Direct detection of Shiga toxin producing by *Escherichia coli* by real-time PCR

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are defined as strains of *E. coli* that produce Shiga toxins (stx), which known as important causes of diarrhea in sheep and cattle. This study was conducted to determine of Shiga toxin 1 producing *E. coli* isolates from diarrheal samples of sheep and cattle. Samples were collected from different fields in Diwanyia city. A total of 50 diarrhea samples (25 of sheep and 25 of cattle) were subjected to bacterial DNA extraction by using (AccuPrep® Stool DNA Extraction Kit). The extracted DNA subjected to Real-Time PCR technique for detection of Shiga toxin 1 (stx1) gene. Results display that sheep are more prevalence to shedding the Shiga toxin 1 producing *E. coli* in (5/25) (20%), while in cattle (2/25) (8%) positive samples. In conclusion the using of Real-Time technique was shown high specific and rapid method in direct detection of (stx1) gene and most the sheep and cattle which infected by diarrhea carried the Shiga toxin 1 producing *E. coli*.

Key words: STEC, stx1, cattle, sheep, real-time PCR.

الكشف المباشر عن سموم الشيكا المنتجة بواسطة الاشريشيا كولاي بتقنية الوقت الحقيقي لتفاعل سلسلة البلمرة

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الخلاصة

تعرف الاشريشيا كولاي المنتجة لسموم الشيكا بانها العزلات التي تنتج سموم الشيكا والتي تعرف بانها من الاسباب الهامة للإسهال في الاغنام والابقار. اجريت هذه الدراسة لتحديد سموم الشيكا 1 المنتجة من عزلات الايشريشيا كولاي في عينات الاسهال في الاغنام والابقار والتي تم جمعها من حقول مختلفة في مدينة الديوانية. تم اخذ 50 عينة اسهال (25 من الاغنام و25 من الابقار) وتم استخلاص الحامض النووي باستخدام AccuPrep® Stool DNA Extraction Kit. وتم اخضاع الحامض النووي المستخلص لتقنية الوقت الحقيقي لتفاعل سلسلة البلمرة للكشف عن جينات سموم الشيكا . اظهرت النتائج ان الاغنام هي الاكثر بطرح الجرثومة المنتجة لسموم الشيكا (5\25) بنسبة (20%)عينات ايجابية فيما اظهرت الابقار (2\25) وبنسبة (8%) عينات ايجابية. نستنتج من الدراسة ان استخدام تقنية الوقت الحقيقي لتفاعل سلسلة البلمرة ابدى انها تقنية عالية النوعية وسريعة في الكشف عن جين سموم الشيكا1. وان معظم الاغنام والابقار المصابة بالإسهال كانت حامله للعزلة .

الكلمات المفتاحية: الاشريشيا كولاي ، سموم الشيكا ا ، ابقار ، اغنام ، تقنية التفاعل الحقيقي

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is an important pathogen worldwide, that are causes problem for veterinary public health (1). Their zoonotic relevance is associated with human infections, diarrhea, bloody diarrhea, hemorrhagic colitis, and haemolytic uraemic syndrome (2,3). The main reservoir for STEC are ruminants, especially cattle and sheep. The infection spreads to humans by consumption of contaminated food or water (4). A range of virulence factors is associated with the severity of STEC infection (5). Cattle derived foods can be contaminated and represent an

important source of infection as well bovine carriers of STEC show no sign of clinical disease whilst shedding this pathogens, the pathogen (STEC) has been isolated from other animals, including deer, goats, horses, dogs, and pigs (6). STEC shedding has also been demonstrated in small ruminants and that may represent an equally serious risk for people as cattle infections (7,8). These bacteria are carried in the gastrointestinal tract and do not cause disease in mature ruminants even when administered at high doses (9). One of the major virulence factors of STEC is Shiga toxins (Stx1) and (Stx2) that encoded by Stx genes, these genes carried in the genome of temperate bacteriophages encode two distinct Stx. The Stx1 and Stx2 toxins possess similar biological activities, but are distinct in the immunological features (10). These toxins are characterized by the production of cytotoxins that deactivate the protein synthesis within host cells (11). STEC strains constitute a heterogeneous group of organisms in serological and biochemical characters and are detected by methods that reliably target the toxins or the genes that encoding these toxins (12). PCR assays have been used widely to detect small numbers of STEC present in stool specimens, enrichment broths, or primary fecal cultures (13). Real-time PCR techniques have facilitated the development of assays that offer rapid, simultaneous amplification and sequence-specific detection of two target genes in a single reaction tube. Real-time PCR, offers several advantages over traditional end-point PCR methods (14).

