African Journal of Biotechnology Vol. 9(28), pp. 4356-4363, 12 July, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Multiplex polymerase chain reaction for detection and characterization of shiga toxigenic *Escherichia coli* (STEC)

Moussa, I. M.[1]*, Ashgan, M. H.[2], Alwathnani, H. A.[3], Mohamed, Kh. F.[4] and Al-Doss, A. A.[1]

[1]Center of Excellence in Biotechnology, King Saud University, P. O. 2460 Riyadh, King Saudi Arabia, Saudi Arabia.
[2]College of Applied Studies and Community Services, King Saud University, Saudi Arabia.
[3]Department of Botany and Microbiology, College of Science, King Saud University, Saudi Arabia.
[4]Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Egypt.

Accepted 21 June, 2010

Escherichia coli **is ubiquitous in the cow's environment that is contaminated by feces, and it is also a frequent cause of bovine mastitis. Thus, the present study was targeted at the rapid detection and characterization of shiga toxigenic *E. coli* (STEC) in bovine fecal and milk samples. Twenty two strains of *E. coli* (39.29%) were isolated from 56 diarrheic calves, while only 5 strains (20.83%) were isolated from apparently normal contact calves. Moreover, 20 strains of *E. coli* (25%) were isolated from milk samples collected from 80 animals suffering from mastitis and subclinical mastitis. *E. coli* serovars yielded from bacteriological examination of milk samples were similar to that of fecal samples. Serogroup-specific multiplex polymerase chain reaction (PCR) assay could detect all the bacteriologically positive samples as well as 4 strains (7.98%), O157:H7 and 3 strains (5.36%), O111 from diarrheic calves and 2 strains (8.33%), O111 from normal calves. Such samples were proved to be negative by bacteriological examination. Multiplex PCR for detection of genes encoding accessory STEC virulence factors, such as shiga toxin type-2 (*stx2)* and intimin gene (*eaeA*) revealed the specificity of such gene to O157:H7 serovars and small number of other sero-groups.**

Key words: *Escherichia coli,* shiga toxigenic, diarrheic calves, multiplex PCR, intimin gene, O157:H7 serovars.

INTRODUCTION

Shiga toxigenic *Escherichia coli* (STEC) 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 to humans. For example, those belonging to serogroup O111 and O157:H7. *E. coli* serotype O157:H7 has emerged as an important agent of public health concern with many outbreaks and numerous sporadic cases of hemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS) and diarrheal illness in different setting (Caprioli et al., 1994; Griffin, 1995; Minami, 1997; Paton et al., 1996) and has been isolated from cattle and foods of animal origin and shown to be transmitted through contaminated food (Borczyk et al., 1987). It has been recognized for a number of years that STEC strains causing human disease may belong to a very broad range of O antigen sero-groups (Karmali, 1989). However, many of the STEC strains found in the gastrointestinal tracts of domestic animals (the principle source of human infections) may have a low degree of virulence factors such as intimin

*Corresponding author. E-mail: imoussa1@ksu.edu.sa-moussaihab @gmail.com. Tel: 00966560749553. Fax: 00966-14678456.

Abbreviations: STEC, Shiga toxigenic *Escherichia coli*; *stx2*, shiga toxin type-2; **PCR,** polymerase chain reaction; **HC,** hemorrhagic colitis; **HUS,** haemolytic uraemic syndrome; **TSB,** tryptic soy broth; **DDW,** deionized distilled water.

Table 1. Standard bacterial strains used for determination of primers specificity.

Number	Source	Bacterial strains
1	ATCC* 35150	*E. coli* serotype O157: H7
2	Local source	*E. coli* serotype O111: K58
1	Local source	*E. coli* serotype O86: K61
2	Local source	*E. coli* serotype O126: K58
1	ATCC* 9111	*Klebsiella pneumoniae*
1	ATCC* 13076	*Salmonella Enteritidis*
1	ATCC* 11511	*Salmonella Typhimurium*
1	ATCC* 29737	*Staphylococcus aureus*

ATCC*: American type culture collection.

(encoded by eaeA) and the plasmid-encoded enterohemolysin (encoded by enterohemorrhagic *E. coli* (EHEC) hlyA) (Bentin et al., 1995; Schmidt et al., 1995; Schmidt and Karch, 1996).

