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Molecular Detection and Characterization of *Escherichia coli* O157:H7 and O111 Associated with Milk Samples by Multiplex PCR

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Shiga toxigenic *Escherichia coli* O157: H7 and O111 are capable of causing severe gastrointestinal disease in humans and animals. The present study aims to detect and characterizes *E. coli* O157:H7 and O111 in the milk samples collected from cattle with clinical and subclinical mastitis. Out of 240 examined milk samples, 48 *E.coli* isolates (20%) obtained clinical mastitic cases, while, 22 (13.75%) out of 160 examined milk samples obtained from subclinical mastitic animals. 22 strains (19.7%) O111, 12 strains (5%) O157: H7, 8 strains (3.33%) O128 and 6 strains (2.5%) O119 were recovered from clinical mastitic cases. While serotyping of *E. coli* received from subclinical mastitis revealed, 11 strains (6.87%) O111, 5 strains (3.13%) O157: H7, 4 strains (2.5%) O128 and 2 strains (1.25%) O119. Multiplex PCR using O157 and O111 primers revealed the ability detect all the bacteriologically positive samples. Moreover, the multiplex PCR could detect 7 strains of *E. coli* O111 and 5 strains of O157 from animals with subclinical mastitis, such samples were proved to be negative for O157 and O111 by bacteriological examination which indicated the ability of the sero-group-specific multiplex PCR assay to detect a very low concentration by STEC organisms.

Key words: Shiga toxin, *E. coli*, Multiplex PCR, *Escherichia coli* O157: H7 , Milk samples.

Escherichia coli O157: H7 is an enteric pathogen frequently found in ruminant animals, cattle may normally harbor the organism and are considered to be the primary reservoir of *E. coli* O157: H7 associated with human disease.[1-3]

Escherichia coli O157: H7 and O111 are members of Shiga toxigenic *Escherichia coli* (STEC) which comprise a diverse group of organisms capable of causing severe gastrointestinal disease in humans and animals. Within the STEC family, certain strains appear to be of greater virulence for humans, for example, those belonging to serogroup O111 and O157:H7. *Escherichia coli* serotype O157:H7 has been emerged as an important agent of public health concern with many outbreaks and nu-merous sporadic cases of hemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS), and diarrheal illness. STEC producing Shiga toxin type 2 (Stx2, encoded by *stx2* gene) appear to be more commonly responsible for serious complication such as HUS than those producing only Shiga toxin type 1 (Stx 1, encoded by *stx 1*gene).[3,4] Furthermore, STEC belonging to sero-group O157: H7and to a lesser extent, sero-group O111 are responsible for the vast majority of HUS outbreaks.[6,7]

Molecular approaches for bacterial detection avoid the need for culture and can be designed to be specific for detection and characterization of STEC *E. coli* specially strain O157 and O111.[8,9] For this reason, there is an increasing demand for improved diagnostic procedures for the detection of STEC in milk samples. Paton and Paton[10] developed multiplex

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polymerase chain reaction (multiplex- PCR) as-says for the simultaneous detection of (i) shiga toxin type 2 (stx2) and intimin (eaeA) genes and (ii) portions of the rfb (O-antigen-encoding) regions of *E. coli* O111 and O157:H7 for the detection and genetic characterization of STEC. Thus, the present study aims to detect and characterizes the *E. coli* O157:H7 and O111 in the milk samples collected from cattle with clinical and subclinical mastitis.

MATERIALS AND METHODS

Samples Collection

Milk samples

During the period of 25th May to 25th October 2012, Milk samples from 10 cow farms located in different Egyptian governorates were aseptically collected from 160 quarters with subclinical mastitis and 240 quarters with clinical mastitis using sterile graduated plastic centrifuge tubes 50 ml capacity. Milk samples were transferred to the labora-tory in a cold chamber container to be cultured without delay.

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 fer-menting 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*.[11]

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* an-tisera (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 serologi-cal 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*.[12] Meanwhile, the extractions of DNA from milk samples were carried out according to Riffon *et al*.[13] and Meiri-Bendek *et al*.[14] One ml of each culture was centrifuged at 5000 rpm / 5 minutes, and then the sedi-ment 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 centrifu-gation at 15,000 rpm for 5 minutes, 10 ml of the supernatant was directly used for PCR.

PCR design and amplification conditions according to Paton and Paton (1998)

PCR primer pairs were designed with reference to published sequence data for shiga toxin type 2 (*stx2*)[15], intimin gene (*eaeA*)[16] and portion of rfb regions of *E. coli* O111[17] and *E. coli* O157.[18] 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 were tested with multiplex-PCR using spe-cific primer pairs of *E. coli* O157 and O111 and with multiplex PCR using the oligo-nucleotide primers specific for *(stx2)* and *(eae A)* genes. Concurrently the crude DNA extracted from milk samples were tested by the same primer pairs. All reactions were carried out in a final volume of 50ml in micro--amplification tube (PCR tubes). The reaction mixture consisted of 10ml (200mg) of the extracted DNA template from the bacterial cultures or 5ml of the extracted DNA tem-plate from the milk samples, 5l of 10X PCR buffer (BIO-TOOLS) (75mM Tris-HCI, pH 9.0, 2mM $MgCI_2$, 50mM KCI,20 mM $(NH_4)_2 SO_4$, 1ml dNTPs *(40*m*M)* (BIOTOOLS), 1ml (l U Amplitaq DNA polymerase) (BIOTOOLS), 1ml (50 pmol) from the forward and reverse primers. The volume of the reaction mixture was completed to 50ml using deionized distilled water (DDW). 40ml 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 an-nealing 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. The PCR products were visualized by agrose gel electrophoresis according to Sambrook *et al*.[12]

