meat product samples as well as swabs from children stools were aseptically added to 90 milliliters of modified Tryptic Soya Broth (TSB/CM129, Oxoid, UK) supplemented with 20 mg/L Novobiocin (Sigma, Germany). The meat samples were homogenized into a stomacher bag for at least 2 min. into a stomacher (Colworth, 400) and then they were incubated at 37°C for 24 h.

Selective plating:
Loopful from the incubated broth was streaked onto the surface of Eosin Methylene Blue agar (EMB) (Oxoid, CM69) to presumptively identify isolates as Gram-negative enteric bacteria and presumptive *E. coli* (green-metallic colonies), and onto Sorbitol MacConkey agar (SMAC) (Oxoid, CM813) to test for sorbitol non-fermenting bacteria (colorless colonies). After 18 to 24 h at 37°C, characteristic colonies from EMB agar and SMAC agar were transferred onto Trypticase Soy agar (TSA) (Oxoid CM131) for further identification.

Identification of isolates:
Isolated purified strains were identified morphologically by Gram's stain and biochemically confirmed as *E. coli* according to Varnam and Evans (1991) by the conventional IMViC, Urea hydrolysis, Triple sugar iron agar and fermentation of sugars (sorbitol).

Serological identification of *E. coli* O157:H7 (Chan *et al.,* 2005):
The biochemically identified non sorbitol fermenting colonies from SMAC were subjected to slide agglutination with the *E. coli* O157 latex test kit (Oxoid, DR620 M) and the agglutinating colonies were further processed for definite confirmation.

PCR assay:
Target genes and oligonucleotide sequences used for PCRs are listed in Table 1, and three pairs of primers were chosen according to (Radu *et al*., 2000). The suspected bacterial colony was subcultured onto TSB and incubated overnight at 37°C for DNA extraction according to manufacturer's instructions (QIAGEN Lot No. 136243308). PCR reactions were performed with Thermal cycler (Biometra,T professional) using a total volume of 25 µl. The optimal amplification reaction mixture contained 12.5 µl of master mix, 1 µl of forward primer (10 pmol), 1 µl of reverse primer (10 pmol), 7 µl of template DNA, 3.5µl of nuclease-free water. Go Taq® Green Master Mix is a premixed ready-to-use solution (Promega, USA):608-274 - 4330).

The PCR samples were subjected to amplification according to the following program: initial denaturation at 94°C for 5 min. and then 40 cycles comprising denaturation at 94°C for 40 seconds, annealing at 50°C for 40 seconds; and an extension at 72°C for 1 min. Following this, a final extension at 72°C for 5 minutes was carried out. The electrophoresis products were visualized by UV transilluminator (Biometra) and photographed by Gel Documentation System including BioDocAnalyze (BDA) Software (Biometra) for measuring and analyzing the PCR results.

Table 1: Primers used to amplify fragments of genes responsible for expression of different virulence factors genes and a species specific gene for *E. coli*

Target gene	Primer sequence	Amplicon product (bp)
SLT-I	SLTIR5'CAGTTAATGTGGTGGCGAAGG-3' SLTIF5'CACCAGACAATGTAACCGCTG-3'	384
SLT-II	SLTIIR5'ATCCTATTCCCGGGAGTTTACG-3' SLTIIF5'GCGTCATCGTATACACAGGAGC-3'	584
FlicH7	FLICH7R5'GCGCTGTCGAGTTCTATCGAGC-3' FlicH7F5'CAACGGTGACTTTATCGCCATTCC-3'	625

RESULTS

The obtained results are recorded in Tables 2- 4 and Fig. 1&2

Table 2: Isolation rate of *E. coli* from different meat product and children stool samples.

Type of samples	No. of examined samples	*Positive samples*	
		No.	%
Frozen beef burgers	20	9	45
Frozen sausages	20	6	30
Cooked beef burgers	20	1	5
Cooked sausages	20	1	5
Children stools	20	12	60
Total	100	29	29

Table 3: Incidence of *E. coli* O157 in different meat product and children stool samples by Latex Agglutination test.