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) (Kleanthous et al., 1990; Ostroff et al., 1989). Furthermore, STEC belonging to sero-group O157:H7 and to a lesser extend, sero-group O111 are responsible for the vast majority of HUS outbreaks (Griffin et al., 1994; Reilly, 1997). Although, there have been several reports on the laboratory approach to detect STEC, the common practice is to screen specimen on sorbitol MacConkey agar and to test the non sorbitol fermenting colonies for *E. coli* O157 by biochemical parameters and by serotyping with O157:H7 antisera (Gransden et al., 1986; Griffin et al., 1988). For this reason, there is an increasing demand for improved diagnostic procedures for the detection of STEC in the bovine fecal and milk samples. Paton and Paton (1998) developed multiplex polymerase chain reaction (multiplex- PCR) assays 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 in the feces of patient suffering from HUS. Thus, the present study was targeted on the rapid detection and characterization of STEC in bovine fecal and milk samples using multiplex-polymerase chain reaction (PCR).

MATERIALS AND METHODS

Samples Collection

Fecal samples

Aliquots of 5 g of rectal feces were collected from 56 diarrheic calves and 24 apparently healthy contact calves. Calves age ranged from 1 to 4 months. Samples were collected during the period of October 2008 to the end of March 2009 from different farms in Menofia, Suez, Ismailia and Kafr EL-Sheikh Governorates.

Milk samples

Milk samples from the same farms were aseptically collected from 32 quarters with subclinical mastitis and 48 quarters with clinical mastitis using sterile graduated plastic centrifuge tubes of 50 ml capacity. Fecal and milk samples were transferred to the laboratory in a cold chamber container to be cultured without delay.

Standard bacterial strains used for determination of primers specificity

A total of 10 standard bacterial strains were used as a control, 6 strains belonging to *E. coli,* while the other 4 strains belonging to bacteria other than *E. coli,* as described in Table 1.

Isolation and identification of E. coli

Milk samples were centrifuged at 3000 rpm for 15 min and after centrifugation, the supernatant as well as the sediment were cultured. Both fecal and milk samples were primarily 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. (2002).

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 (Difco code 2970-47-7) and H7 antisera (Difco code 2159-47-0) were used for serological identification of *E. coli* O157:H7.

Table 2. PCR primers used for multiplex PCR.

Primer name	Sequence (5 - 3)	Specificity	Amplicon size bp
Assay 1			
O157F O157R	CGG ACA TCC ATG TGA TAT GG TTG CCT ATG TAC AGC TAA TCC	*E. coli* serovar O157: H7	259
O111F O111R	TAG AGA AAT TAT CAA GTT AGT TCC ATA GTT ATG AAC ATC TTG TTT AGC	*E. coli* serovar O111	406
Assay 2			
eaeA F eaeA R	GAC CCG GCA CAA GCA TAA GC CCA CCT GCA GCA ACA AGA GG	Intimin gene	384
stx2 F stx2 R	GGC ACT GTC TGA AAC TGC TCC TCG CCA GTT ATC TGA CAT TCT G	Shiga toxin type 2	255

Extraction of DNA

The DNA of the standards strains and of the other bacterial isolates yielded from bacteriological examination were extracted by hexadecyl trimethyl ammonium bromide (CTAB), according to Sambrook et al. (1989). Meanwhile, the extractions of DNA from milk samples were carried out according to Riffon et al. (2001) and Meiri-Bendek et al. (2002). Broth enrichment of the fecal samples was carried out on tryptic soy broth (TSB) at 37°C for 6 h as described by Paton and Paton (1998). One mille of each culture was centrifuged at 5000 rpm/5 min, 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 min, and after centrifugation at 15,000 rpm for 5 min, 10 µl 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 *stx2* (Jackson et al., 1987), intimin gene (*eaeA*) (Yu and Kaper, 1992), portion of rfb regions of *E. coli* O111 (Bastin and Reeves, 1995) and *E. coli* O157 (Bilge et al., 1996). 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 2. The extracted DNA of the standard strains and of the bacterial isolates yielded from bacteriological examination were tested with multiplex-PCR using specific primer pairs of *E. coli* O157 and O111 and with multiplex PCR using the oligonucleotide primers specific for *stx2* and *eae A* genes. Concurrently, the crude DNA extracted from milk samples and primary fecal cultures were tested by the same primer pairs. All reactions were carried out in a final volume of 50 µl in micro-amplification tube (PCR tubes). The reaction mixture consisted of 10 µl (200 µg) of the extracted DNA template from the bacterial cultures or 5 µl of the extracted DNA template from the milk samples and primary fecal cultures, 5 µl of 10X PCR buffer (BIOTOOLS) (75mM Tris-HCI, pH 9.0, 2mM $MgCl_2$, 50mM KCI,20 mM $(NH_4)_2$ SO_4, 1 µl dNTPs (*40µM*) (BIOTOOLS), 1 µl (l U Amplitaq DNA polymerase) (BIOTOOLS), 1 µl (50 pmol) from the forward and reverse primers. The volume of the reaction mixture was completed to 50 µl using deionized distilled water (DDW). 40 µl 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 stored in a thermal cycler at 4°C until they were collected.

