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Identification of Virulence Genes in Isolated *Escherichia coli* from Diarrheic Calves and Lambs by Multiplex Polymerase Chain Reaction

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Abstract: The purpose of this study was to determine the presence of selected virulence genes in *Escherichia coli* isolated from diarrheic calves (Tehran province) and lambs (Ghume provinces) of Iran. We examined 40 isolates (29 from calves and 11 from lambs). All 40 isolates were tested for the presence of *stx1, stx2, eae, espB* and *hly* genes by multiplex polymerase chain reaction in two protocols. In the first protocol the isolates were tested with *EC* and *hly* primers and in the second protocol the isolates were examined with *eae, stx1, stx2* and *espB* primers. Multiplex PCR showed that from 29 strains isolated from diarrheic calves, 4 of isolates (13.7%) were *stx1* positive, 16 isolates (55.17%) carried *stx2* and 2 isolates (6.89%) had *espB* gene. 1 isolate (3.44%) possessed *eae*. Among 11 strains isolated from diarrheic sheep, 9 isolates (81.81%) carried *stx2* and 2 isolates (18.18%) had *espB* gene. Six isolates (54.54%) possessed *eae* and none of them was *stx1* positive. The *hly* gene was not detected in any of the isolates. The findings of this study indicated that the *stx2* may be widespread among pathogenic *Escherichia coli* in Iran.

Key words: Virulence genes, *Escherichia coli*, multiplex PCR, *stx*1, *stx*2, *eae*, *espB*, *hly* genes

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) have emerged as food poisoning pathogens which can cause sever disease in humans, such as Hemorrhagic Colitis (HC) and Hemolytic Uremic Syndrome (HUS) (Armstrong *et al*., 1996; Paton and Paton, 1998). STEC are defined by production of shiga toxins (stx_1 and stx_2) or Vero toxins (Vt_1 and Vt_2), encoded by the *stx* genes of temperate, lambdoid bacteriophage that remain integrated in the *E. coli* chromosome (O'Brien and Holmes, 1987). STEC may carry either *stx* subtype *stx1*, *stx2* or both *stx1* and *stx2*. In addition to toxin production, virulence-associated factor expressed by STEC is a protein called intimin (94-97 kDa protein) which is responsible for intimate attachment of STEC to the intestinal epithelial cells, causing attaching and effacing (AE) lesions in the intestinal mucosa. Intimin is encoded by the chromosomal *eae* gene which is located in the pathogenecity island termed the locus for enterocyte effacement (LEE) (Jerse *et al*., 1990; McDaniel *et al*., 1995; McDaniel and Kaper, 1997). The LEE encodes a type III secretion system, a series of protein secreted by this system called Esps, intimin and the receptor for intimin which is translocated into host cells (Frankel *et al*., 1998; Nataro and Koper, 1998). Secretion of the *espB* protein is essential for attachment and signal transduction in host cells and A/E lesions (Donnenberg *et al*., 1993; Foubister *et al*., 1994). The *espB* gene is located approximately 5 kb downstream of the *eae* gene (Donnenberg *et al*., 1993). In addition, one of the main agents of urinary tract infections in humans is *E. coli* which produces Hly (haemolysin) in urinary tract, causing release of iron for the bacteria.

Domestic ruminants especially cattle and sheep have been found to be the principal reservoir of STEC that are transmitted to human through foods contaminated with fecal material and cause human infections (Armstrong *et al*., 1996; Blanco *et al*., 1996; Blanco *et al*., 1997; Zschock *et al*., 2000; Cid *et al*., 2001; Osek and Gallien 2002).

The aim of this study was to detection of the STEC-associated virulence genes *stx1*, *stx2*, *eae*, *espB* and *hly* in *Escherichia coli* isolated from diarrheic calves

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and lambs, using multiplex PCR with the purpose of determining whether they can be a potential source of STEC pathogenic for human.

MATERIALS AND METHODS

Bacterial strains: Among 150 *Escherichia coli* isolated from diarrheic calves around Tehran (Iran) and 22 *Escherichia coli* isolated from diarrheic lambs during 2004-2005, 40 isolates (29 isolates from calves and 11 isolates from lambs) which were positive in serotyping with monovalent and polyvalent antiserums, were selected and tested for the presence of the selected genes. All isolates which had been stored at 4°C, were recultured on nutrient agar, then subcultured on blood agar, Mc Conkey agar, EMB agar and TSI agar (Merck) and incubated for 24 h at 37°C. Reference *E. coli* strains used as positive controls were *Escherichia coli* O157 (Strain No. 84-4, Tarbiat Modarres University) for the first protocol (*eae*, *stx1*, *stx2*, *espB*) and *Escherichia coli* ATTC 35218 and NTCC 11954 for the second protocol (*alr*, *hly*). Sterile dionised water was as negative control.