Type of samples	No. of examined samples	*Positive samples*	
		No.	%
Frozen beef burgers	20	2	10
Frozen sausages	20	3	15
Cooked beef burgers	20	1	5
Cooked sausages	20	1	5
Children stools	20	4	20
Total	100	11	11

Table 4: Characterization of *E. coli* O157 virulence factors (SLT1 & SLT2) and flagellar (H7) genes by PCR from different samples.

Type of samples	No. of samples	No. of *E. coli* O157		*SLtx-I contain isolates*		*SLtx-II contain isolates*		*H7 gene*	
		No.	%	No.	%	No.	%	No.	%
Frozen beef burgers	20	2	10	-	-	-	-	-	-
Frozen sausages	20	3	15	1	5	1	5	-	-
Cooked beef burgers	20	1	5	-	-	-	-	-	-
Cooked sausages	20	1	5	-	-	-	-	-	-
Children stools	20	4	20	-	-	-	-	-	-
Total	100	11	11	1	1	1	1	0	0

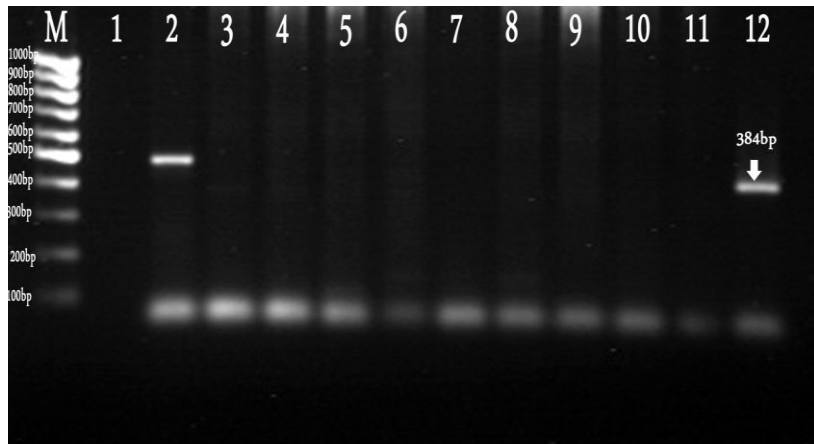

Figure (1): PCR products of Shiga toxin I (stx-I) on agarose gel 2% stained by Etbr. Lane M: 100bp DNA ladder; Lane 1: Negative control; Lane 2-11: Negative *E. coli* O157 for Shiga toxin I production; Lane 12: Shiga toxin I gene-positive strains isolated from frozen sausage samples.

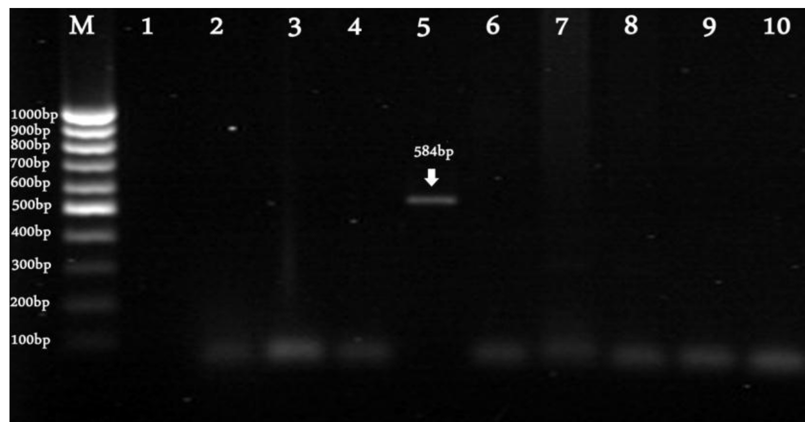

Figure (2): PCR products of Shiga toxin II (stx-II) on agarose gel 2% stained by Etbr. Lane M: 100bp DNA ladder; Lane 1: Negative control; Lane 2-4 & 6-10: Negative *E. coli* O157 for Shiga toxin II production; Lane 5: Shiga toxin II gene-positive strains isolated from frozen sausage samples.

DISCUSSION

Because the reservoir of *E. coli* is the intestinal tract of both man and animals, the presence of such organism in foods and water is used as indicator of faecal pollution either directly or indirectly (ICMSF, 1978).