Agarose gel electrophoresis according to Sambrook et al. (1989)

The PCR products were visualized by agarose gel electrophoresis. Samples (10 µl) of final PCR products were mixed with 2 µl gel loading buffer 6X stock (bromophenol blue 0.25%; Xylene cyanol 0.25% and glycerol 30%) and then loaded onto a 1.5% agarose gel containing ethidium bromide at concentration of 0.5 µg/ml. The gel was subjected to electrophoresis in 1 X TAE (Sambrook et al., 1989) for two hours at 120 V.A. 100 bp ladder (Life Technologies, Gent, Belgium) was inoculated in the gel as a molecular weight standard.

Analysis of data

The sensitivity and specificity of PCR were calculated according to Timmreck (1994) taking the bacteriological examination as a gold standard.

RESULTS

Isolation and identification of *E. coli* organisms

Bacteriological examination of the fecal samples collected from diarrheic and apparently healthy contact calves revealed the presence of *E. coli* organisms in both of them. *E. coli* were isolated from 22 (39.29%) out of 56 examined diarrheic calves and from 5 (20.85%) out of 24 apparently healthy calves as shown in Table 3.

Culturing of *E. coli* isolates on MacConkey sorbitol agar media revealed 22 strains that fermented sorbitol while the other 5 strains did not ferment sorbitol; such strains were identified as O157:H7 by using diagnostic antisera

Table 3. *E. coli* serovars isolated from fecal samples of diarrheic and contact healthy calves.

Bovine fecal samples	No. of examined samples	*E. coli* serovars						
		O157:H7	O 111	O159	O48	O26	O128	Total
Diarrheic calves	56	5 (8.93%)	7 (12.5%)	1 (1.79%)	2 (3.5%)	3 (5.36%)	4 (7.14%)	22 (39.29%)
Apparently normal	24	0	0	0	1 (4.17%)	2 (8.33%)	2 (8.33%)	5 (20.83%)

Table 4. *E. coli* serovars isolated from milk samples received from animals with subclinical and clinical mastitis.

Milk samples	No. of samples	*E. coli* serovars				
		O111	O157H7	O157H7	O119	Total
Clinical mastitis	48	6 (12.50%)	3 (6.25%)	3 (6.25%)	1 (2.08%)	12 (25%)
Sub clinical mastitis	32	3 (9.38%)	2 (6.25%)	2 (6.25%)	2 (6.25%)	8 (25%)

and isolated from fecal samples of diarrheic calves only. Serotyping of the other *E. coli* isolates yielded from bacteriological examination of the fecal samples of diarrheic calves revealed 7 strains (12.50%) O111, 4 strains (7.14%) O128, 3 strains (5.36%) O26, 2 strains (3.57%) O48 and one strain (1.79%) O159. While serotyping of the *E. coli* isolates from bacteriological examination of the fecal samples of apparently healthy calves revealed 2 strains (8.33%) O128, two strains (8.33%) O26 and one strain (4.17%) O48 as shown in Table 3.