Detection of virulence genes by multiplex PCR: For multiplex PCR amplification, 40 *Escherichia coli* isolates and positive control strains were cultured on LB agar for 24 h at 37°C. To extract bacterial DNA, 6 to 8 colonies of each culture were picked and suspended in 100 μL of sterile deionized water, incubated at 100°C for 10 min to release the DNA and centrifuged at 6000×g for 5 min. The supernatant was used in the PCR reaction as the template DNA. Base sequence and predicted size of amplified product for each oligonucleotide primers (CinnaGene Inc, Iran) used in this study are shown in Table 1. Primers were used in two different protocols. In the fist protocol, *EC* and *hly* primers and in the second protocol, *stx1*, *stx2*, *eae* and *espB* primers were included. *EC* primers confirmed the isolates as *E. coli* (for alanine racemase gene). Amplification reactions were performed in a 25 μL volume containing 2.5 μL of 10X PCR buffer, 1 μL of 50 mM $MgCl_2$, 1.5 μL of 10 mM deoxynucleoside triphosphate (CinnaGene Inc, Iran), 1 μL of each primer, 0.5 μL of *Taq* DNA polymerase (CinnaGene Inc, Iran), 2 μL of the template DNA and 13.5 μL (9.5 μL in the second protocol) of sterile dionised water. Using a thermal cycler (Techne, UK), the conditions for the multiplex PCR were programmed as follows: 94°C for 10 min for initial denaturation of DNA followed by 30 cycles of 94°C for 1 min, 48°C for 1 min (64°C in the second protocol) and 72°C for 1 min.

The amplified products were visualized by gel electrophoresis using 10 μL of the final reaction mixture on a 1.2% agaros gel in TBE buffer. The samples were electrophoresed for 1 h at 100 V. Amplified DNA fragments of specific sizes were located by UV illuminator after staining with ethidium bromide. Molecular size markers (Gene ruler 100 bp DNA ladder plus, Fermentas) were included in each gel.

RESULTS

To determine the prevalence of selected virulence genes among ruminants *Escherichia coli*, we examined 40 isolates (29 from diarrheic calves and 11 isolates from diarrheic lambs) by multiplex PCR amplification (Table 2 and 3). All isolates were positive

Table 1: Primers sequences used in multiplex PCR

Gene	Primer	Oligonucleotide Sequence (5-3)	Number of nucleotide	Fragment size (bp)	Ref.
stx1	vt1	CGC TGA ATG TCA TTC GCT CTG C	22	302	Rey (2002)
	vt2	CGT GGT ATA GCT ACT GTC ACC	21		
stx2	vt1	CCT CGG TAT CCT ATT CCC GG	20	516	Rey (2002)
	vt2	CTG CTG TGA CAG TGA CAA AAC GC	23		
eae	eae1	GAG AAT GAA ATA GAA GTC GT	20	775	Rey (2002)
	eae2	GCG GTA TCT TTC GCG TAA TCG CC	23		
espB	espB1	GGC GTT TTT GAG AGC CA	17	260	Cid (2001)
	aspB2	GAT GCC TCC TCT GCG A	16		
hly	hly1	AAC AAG GAT AAG CAC TGT TCT GGC T	25	1177	Yamamoto (1995)
	hly2	ACC ATA TAA GCG GTC ATT CCC GTC A	25		
alr	E.c1	CGT GAA GAG GCT AGC CTG GAC GAG	24	366	Yokoigawaka (1999)
	E.c1	AAA ATC GGC ACC GGT GGA GCG ATC	24		

Table 2: Occurrence of virulence factors among different serotypes of *E. coli* isolated from diarrheic lambs

Sample	Serotyping	*alr*	*espB*	*stx1*	*stx2*	*eae*	*hly*
1	[b] Poly 3 +	+	+	-	+	+	-
4	Poly 3 +	+	-	-	+	-	-
5	O2 +	+	-	-	-	-	-
6	Poly 3 +	+	+	-	+	-	-
8	O2 +	+	-	-	+	+	-
9	Poly 3 +, [c] Poly 4 +	+	-	-	+	+	-

Table 2: Continued

Sample	Serotyping	*alr*	*espB*	*stx1*	*stx2*	*eae*	*hly*
11	[a] Poly 2 +' O157 +	+	-	-	+	+	-
12	O2 +	+	-	-	+	-	-
15	Poly 4 +	+	-	-	+	+	-
16	O2 +	+	-	-	-	-	-
21	O2 +	+	-	-	+	+	-