A total of 80 frozen and cooked beef burger and sausage samples as well as 20 children stool samples were analyzed and determined the levels of *E. coli* in the examined samples. According to the data in Table (2), Twenty nine *E. coli* strains were isolated, resulting in an overall prevalence of 29%. The highest isolation frequencies of *E. coli* among the different samples were from children stools (60%), frozen beef burgers (45%), frozen sausages (30%), with lower isolation frequencies from cooked beef burgers and cooked sausages (5% for each product). Regarding frozen sausage, similar results (30%) was obtained by Badri *et al*. (2009) in Casablanca. While lower levels were obtained in Egypt by Hassanien (2004), who showed that, 20% of frozen sausage and 12% of frozen beef burgers were contaminated *with E. coli*. In Iran Kalantari *et al*. (2012) detected *E. coli* in 14 (10.5%) of sausage sandwich samples and in 22 (16.4%) of hamburger sandwich samples. On the other hand, *E. coli* was isolated from children stool with a percentage higher than that isolated by Ali *et al*. (2010) who isolated *E. coli* with a percentage of 7.14% from cases of acute diarrhea.

The differences in contamination levels could be affected by the national or geographic characteristics of meat sources, processing environments, and different methodologies such as numbers, amounts and periods of samples tested (Kegode *et al.,* 2008).

A number of reports from different countries report the presence of *E. coli* O157 isolates in a variety of foods of domestic animal origin. In Turkey, Noveir *et al*. (2000) reported that 0.4% of the 225 ground beef, 2% of the 50 hamburger, 1% of the 101 sausage samples were contaminated with *E. coli* O157. In a

study reported by Silveira *et al*. (1999) concerning the investigation of *E. coli* O157 in Brazil, 886 hamburger samples were negative. Chinen *et al.* (2001) reported that 4.8% of 83 fresh pork sausage and 3.3% of 30 dry sausage samples examined in Argentina were contaminated with *E. coli* O157.

March and Ratnam, (1989) and Silveira *et al*. (1999) stated that the latex test is simple, highly efficient and reliable test in detecting *E. coli* O157:H7 with 100% sensitivity and specificity. The current study showed that eleven (11%) of the 100 samples of different meat product and children stool were contaminated with *E. coli* O157 (Table 3). The prevalence of this organism was frequently higher in children stools (20%), frozen sausages (15%) and frozen beef burgers (10%), with lower isolation frequencies from cooked beef burgers and cooked sausages (5% for each product). Regarding frozen beef burgers, these findings corroborate those of Mattar and Vasquez (1998) who reported that 8.7% of hamburger samples tested were contaminated with *E. coli* O157 in Colombia. Also, this result closely agrees with a previous study of Keles *et al*. (2006) in which *E. coli* O157 was isolated from frozen hamburger balls with an isolation rate of 12% in Turkey. In the present study, the incidence of *E. coli* O157 in frozen sausage was higher than those reported by Nastasijevic *et al.* (2009) in Serbia (2.1%) and in Egypt, Abd El-Atty and Meshref (2007) reported a prevalence of 2% in sausage. Our incidence of *E. coli* O157 in frozen sausage corroborate those of Beneduce *et al.* (2008) since both fall within a similar percentage range. The presence of *E. coli* O157 in sausage was attributed to contamination from faeces of infected animals as well as the unsatisfactory hygienic measures during manufacturing and handling (Abd El-Atty and Meshref, 2007). The result of the current study regarding to the incidence of *E. coli* O157 in children stool was higher than those reported by Hassanein *et al*. (2012) and Abd Al-Azeem *et al.* (2013) who isolated the bacterium from (1.7%) and (13.3%) from human patients with diarrhea. Most significant of this study is the fact that *E. coli* O157 was isolated from ready to eat beef burgers and sausages samples. Contamination by this organisms therefore could be as a result of improper handling of the product after preparation and most especially from vectors like flies and hands of the sellers themselves.

STEC infections in humans are primarily regarded as food-borne, and numerous outbreaks have been attributed to consumption of STEC contaminated foodstuff (Mead *et al*., 1999; Rangel *et al.,* 2005 and (EFSA), 2009). Food may become contaminated with STEC at all stages of production and retail but in most cases the source of STEC in foodstuff remains unknown (Mead *et al*., 1999; (CDC), 2009 and (EFSA), 2009).

