

Molecular Detection and Characterization of Shiga Toxogenic *Escherichia coli* Associated with Dairy Product

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Raw, unpasteurized milk can carry dangerous bacteria such as *Salmonella*, *E. coli*, and *Listeria*, which are responsible for causing numerous foodborne illnesses. The objective of this study was molecular characterization of shiga toxogenic *E. coli* in raw milk collected from different Egyptian governorates by multiplex PCR. During the period of 25th May to 25th October 2012, a total of 320 bulk-tank milk samples were collected from 10 cow farms located in different Egyptian governorates. Bacteriological examination of milk samples revealed the presence of *E. coli* organisms in 65 samples (20.3%), serotyping of the *E. coli* isolates revealed, 35 strains (10.94%) O111, 15 strains (4.69%) O157: H7, 10 strains (3.13%) O128 and 5 strains (1.56%) O119. Multiplex PCR for detection of shiga toxin type 2 and intimin genes revealed positive amplification of 255 bp fragment of shiga toxin type 2 gene and 384 bp fragment of intimin gene from all *E. coli* serovar O157: H7, while from serovar O111 were 25 (71.43%), 20 (57.14%) and from serovar O128 were 6 (60%), 8 (80%), respectively. The results of multiplex PCR as-say are useful for identification of STEC possessing the *eaeA* and *stx2* genes.

Key words: Raw milk, *E. coli*, multiplex PCR, Shiga toxin type 2, intimin gene.

Raw milk is milk from cows, sheep, or goats that has not been pasteurized to kill harmful bacteria.¹ This raw, unpasteurized milk can carry dangerous bacteria such as *Salmonella*, *E. coli*, and *Listeria*, which are responsible for causing numerous foodborne illnesses.^{2,3} In addition, CDC reported that unpasteurized milk is 150 times more likely to cause foodborne illness and results in 13 times more hospitalizations than illnesses involving pasteurized dairy products.

These harmful bacteria can seriously affect the health of anyone who drinks raw milk, or eats foods made from raw milk.⁴ However, the bacteria in raw milk can be especially dangerous to people with weakened immune systems, older adults, pregnant women, and children. In fact, the CDC analysis found that foodborne illness from raw milk especially affected children and teenagers.

Enteropathogenic *Escherichia coli* (EPEC) are a major cause of infantile diarrhea^{5,6} and it's considered to be one of the world's leading causes of morbidity and mortality.^{2,3} Transmission of EPEC is through fecal-oral route, with contaminated hand or contaminated foods.^{7,8} The

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main mechanism of EPEC pathogenesis is a lesion called 'attaching and effacing' (A/E), which is characterized by intimate adherence of bacteria to the intestinal epithelium.⁹ Food-infections most commonly occur due to the consumption of milk and dairy products, and 53% of cases of food-borne infections caused by EPEC.¹⁰ Shiga toxin-producing *E. coli* (STEC), the causative agent of gastroenteritis that produce one or two potent toxins called Shiga toxin (Stx1, Stx2), may be able to produce intimin protein.^{11,12} For this reason, *E. coli* strains with the *eaeA* genotype which lack Shiga toxin gene (*stx1* and/or *stx2*) are classified as EPEC.¹⁵ Although some comprehensive studies have been conducted in Egypt to detect the contribution of shiga toxigenic *E. coli* as a cause of infectious diarrhea, unfortunately there is no relevant data about food contamination with shiga toxigenic *E. coli*. Thus, the objective of this study was molecular characterization of shiga toxigenic *E. coli* in raw milk collected from different Egyptian governorates by multiplex PCR.

Samples

During the period of 25th May to 25th October 2012, a total of 320 bulk-tank milk samples were collected from 10 cow farms located in different Egyptian governorates. The samples were placed on ice and transported immediately to the laboratory.

Isolation and identification of *E. coli*

Milk samples were centrifuged at 3000 rpm for 15 minutes and after centrifugation the supernatant as well as the sediment were cultured. Milk samples were primary cultured on MacConkey agar medium, incubated aerobically at 37°C. After overnight incubation, a part of single typical well isolated lactose fermenting colonies were tested for sorbitol fermentation by culturing on sorbitol MacConkey agar and sorbitol phenol red agar media, incubated at 37°C overnight. Morphological, cultural and biochemical examination were carried out according to methods described by Quinn et al.¹⁴

Serotyping of *E. coli*

Isolates that were primarily identified by biochemical tests as *E. coli* were subjected to serological identification using diagnostic polyvalent and monovalent *E. coli* antisera (Welcome *E. coli* diagnostic antisera). Diagnostic *E. coli*-O157 antisera (Dif-co code 2970-47-7) and

H7 antisera (Difco code 2159-47-0) were used for serological identification of *E. coli* O157:H7.

Extraction of DNA

The DNA of the standards strains and of the other bacterial isolates yielded from bacteriological examination was extracted by hexadecyl trimethyl ammonium bromide (CTAB), according to Sambrook et al.¹⁵ Meanwhile, the extractions of DNA from milk samples were carried out according to Riffon et al.,¹⁶ and Meiri-Bendek et al.¹⁷ One ml of each culture was centrifuged at 5000 rpm / 5 minutes, and then the sediment was washed five times with sterilized water and finally suspended in 1.0 ml of sterilized water. The suspension was kept at 95°C for 15 minutes, and after centrifugation at 15,000 rpm for 5 minutes, 10 µl of the supernatant was directly used for PCR.