[a] Poly 2: O26, O55, O111, O119, O126; [b] Poly 3: O 86, O 114, O125, O127, O128; [c] Poly 4: O44, O112, O124, O142; + = Presence of gene; - = Absent of gene

Table 3: Occurrence of virulence factors among different serotypes of *E. coli* isolated from diarrheic calves

Sample	Serotyping	*alr*	*espB*	*stx1*	*stx2*	*eae*	*hly*
10	[b] Poly 3 +	+	-	-	-	-	-
13	Poly 3 +	+	-	-	+	-	-
26	Poly 3 + ' O157	+	-	-	+	-	-
30	[c] Poly 4 +	+	-	-	-	-	-
34	[a] Poly 2+ ' O157	+	-	-	-	-	-
35	Poly 2+ ' O157+	+	-	-	-	-	-
40	Poly 3+ ' K99 +	+	-	-	-	-	-
44	Poly 2 + ' O157+ ' k99+	+	-	-	-	-	-
47	Poly 2+' O157+	+	-	-	+	-	-
49	Poly 2+	+	-	-	+	-	-
53	Poly 4+	+	-	+	+	-	-
54	Poly 3+	+	-	-	-	-	-
66	Poly 2+	+	-	-	-	-	-
72	O2 K12	+	-	-	+	-	-
83	Poly 3+	+	+	+	+	-	-
92	O2 K1	+	-	-	-	-	-
93	O2 K1	+	-	+	+	-	-
96	Poly 4+	+	-	-	-	-	-
98	O157+	+	-	+	+	-	-
99	O157+	+	-	-	+	-	-
100	O157+	+	-	-	-	-	-
101	O2+	+	-	-	+	-	-
111	Poly 3+	+	-	-	+	-	-
112	O157+ 'K99+	+	+	-	+	+	-
129	Poly 2+	+	-	-	-	-	-
135	Poly 4+	+	-	-	+	-	-
137	Poly 2+	+	-	-	-	-	-
138	O12+	+	-	-	+	-	-
143	Poly 4+	+	-	-	+	-	-

[a] Poly 2: O26, O55, O111, O119, O126; [b] Poly 3: O 86, O 114, O125, O127, O128; [c] Poly 4: O44, O112, O124, O142; + = Presence of gene; - = Absent of gene

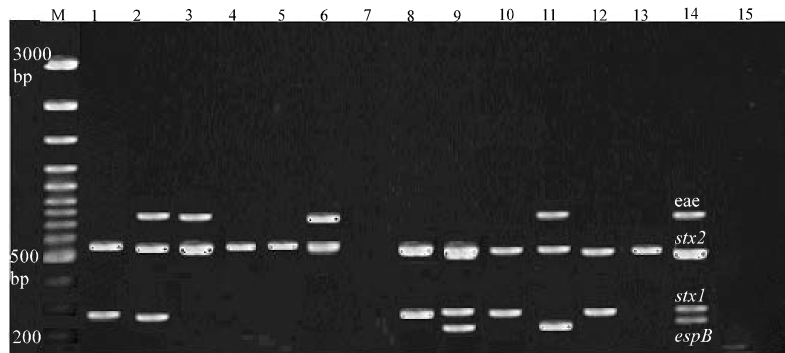

Fig. 1: Multiplex PCR of isolated *Escherichia coli* from calves and lambs, using primer set *espB*: 260 bp, *stx1*: 302 bp. *stx2*: 515 bp and *eae*: 775 bp. Lane M: 100 bp Marker (fermantase). Lane 1 to 6: *Escherichia coli* isolate from diarrheic lambs (6, 1, 8, 4, 12, 21 samples); Lane 8 to 13: *Escherichia coli* isolates from diarrheic calves (53, 83, 93, 112, 98, 143); Lane 14: *Escherichia coli* O157 (Strain No. 84-4, Tarbiat Modarres University) as positive control; Lane 15: negative control (Water)

with *EC* primer that confirmed the isolates as *E. coli*. From 11 ovine strains just one isolates was O157 positive and the others belonged to other serogroups. Totally 11 isolates, 9 isolates (81.81%) showed *stx2* gene and none of them had *stx1* (Fig. 1). In addition 6 isolates (54.54%) carried *eae* gene and two isolates (18.18%) possessed *espB* gene (Table 2).

Among 29 bovine strains 9 isolates belonged to O157 serogruop and the other were non O157. Totally 29 isolates, 12 isolates (41.37%) carried *stx2* and 4 isolates (13.79%) had both *stx1* and *stx2*. Furthermore 2 isolates (6.89%) showed *espB* and just one isolate (3.44%) had *eae* gene (Table 3).

None of the bovine or ovine isolates had hly (Heamolysis) neither on blood agar nor by multiplex PCR.