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 12 (25%) out of 48 examined milk samples obtained from animals with clinical mastitis and from 8 (25%) out of 32 examined milk samples received from animals with subclinical mastitis as shown in Table 4. Serotyping of the *E. coli* isolates from bacteriological examination of milk samples received from clinical mastitic cases, revealed 6 strains (12.50%) O111, 3 strains (6.25%) O157:H7, 2 strains (4.17%) O128 and one strain (2.08%) O119. While serotyping of *E. coli* received from bacteriological examination of milk samples received from subclinical mastitis, revealed 3 strains (9.38%) O111, 2 strains (6.25%) O157:H7, 2 strains O119 and one strain (3.12%) O128 as shown in Table 4.

Detection of *E. coli* O157:H7 and O111 by multiplex PCR using O157F, O157R and O111F, O111R

The specificity of the oligonucleotide primers were tested with the extracted DNA of the standard strains and with the extracted DNA of *E. coli* isolates yielded from the bacteriological examination. 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, for either of the standard strains or of the yielded isolates, but no amplification could be observed from the other *E. coli* isolates. The extracted DNA of the fecal cultures and the milk samples were tested by multiplex PCR using O157F, O157R and O111F, O111R primers. Results observed in Table 5 and Figure 1 revealed positive amplification of 406 bp fragment of *E. coli* serovar O111 from 10 fecal samples (17.86%) obtained from diarrheic calves, 2 fecal samples (8.33%) obtained from healthy contact calves, 8 milk samples (16.67%) obtained from animals with clinical mastitis and 5 milk samples (12.63%) obtained 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 9 fecal samples (16.07%) obtained from diarrheic calves, 4 milk samples (8.33%) obtained from animals with clinical mastitis and 2 milk samples (6.25%) obtained from animals with subclinical mastitis as shown in Table 5 and Figure 1.

Detection of stx2 and intimin (eaeA) genes by multiplex PCR using stx2F& stx2R and eaeA F& eae AR primers

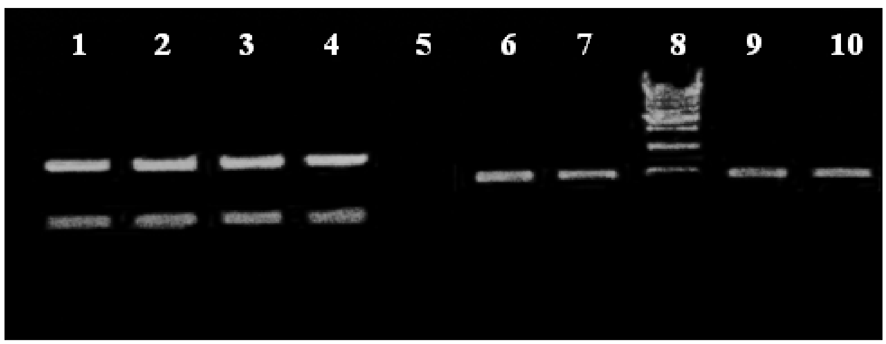
The *E. coli* serovars recovered by bacteriological examination were tested by multiplex PCR using stx2 F&stx2 R and eae A F& eae AR primers. Results observed in Table 6 and Figure 2 revealed positive amplification of 255 bp fragment of shiga toxin type 2 gene and 384 bp fragment of intimin gene from all *E. coli* serovar O157:H7, while from serovar O111, were 9 (56.25%), 6 (37.5%) and from serovar O128, were 5 (55.56%) and 2 (22.22%), respectively. Amplification of 255 bp fragment of stx2 was

Table 5. Comparison between the bacteriological examination and the multiplex PCR using O157F, Ol57R and O111F, O111R primers.

Examined samples	Bacteriological examination				Multiplex PCR			
	O157:H7		O111		O157:H7		O111	
	No.	%*	No.	%*	No.	%*	No.	%*
Bovine fecal samples								
Diarrheic calves (56)	5	8.9	7	12.5	9	16.07	10	17.86
Contact normal calves (24)	0	0	0	0	0	0	2	8.33
Bovine milk samples								
Clinical mastitis (48)	3	6.25	6	12.5	4	8.33	8	16.67
Sub clinical mastitis (32)	2	6.25	3	9.38	2	6.25	5	12.63

% was calculated according to the number of examined samples.