In this study, 11 *E. coli* O157 isolates were characterized by PCR technique and the virulence factor coding genes were detected. Sltx-1 gene was detected in one out of the three frozen sausage isolates; also, Sltx-2 gene was detected in one out of the three frozen sausage isolates (Table 4, Figure 1&2). Similar occurrences were observed in studies conducted by Beneduce *et al.* (2008) who confirmed two isolates by molecular methods as shiga toxin-producing *E. coli* O157. Conversely, Tafida *et al.* (2014) reported the detection of stx1 gene in 2 out of the 4 *E. coli* O157 isolates tested by PCR; but stx 2 was not detected in any of the isolates tested.

Although *E. coli* O157:H7 is the predominant *E. coli* incriminated in food borne disease, various non-motile O157 variants have been isolated (Feng *et al.,*1998). None motile variant of the O157 serotype have been isolated more frequently worldwide, some have been implicated in illness and theses variant designated (non-motile) NM or H- (Aleksic *et al.,* 1992). *E. coli* O157:H7 and *E. coli* O157: H- (non-motile) continue to be the dominant causes of illness in humans, from the numerous VTEC serotypes identified (Feng, 1993). In this study, none of *E. coli* O157 was found to harbor H7 genes (Table 4). These findings corroborate those of Sarimehmetoglu *et al*. (1998) and Noveir *et al*. (2000) who found that none of the isolates was characterized as H7 strain.

It is important to realize that management of meat safety risks should be based on an integrated effort and approach that applies to all sectors, from the producer through the processor, distributor, packer, retailer, food monitoring authorities and consumer. The report of the presence of *E. coli* O157 in this study should prompt relevant authorities to bear in mind that most food borne illnesses may be due to mishandling of foods, while animal-borne pathogens introduced into the environment lead to illness associated with consumption of contaminated meat. Thus, consumer education and environmental pollution issues should be major targets in efforts to improve meat and food safety.

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دراسة علي سموم الشيجا المنتجة بواسطة ميكروب الإيشيريشيا القولونية O157:H7 المعزولة من بعض منتجات اللحوم في محافظة أسيوط كمسبب للإسهال الدموي في الأطفال

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أجريت هذه الدراسة على ٨٠ عينة (عشرون عينة من كل من البيف بيرجر المجمد، السجق المجمد ، ساندوتشات البيف بيرجر وساندوتشات السجق) التي تم جمعها من مختلف المحلات والسوبر ماركت ومطاعم الوجبات الجاهزة بمحافظة أسيوط وذلك لمعرفة مدى تواجد ميكروب الإيشيريشيا القولونية وبالأخص العترة O157:H7 في هذه العينات، والكشف عن سموم الشيجا (**stx2** و **stx1**) فى العترات المعزولة. بالإضافة إلى فحص٢٠ عينة من المسحات الشرجية من الأطفال الذين يعانون من حالات إسهال وحمى في مستشفى طب الأطفال، جامعة أسيوط. وقد أسفرت النتائج عن تواجد ميكروب الإيشيريشيا القولونية بنسبة٤٥% ، ٣٠% ، ٥% و٥% في عينات البيف بيرجر المجمد ، السجق المجمد ، ساندويتشات البيف بيرجر وساندويتشات السجق على التوالي. وبالنسبة للمسحات الشرجية للأطفال فقد تم عزل الميكروب بنسبة ٦٠%. كما تم عزل العتره *E. coli* O157 من ١١(١١%) من اجمالى عدد العينات التى تم فحصها بنسبة ١٠% ، ١٥%، ٥%، ٥% و ٢٠% في عينات البيف بيرجر المجمد، السجق المجمد، ساندوتشات البيف بيرجر، ساندوتشات السجق والمسحات الشرجية للأطفال على التوالي. ولم يستدل على وجود الانتجين السوطى ٧ فى العترات المعزولة. وبالنسبة لسموم الشيجا (**stx2** و **stx1**) وجدت فى عترتين من *E. coli* O157 المعزوله من عينات السجق المجمد. ولقد نوقشت الأهمية الصحية للميكروب وسمومه ووضعت التوصيات اللازمة لسلامة المنتج والمستهلك.