DISCUSSION

Verotoxins or shigatoxins are cytotoxins produced by some enterohemorrhagic *Escherichia coli* (EHEC or STEC). VT1 (Stx1) and VT2 (Stx2) are two major types of Verotoxins that have been recognized. There is little information concerning the isolation and characterization of STEC strains and virulence gene in Iran. In the other countries there were some studies about virulence genes in *E. coli* especially by using multiplex PCR (Parriera *et al*., 2002; Call *et al*., 2001; Yamamoto *et al*., 1995).

Several reports have indicated the presence of verotoxins in *E. coli* which were isolated from ruminants like cattle (or calves) and sheep (or lambs) (Monserrat *et al*., 2003).

The *eae* gene encodes a protein named intimin which is responsible for intimate attachment of *E. coli* to the enterocytes causing attaching and effacing (A/E) lesions in the intestinal mucosa (Agin and Wolf, 1997). The carriage of *eae* gene sequence has been detected in *E. coli* isolates from ruminant sources in recent study. The EspB protein which is encoded by *espB* gene also helps bacteria to attach to the enterocytes (McDaniel and Kaper, 1997). Both *eae* and *espB* genes are part of a pathogenicity island termed the locus for enterocyte effacement (LEE). The presence of *espB* has been demonstrated in attaching and effacing *Escherichia coli* (AEEC) strains isolated from humans and animals (Cid *et al*., 2001; Orden *et al*., 2003).

China *et al*. (1999) found that among 191 *E. coli* isolates from diarrheic calves, 48% were EHEC and 44% were *eae* positive. Cid *et al*. (2001) detected *eae* and *espB* genes in 50 strain of 398 strains isolated from lambs. None of the 398 isolates carried *stx* genes. In Australia, a survey of 505 dairy cattle at a slaughterhouse found only 4 STEC O157 isolates which did not have *stx* gene and all of them carried *eae* gene (Hallaran and Sumner, 2001). In Spain, Rey *et al*. (2003) detected STEC O157: H 7 in 1% and non-O157STEC in 35% of 253 sheep samples. They showed with PCR that 43% of strains carried *stx1* gene, 4% possessed *stx2* and 53% had both *stx1* and *stx2* genes. Furthermore, *eae* gene was detected in 4% of STEC strains. Osek (2003), detected *stx* gene in 12.5% of 202 bovine isolates using multiplex PCR. 10 isolates were STEC O157. In addition 20 isolates carried *eae* gene. In India, 62 bovine and human STEC O157 isolates which 19% carried *stx2* and 36.5% possessed *stx1*. 44.5% had both *stx1* and *stx2*. Furthermore, just 6.6% had *eae* gene which was contributed with *stx1*. In Switzerland, all of the 11 STEC O157:H45 carried *eae* gene, none of them possessed *stx* gene.

In this study totally 10 isolates (25%) of 40 isolates were O157 serogroup in serotyping which nine of them were isolated from diarrheic calves and one was from diarrheic lamb. From 10 O157 isolates, 6 isolates (60%) carried *stx2* gene and 3 isolates (30%) presented both *stx1* and *stx2*. Furthermore, just two isolates (20%) carried *eae* gene which was contributed with *stx*2 and one (10%) isolate present *espB*. No isolates had *hly*.

These data indicate that domestic animals and birds constitute a natural reservoir of attaching and effacing *Escherichia coli* strains and some of these are known as human pathogens. Numerous researchers have underlined the strong association between the carriage of *eae* gene and the capacity of *stx*-positive strains to cause severe human diseases specially Hemolyitc Uremic Syndrome (HUS). In some studies, although the *eae* gene was present only in minimal portion of animals, (Rey *et al*., 2003; Osek 2003; Khan *et al*., 2002), in this study *eae* gene was found in 7(28%) isolates. The *stx*-positive isolates especially in ovine *stx*-positive isolates (66.6%) indicating that it is probably some of these strains in Iran show high virulence for human. However, production of intimin is not essential for pathogenesis, because a number of sporadic cases of HUS are caused by *eae*-negative non-O157 STEC strains.

The production of different types of hemolysins has been frequently contributed to *E. coli* from intestinal and extra intestinal diseases (Yamamoto *et al*., 1995; Rey *et al*., 2003). It causes the release of the ferro from cells, providing iron for the bacteria. Epidemiological studies have shown that α-hemolysin correlates with *E. coli* isolates associated with uropathogenic infection and sepsis (Yamamoto *et al*., 1995). However, most studies have reported the animal *E. coli* as non-hemolytic (Call *et al*., 2001; Emery *et al*., 1992; Fantinatti *et al*., 1994; Anjum *et al*., 2003) that corresponds with the present findings.

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