RESULTS AND DISCUSSION

Bacteriological examination of milk samples collected from clinical and subclinical mastitic cases revealed the presence of *E. coli* organisms in both of them. *E. coli* were isolated from 48 (20%) out of 240 examined milk samples obtained from animals with clinical mastitis and from 22 (13.75%) out of 160 examined milk samples received from ani-mals with subclinical mastitis as shown in Table (2). Serotyping of the *E. coli* isolates yielded from bacteriological examination of milk samples received from clinical mastitic cases revealed, 22 strains (19.7%) O111, 12 strains (5%) O157: H7, 8 strains (3.33%) O128 and 6 strains (2.5%) O119. While serotyping of *E. coli* received from bacteriological examination of milk samples re-ceived from subclinical mastitis revealed, 11 strains (6.87%) O111, 5 strains (3.13%) O157: H7, 4 strains (2.5%) O128 and 2 strains (1.25%) O119 as shown in Table 2. These results indicate that *Escherichia coli* is ubiquitous in the cow's environment that is contaminated by feces.[19,20] Moreover, give an idea about the frequent cause of bovine mastitis as the *E. coli* serotypes in mastitic milk were similar to that of fecal isolates

Table 1. PCR primers used for multiplex PCR

Primer name	Sequence (5 - 3)	Specificity	Amplicon size bp
Multiplex1O157F	CGG ACA TCC ATG TGA TAT GG	*E. coli* serovar	259
O157R	TTG CCT ATG TAC AGC TAA TCC	O157: H7	
O111FO111R	TAG AGA AAT TAT CAA GTT AGT TCC ATA GTT ATG AAC ATC TTG TTT AGC	*E. coli* serovar O111	406
Multiplex2eaeA F	GAC CCG GCA CAA GCA TAA GC	Intimin gene	
eaeA R	CCA CCT GCA GCA ACA AGA GG	384	
stx2 Fstx2 R	GGC ACT GTC TGA AAC TGC TCC TCG CCA GTT ATC TGA CAT TCT G	Shiga toxin type 2	255

Table 2. *E. coli* serovars isolated from milk samples received from animals

Milk samples	No. of samples	*E. coli* serovars				
		O111	O157H7	O128	O119	Total
Clinical mastitis	240	22 (9.17%)	12 (5%)	8 (3.33%)	6 (2.5%)	48 (20%)
Sub clinical mastitis	160	11 (6.87%)	5 (3.13%)	4 (2.5%)	2 (1.25%)	22 (13.75%)

Table 3. Comparison between the bacteriological examination and the multiplex PCR using O157F, Ol57R and O111 F, O111R primers

Examined samples	Bacteriological examination				Multiplex PCR			
	O157:H7		O111		O157:H7		O111	
	No.	%*	No.	%*	No.	%*	No.	%*
2) Bovine milk samples								
Clinical mastitis (240)	12	5	22	9.17	15	6.25	25	10.42
Sub clinical mastitis (160)	5	3.13	11	6.87	7	4.38	15	9.37

%: was calculated according to the number of examined samples

frequently isolated from cow's environment. It is known that the O157:H7 serotypes of *E. coli* isolated from raw milk samples.[21,22] This can cause severe diseases such as bloody diarrhea, (HUS) and hemorrhagic colitis in human[23,24], although other fecal *E. coli* serotype are known to cause bovine mastitis.[20]

Comparison between bacteriological examination and multiplex PCR using O157F, O157R and O111F, O111R primers, results observed in Table (3) revealed the ability of multiplex- PCR to detect all the bacteriologically posi-tive samples. Amplification of 406 bp fragment of *E. coli* serovar O111 and 259 bp fragment of *E. coli* serovar O157: H7, were observed with the extracted DNA of *E. coli* O111 and *E. coli* O157: H7 respectively of the yielded isolates, but no amplification could be observed from the other *E. coli* iso-lates. Results observed in Table (3), revealed positive amplification of 406 bp fragment of *E. coli* serovar O111 from 25 milk samples (10.42%) obtained from animals with clinical mastitis and 15 milk samples (9.38%) ob-tained from animals with sub clinical mastitis. Moreover, amplification of 259 bp fragments of *E. coli* serovar O157: H7 were observed with the extracted DNA of 15 milk samples (6.25%) obtained from animals with clinical mastitis and 7 milk samples (4.38%) obtained from animals with subclinical mastitis as shown in Table 3. The obtained results indicate the ability of multiplex PCR to detect 3 strains O157 and another 3 strains of *E. coli* O111 from animals with clinical mastitis and 4 strains of O111 and 2 strains of O157 from animals with subclinical mastitis, such samples were proved to be negative for O157 and O111 by bacteriological examination which indicated the ability of the sero-group- specific multiplex PCR assay to detect a very low concentration by STEC organisms which cannot be detected by bacteriological examination.[1-3] The obtained results confirm the higher sensitivity of the multiplex-PCR as mentioned by Paton and Paton[10]. Multiplex-PCR has also been used for the detection of genes encoding accessory STEC virulence factors, such as *eaeA* and *stx2* genes. The *E. coli* serovars recovered by bacteriological examination were tested by multiplex PCR using stx2 F&stx2 R and eae A F& eae AR primers. Results observed in Table (3), 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 30 (75%), 18 (45%) and from serovar O128 were 8 (66.67%), 6 (50%), respectively. Amplification of 255 bp fragment of shiga toxin type-2 ob-served and 384 bp fragment of intimin gene observed only with 4 strains 50% and 2 strains (25%) of *E. coli* serovar O119, respectively. These results confirm that multiplex PCR as-say is useful for identification of STEC *possessing* the *eaeA* and *stx2* genes as well as the specific identification of *E. coli* O157:H7 *and E. coli O111*.

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