PCR design and amplification conditions according to Paton and Paton¹⁸

PCR primer pairs were designed with reference to published sequence data for shiga toxin type 2 (*stx2*)¹⁹, intimin gene (*eaeA*).²⁰ Details of the nucleotide sequence, the specific gene region amplified, and the size of the PCR product for each primer pair are listed in Table 1. The extracted DNA of the standard strains and of the bacterial isolates yielded from bacteriological examination was tested with multiplex-PCR using the oligo-nucleotide primers specific for (*stx2*) and (*eaeA*) genes. Concurrently the crude DNA extracted from milk samples were tested by the same primer pair. All reactions were carried out in a final volume of 50ml in micro-amplification tube (PCR tubes). The reaction mixture consisted of 10µl (200mg) of the extracted DNA template from the bacterial cultures or 5µl of the extracted DNA template from the milk samples, 5µl of 10X PCR buffer (BIO-TOOLS) (75mM Tris-HCl, pH 9.0, 2mM MgCl₂, 50mM KCl, 20 mM (NH₄)₂ SO₄, 1µl dNTPs (40µM) (BIO-TOOLS), 1ml (1 U Amplitaq DNA polymerase) (Qiagen), 1ml (50 pmol) from the forward and reverse primers. The volume of the reaction mixture was completed to 50µl using deionized distilled water (DDW). 40µl paraffin oil was added and the samples were subjected to PCR cycles, each consisting of 1 min of denaturation at 95°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. Final extension was carried out at 72°C for 10 min, and

the PCR products were visualized by agrose gel electrophoresis according to Sambrook et al.¹⁵

RESULTS AND DISCUSSION

Bacteriological examination of milk samples collected from different Governorates revealed the presence of *E. coli* organisms 65 (20.3%) out of 320 examined milk samples obtained from different governorates the prevalence of STEC in raw milk samples was 20.3%; however, the presence of this pathogen in milk proved to be variable in different regions these variations may be due to geographical location, season, farm size, number of animals on the farm, hygiene status, farm management practices, variation in sampling, variation in types of samples evaluated, and differences in detection methods(6, 9,10) as shown in Table 2. Serotyping of the *E. coli* isolates yielded from bacteriological examination of milk samples revealed, 35 strains (10.94%) O111, 15 strains (4.69%) O157: H7, 10 strains (3.13%) O128 and 5 strains (1.56%) O119. The *E. coli* serovars yielded from bacteriological examination of milk samples were similar to the *E. coli* serovars yielded from

fecal samples, the obtained results indicated that the serotypes causing bovine mastitis were similar to the serotype causing diarrhea or even associated with the fecal samples of apparently healthy calves. Our result confirm the conclusion of Padhye and Doyle²¹; Harmon et al.,²² and Garber et al.,²³ who mentioned that *E. coli* serovars that causing bovine mastitis were similar to that of fecal isolates.

For detection of shiga toxin type 2 (*stx2*) and intimin (*eaeA*) genes by multiplex PCR using *stx2*F& *stx2*R and *eaeA* F& *eaeA* R primers, the *E. coli* serovars recovered by bacteriological examination were tested by multiplex PCR using *stx2* F & *stx2* R and *eaeA* F& *eaeA* R primers. Results observed in Table (2) and Figure (1) revealed positive amplification of 255 bp fragment of shiga toxin type 2 gene and 384 bp fragment of intimin gene from all *E. coli* serovar O157: H7^{18,24}, while from serovar O111 were 25 (71.43%), 20 (57.14%) and from serovar O128 were 6 (60%), 8 (80%), respectively. No amplification could be observed with *E. coli* serovar O119 which confirm that multiplex PCR as-say are useful for identification of STEC possessing the *eaeA* and *stx2* genes.

Table 1. PCR primers used for multiplex PCR

Primer	Sequence (5 - 3)	Specificity	Amplicon size
Assay 2 <i>eaeA</i> F/ <i>eaeA</i> R	GAC CCG GCA CAA GCA TAA GC CCA CCT GCA GCA ACA AGA GG	Intimin gene	384
<i>stx2</i> F <i>stx2</i> R	GGC ACT GTC TGA AAC TGC TCC TCG CCA GTT ATC TGA CAT TCT G	Shiga toxin type 2	255

Table 2. Characterization of the recovered *E. coli* serovars by multiplex PCR using *stx2* F, *stx2* R and *eaeA* F, *eaeA* R primers

<i>E. coli</i> Serovars	Multiplex PCR			
	intimin (<i>eaeA</i>) gene		Shiga toxin type-2 (<i>stx2</i>) gene	
	No.	%	No.	%
O157:H7 (15 strains)	15	100	15	100
O111 (35 strains)	25	56.25	20	37.5
O128 (10 strains)	6	60	8	80
O119 (5 strain)	0	0	0	0

%: was calculated according to the number of examined samples

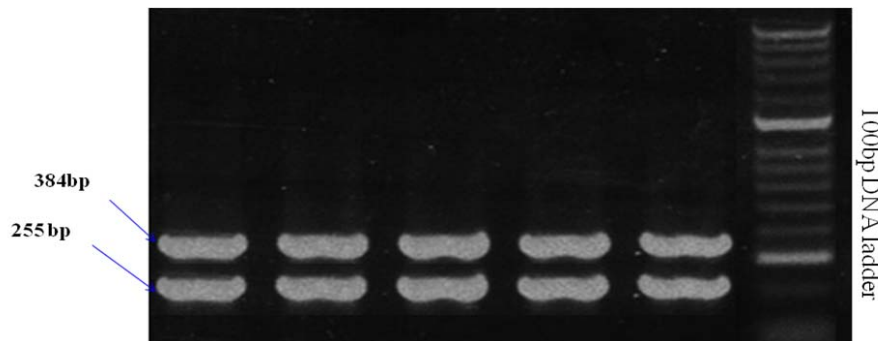


Fig. 1. Agarose gel electrophoresis showing amplification of 384 bp fragments and 255bp fragments of intimin and shiga toxin type two genes of *E. coli* serovar O157:H7, respectively

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