Materials and methods

Feces samples collection

Fifty (50) feces diarrheic samples (25 of sheep, and 25 of cattle) were collected from different farms in Al-Diwanyia city. The samples were collected in 25ml sterile containers transported into laboratory and stored in a refrigerator until use for genomic DNA extraction.

Genomic DNA extraction

Bacterial genomic DNA was extracted from fecal samples by using (AccuPrep® stool DNA Extraction Kit. Bioneer. Korea). 200mg fecal sample was placed in 1.5ml micro centrifuge tube and 20µL 10mg/ml Proteinase K and 400µL stool lysis buffer was added and mixed by vortex, then incubated at 60°C for 10 minutes. Then the tubes transferred into centrifuged at 10000rpm for 5 min, after that, the supernatant was transferred in two new 1.5ml microcentrifuge tube and genomic DNA extraction was done according to company instruction. After that, the extracted DNA was checked by Nanodrop spectrophotometer, then store in -20°C at refrigerator until perform PCR assay.

Real-Time PCR

Real-Time PCR technique was performed by using qPCR Syber green dye kit for detection and amplification of (shiga toxin 1 gene) virulence factors gene in *Escherichia coli*. The primes were designed in this study by using NCBI-GenBank recorded sequence for *Escherichia coli* partial stx1 gene for shiga toxin 1, strain EHEC FE94076 GenBank: (FR875155.1) and by using primer3 plus design online. The primers were provided by (Bioneer company. Korea) (Table 1).

Table (1): The nucleotide sequences of primers used for PCR amplification.

Primer		Sequence	Amplicon
Stx1	F	TGCGGTTACATTGTCTGGTG	87bp
	R	TGGCGATTTATCTGCATCCC	

The real-time PCR amplification reaction was done by using (AccuPower^{TM} 2X Green star qPCR master mix kit, Bioneer. Korea) and the qPCR master mix were prepared for each sample according to company instruction (Table 2). These qPCR master mix reaction components that mentioned in table (2) was placed in sterile white qPCR strip tubes and transferred into Exispin vortex centrifuge for 3minutes, the place in MiniOpticon Real-Time PCR system and applied the following thermo cycler conditions (Table 3).

Table (2): The qPCR master mix components according to company instruction.

qPCR master mix	Volume
Genomic DNA template	2.5µL
2X Green star master mix	25µL
Stx1 gene Forward primer (10pmol)	1µL
Stx1 gene Reverse primer (10pmol)	1µL
DEPC water	20.5µL
Total volume	50µL

Table (3): The thermo cycler conditions in the Real-Time PCR system.

qPCR step	Temp.	Time	Repeat cycle
Initial Denaturation	95 °C	3 minute	1
Denaturation	95 °C	10 sec	45
Annealing\ Extension	57.2 °C	30 sec	
Detection(scan)			
Melting	60-95°C	0.5 sec	1

Bacteriological examination of clinical specimens:

The clinical samples (feces samples from diarrheic sheep and cattle) were immediately inoculated on MacConkey's agar plates and incubated at 37 ºC for 18-24 hr. randomly selected colonies from MacConkey's agar plates were sub cultured on eosin methylene blue (EMB) agar plates to observe the characteristic metallic sheen of *E. coli*. The pure colonies were picked up on nutrient agar slants as pure culture and subjected for standard morphological and biochemical tests.

Results

Real-Time PCR technique based SYBR Green dye for detection of Shiga toxin-producing *E. coli* (STEC) were seen in (5/25) (20%) and (2/25) (8%) positive samples in sheep and cattle respectively, by amplification of stx1 gene in extracted DNA from feces samples (Fig. 1). The specificity of stx1 gene primers that amplification by Syber green based real-time PCR was determined by dissociation curve (Melt Curve). Where the positive amplification product samples show specific amplification melt peak mainly at (82°C) without primer dimer or nonspecific products (Fig. 3).

Bacterial isolation and characterization

Bacteriological examination of feces samples revealed the presence of Gram-negative bacilli. In biochemical tests, the isolates were identified as *E. coli* (Fig . 4).