406 bp



259 bp

Figure 1. Agarose gel electrophoresis showing the amplification of 406 bp fragments of *E. coli* serovar O111 (lanes 1, 2, 3, 4) and amplification of 259 bp fragment of *E. coli* serovar O157:H7 (lanes 6, 7, 9, 10). Lane 8 shows 250 bp ladder.

Table 6. Characterization of the recovered *E. coli* serovars by multiplex PCR using stx2F, stx2R and eae AF, eae AR primers.

E. coli serovars	Multiplex PCR			
	intimin (*eae A*) gene		Shiga toxin type-2 (*stx2*) gene	
	No.	%	No.	%
O157:H7 (10 strains)	10	100	10	100
O111 (16 strains)	6	37.5	9	56.25
O128 (9 strains)	2	22.22	5	55.56
O26 (5 strains)	0	0	3	60.00
O48 (3 strains)	0	0	1	33.33
O159 (1 strain)	0	0	0	0
O119 (1 strain)	0	0	0	0

% was calculated according to the number of examined samples.

observed only with 3 strains (60%) of *E. coli* serovar O26 and one strain (33.33%) of *E. coli* serovar O48 but no amplification could be observed with *E. coli* serovars O159 and O119.

DISCUSSION

E. coli is ubiquitous in the cow's environment that is contaminated by feces (Watts, 1989; Jones, 1990). It is

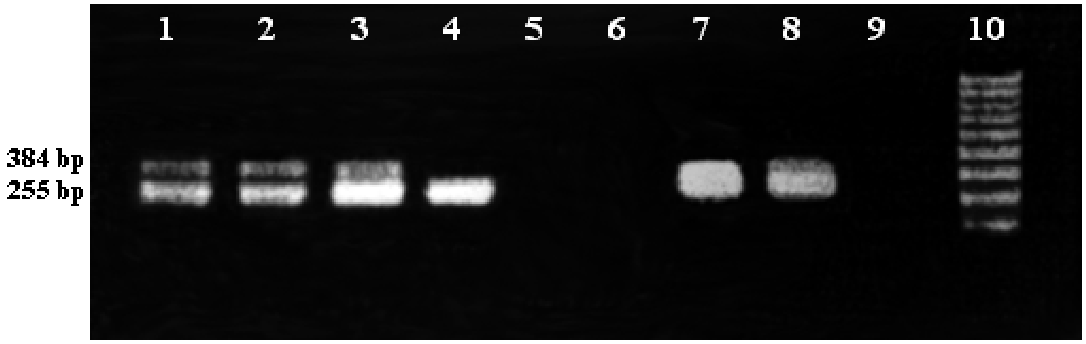


Figure 2. Agarose gel electrophoresis showing amplification of 384 and 255 bp fragments of intimin and shiga toxin type two genes of *E. coli* serovar O157:H7, respectively (lanes 1, 2 and 3). While lanes 7 and 8 show 255 bp fragments of shiga toxin type two gene only. Lane 10 shows 100 bp ladder.

also a frequent cause of bovine mastitis. The *E. coli* serotypes in mastitic milk were similar to fecal isolates. It is known that the O157:H7 serotypes of *E. coli* isolated from raw milk samples (Padhye and Doyle, 1991; Rubini et al., 1999) was of fecal origin (Garber et al., 1999; Harmon et al., 1990). This can cause severe diseases such as bloody diarrhea (HUS) and hemorrhagic colitis in human (Karmali et al., 1985; Salmon et al., 1989), although other fecal *E. coli* serotype are known to cause bovine mastitis (Jones, 1990; Bisping and Amtsberg, 1988).

In this study, 22 strains of *E. coli* (39.29%) were isolated from 56 diarrheic calves and only 5 strains (20.83%) of *E. coli* were isolated from apparently normal contact calves. *E. coli* serovar O157:H7 was isolated from 5 fecal samples (8.9%) of diarrheic calves. This result supports the view of association between *E. coli* O157:H7 infection and diarrheal illness (Capriolo et al., 1994; Griffin, 1995; Minami, 1997). *E. coli* serovar O157:H7 cannot be isolated from apparently healthy contact calves but the lack of isolation of O157 from apparently healthy bovine calves disprove the idea that these apparently healthy animals are likely to harbor the organism, and reinforces the observation that *E. coli* was the most frequently reported types in *E. coli* strains that cause diarrheal disease in animals (Erganis et al., 1989); also it confirms the result of Wells et al. (1991) that *E. coli* O157 cannot be isolated from 47 healthy dairy cow in Germany. *E. coli* serovars O111 (12.5%), O128 (7.14%), O26 (5.3%), O48 (3.5%) and O159 (1.79%) were isolated from fecal samples of diarrheic calves, such *E. coli* serovars are STEC and most frequently isolated from fecal samples of diarrheic calves (Karmali, 1989; Paton and Paton, 1998). Twelve strains of *E. coli* (25%) were isolated from milk samples received from animals with clinical mastitis O111 [6 strains (12.5%), 3 strains O157:H7 (6.25%), 2 strains O128 (4.17%) and one strain O119 (2.08%)]. Moreover, 8 strains (25%) were isolated from milk samples received from animals with subclinical mastitis [3 strains (9.38%) O111, 2 strains (6.25%) O157:H7, 2 strains (6.25%) O119 and one strain (3.13%) O128].

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 Doyle (1991), Harmon et al. (1990) and Garber et al. (1999) who mentioned that *E. coli* serovars that causes bovine mastitis were similar to that of fecal isolates. Comparison between bacteriological examination and multiplex PCR using O157F, O157R and O111F, O111R primers (Table 5) revealed the ability of multiplex- PCR to detect all the bacteriologically positive samples. Moreover, it could detect 4 strains (7.98%) O157 and 3 strains (5.36%) O111 from fecal samples of diarrheic calves and 2 strains (8.33%) O111 from fecal samples of contact normal calves, such samples were shown to be negative for O157 and O111 by bacteriological examination which indicated the ability of the serogroup specific multiplex PCR assay to detect a very low concentration by STEC organisms which cannot be detected by bacteriological examination (Brian et al., 1992; Paton et al., 1993; Paton et al., 1996). Moreover, the serogroup specific multiplex-PCR could detect one strain (2.08%) O157 and 2 strains (5.17%) O111 from milk samples received from animals with clinical mastitis and two strains (3.25%) O111 from milk samples received from animals with subclinical mastitis. The obtained results confirm the higher sensitivity of the multiplex-PCR as mentioned by Paton and Paton (1998). Multiplex-PCR has also been used for the detection of genes encoding accessory STEC virulence factors, such as *eaeA* and *stx2* genes. Results observed in Table 6 revealed that eaeA gene was detected in all

O157:H7 serovars (100%), 6 strains (37.5%) belong to O111 serotype and 2 strains (22.22%) belonging to O128 which indicated the specificity of such gene to O157:H7 serovars and a small number of other serogroups (Gannon et al., 1993; Paton and Paton, 1998). While shiga toxin type-2 gene was detected in all O157:H7 serovars, 9 strains (56.25%) belonged to O111, 5 strains (55.56%) to O128, 3 strains (60%) to O26 and one strain (33.33%) to O48 which confirmed that multiplex PCR assay are 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.

ACKNOWLEDGEMENT

The authors would like to thank the Ministry of Higher Education represented in the center of Excellence in Biotechnology Research where this work was done.

REFFERENCES

Bastin DA, Reeves PR (1995): Sequence analysis of the O antigen gene (rfb) cluster of *Escherichia coli* O111. Gene, 164: 17-23.

Bentin L, Geier D, Zimmermann S, Karch H (1995). Virulence markers of Shiga-like toxin producing *Escherichia coli* strains originating from healthy domestic animals of different species. J. Clin. Microbiol. 33: 631-635.

Bilge SS, Vary Jr. JC, Dowell SF, Tarr PI (1996). Role of the *Escherichia coli* O157: H7 O side chain in adherence and analysis of an rfb locus. Infect. Immun. 64: 4795-4801.

Bisping W, Amtsberg GG (1988). Colour atlas for the diagnosis of bacterial pathogens in animals. Paul Parey Scientific Publishers, Berlin, pp. 160-169.

Borczyk AA, Karmali MA, Lior H, Duncan MC (1987). Bovine reservoir for verotoxin producing *Escherichia coli* O157: H7. Lancet, 1: p. 98.

Brian MJ, Frosolono M, Murray BE, Miranda A, Lopez EL, Gomez HF, Cleary TG (1992). Polymerase chain reaction for diagnosis of enterohaemorrhagic *Escherichia coli* infection and haemolytic-uremic syndrome. J. Clin. Microbiol. 30: 1801-1806.

Caprioli A, Luzzi F, Rosmini C, Resti A, Edefontf F, Perfumo C, Farina A, Goglio A, Gianviti A, Rizzone G (1994): Community wide outbreak of hemolytic-uremic syndrome associated with non-O157 verocytotoxin-producing *Escherichia coli*. J. Infect. Dis. 169: 208-211.

Erganis O, Ates M, Kay O, Corlu M (1989): Studies on the biochemical, serological, haemagglutination, man nose resistant haemagglutination and enteropathogenic properties of *Escherichia coli* strains isolated from calves in diarrhea in the Korya region. Turk. Veterinerlik eve Hayvancilikis, 2: 108-122.

Gannon VPJ, Rashed M, King RK, Golsteyn Thomas EJ (1993). Detection and characterization of the eae gene of Shiga-like toxin-producing *Escherichia coli* using polymerase chain reaction. J. Clin. Microbiol. 31: 1268-1274.

Garber L, Wells S, Schroeder-Tucker L Ferris K (1999): Factors associated with decal shedding of verotoxin producing *Escherichia coli* O157 on dairy farms. J. Food Prot. 62: 307-312.

Gransden WR, Damm MAS, Anderson JD, Carter JE, Lior H (1986). Further evidence associating hemolytic uraemic syndrome with infection by verotoxin-producing *Escherichia coli* O157: H7. J. Infect. Dis. 154: 522-524.

Griffin PM (1995). *Escherichia coli* O157: H7 and other enterohemorrhagic *Escherichia coli*, p. 739761. In M. 1. Blaster PD, Smith JI, Ravdin HB, Greenberg and Gurrant RI (ed.). Infections of the gastrointestinal tract. Raven Press, New York.

Griffin PM, Bell BP, Cieslak PR, Tuttle J, Barrett TJ, Doyle MP, McNamara AM, Shefer AM and Wells JG (1994): Large outbreak of *Escherichia coli* O157: H7 infections in the western United States: the big picture, p. 7-12. In M. A. Karmali and A. G. Goglio (ed.), recent advances in verocytotoxin-producing *Escherichia coli* infections. Elsevier Science B. V. Amsterdam, the Netherlands.

Griffin PM, Ostroff SM, Tauxe RV, Greene KD, Wells JG, Wells JH, Blake PA (1988). Illnesses associated with *Escherichia coli* O157: H7 infections: a broad clinical spectrum. Ann. Int. Med. 109: 705-712.

Harmon BG, Cathy AB, Tkalcic S, Mueller POE, Parks A, Jain AV, Zhae T, Doyle MP (1990). Fecal shedding and rumen growth of *Escherichia coli* O157:H7 in fasted calves. J. Food Prot. 62: 574-576.

Jackson MP, Neill RJ, O'Brien AD, Holmes R K and Newland JW (1987). Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli*. EMS Microbiol. Lett. 44: 109-114.

Jones TO (1990). *Escherichia coli* mastitis in dairy cattle. A review of the literature. Vet. Bull. 60: 205-220.

Karmali MA (1989). Infection by verotoxin-producing *Escherichia coli*. Clin. Microbiol. Rev. 2: p. 1538.

Karmali MA, Petrie M, Lim C, Fleming PC, Arbus GS, Lior H (1985). The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. J. Infect. Dis. 151: 775-782.

Kleanthous H, Smith HR, Scotland SM, Gross RJ, Rowe B, Taylor CM, Milford DV (1990). Haemolytic uraemic syndromes in the British Islets, 1985-8: association with verocytotoxin producing *Escherichia coli*. Part 2: microbiological aspects. Arch. Dis. Child. 65: 722-727.

Meiri-Bendek I, Lipkin E, Friedmann A, Leitner G, Saran A, Friedmann S, Kashi Y (2002): A PCR·based method for the detection of *S. agalactiae* in milk. Am. Dairy Scl Ass. 85: 1717-1723.

Minami S (1997). Measures for the control of enterhaemorrhagic *E. coli* O157 in Japan, background paper number 9. WHO Consultation on the Prevention and Control of Enterohaemorrhagic *Escherichia coli* (EHEC) infections. World Health Organization, Geneva, Switzerland.

Ostroff SM, Tarr PI, Neill MA, Lewis JH, Hargrett-Bean N, Kobayashi JM (1989): Toxin genotypes and plasmid profiles as determinants of systemic sequelae in *Escherichia coli* O157: H7 infections. J. Infect. Dis. 160: 994-999.

Padhye NV, Doyle MP (1991). Rapid procedure for detecting enterohemorrhagic *Escherichia coli* O157:H7 in food, App. Environ. Microbiol. 57: p. 2698.

Paton AW, Paton JC (1998). Detection and characterization of Shiga toxigenic *E. coli* by using multiplex PCR assays for stxl, stx2, eaeA, enterohaemorrhagic E. coli hlyA, rfb O111 and rfb O157. J. Clin. Microbiol. pp. 598-602.

Paton AW, Paton JC, Goldwater PN, Mauning PA (1993). Direct detection of *Escherichia coli* shiga-like toxin genes in primary fecal cultures using the polymerase chain reaction. J. Clin. Microbiol. 31: 3063-3067.

Paton AW, Ratcliff R, Dolye RM, Seymour-Murray J, Davos D, Lanser JA, Paton JC (1996). Molecular microbiological investigation of an outbreak of hemolytic uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing *Escherichia coli*. J. Clin. Microbiol. 34: 1622-1627.

Quinn PJ, Markey BK, Carter ME, Donnelly WJC, Leonard FC (2002). Veterinary Microbiology and Microbial diseases. Blackwell Scientific Publications, Oxford, London.

Reilly WJ (1997). Verotoxigenic *E. coli* O157 in Scotland, background paper number 4. WHO Consultation on the Prevention and Control of Enterohemorrhagic *Escherichia coli* (ETEC) infections. World Health Organization, Geneva, Switzerland.

Riffon R, Sayasith K, Khalil H, Dubreuil P, Droplet M, Lagace J (2001). Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. J. Clin. Microbiol. 39(7): 2585-

2589.
Rubini S, Cardeti G, Amiti S, Manna G, Onovati R, Caprioli A, Morabito S (1999): Verocytotoxin- producing *Escherichia coli* O157 in sheep milk. Vet. Rec. 144: p. 56.
Salmon RL, Farrell ID, Hutchison JGP, Coleman DJ, Gross RJ, Fry NK, Rowe B, Palmer SR (1989). A christening party outbreak of hemorrhagic colitis and hemolytic uraemic syndrome associated with *Escherichia coli* O157: H7. Epidemiol. Infect. 103: 249-254.
Sambrook J, Fritscgh EF, Maniatis T (1989). Molecular cloning: a Laboratory Manual (2nd edn).Edited by Nolan C. Cold Spring Harbor :Cold Spring Harbor Laboratory Press,New York.
Schmidt H, Karch H (1996). Enterohemolytic phenotypes and genotypes of Shiga toxin producing *Escherichia coli* O111strains from patients with diarrhea and hemolytic-uremic syndrome. J. Clin. Microbiol. 34: 2364-2367.
Schmidt H, Beutin L, Karch H (1995). Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157: H7 strain EDL933. Infect. Immun. 63: 1055-1061.
Timmreck TC (1994). An introduction to epidemiology, Jones and Bartlett Publishers-Boston, London and Singapore.
Watts JL (1989). Etiological agents of bovine mastitis. Vet. Microbiol. 16: 41-66.
Wells JG, Shipman LD, Greene KDJ, Sowers EG, Green GH, Cameron DB, Downes FP, Martin ML, Griffin PM, Ostroff SM, Potter ME, Tauxe RW, Wachsmuth IK (1991). Isolation *Escherichia coli* serotype O157: H7 and other shiga like toxin producing *E. coli* from dairy cattle. J. Clin. Microbiol. 29(5): 985-989.
Yu J, Kaper JB (1982). Cloning and characterization of the eae gene of enterohaemorrhagic *E. coli* O157: H7. Environ. Microbiol. 6: 411-417.