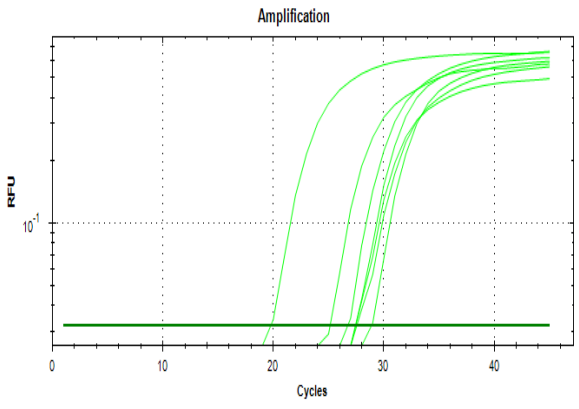

Fig. (1)

Well	Fluor	Content	Sample	End RFU	Call
D03	SYBR	Unkn	Sheep	0.653	(+) Positive
G03	SYBR	Unkn	Sheep	0.611	(+) Positive
B04	SYBR	Unkn	Sheep	0.585	(+) Positive
F02	SYBR	Unkn	Cattle	0.567	(+) Positive
B03	SYBR	Unkn	Sheep	0.547	(+) Positive
B02	SYBR	Unkn	Cattle	0.483	(+) Positive
H06	SYBR	Unkn	Cattle	0.0127	
H05	SYBR	Unkn	Sheep	0.0108	
H04	SYBR	Unkn	Sheep	0.00909	
G04	SYBR	Unkn	Sheep	0.00457	
A06	SYBR	Unkn	Cattle	0.00200	

Fig. (2)

Fig. (1): Real-Time PCR amplification plots for stx1 gene that appeared at 20 to 28 cycle. The samples with amplification at 20 cycles contained very large amount of DNA while the samples with the amplification appeared at 24 cycles contained lower quantity of DNA for *E. coli*.

Fig. (2): Real-Time PCR endpoint analysis that the positive and negative samples in sheep and cattle.

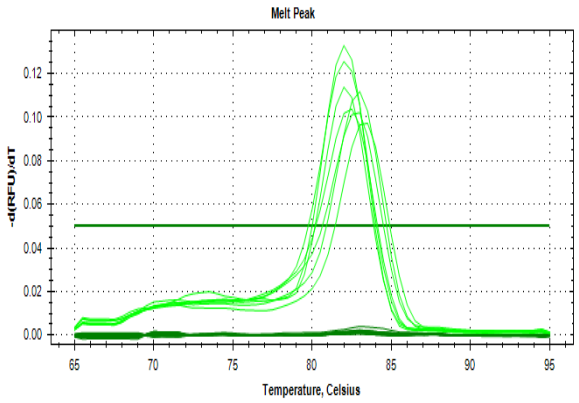

Fig. (3)

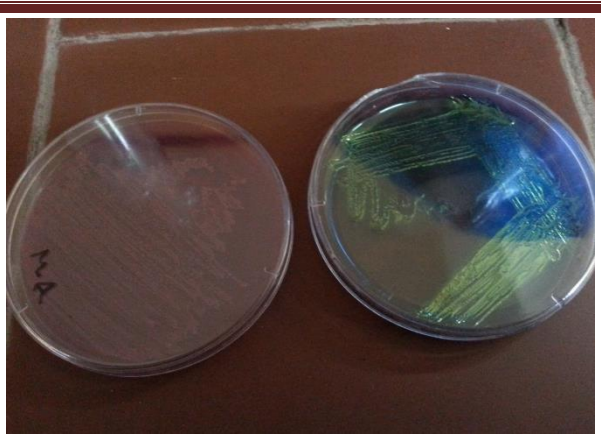

Fig. (4)

Fig. (3): Real-Time PCR Melt curve that shows the melting point for *E. coli* stx1 gene ranged from 81.5°C to 84°C for all samples, and the line from the highest peak to the button was detected that the melting point at 82°C slightly range above or low which represent the specific primers amplification.

Fig. (4): *E. coli* isolation on culture media. Left was MacConkey's agar plate, right was eosin methylene blue (EMB) agar plate, show the characteristic metallic sheen of *E. coli*.

Discussion

The intestinal tract of ruminants, particularly healthy adult cattle and sheep are the principal reservoirs of Shiga toxin-producing *Escherichia coli* (STEC) and only some strains of STEC cause diarrhea (15). This study demonstrated the distribution of STEC in ruminant herds, which represent an important reservoir for strains that pose a potential risk for human infections. The more prevalence of Shiga toxin-producing *Escherichia coli* (STEC) in sheep was appear accepted with (4) who explain that Shiga toxin-producing E. coli in healthy cattle, sheep estimated at 8.7% for sheep and 3.8% for cattle. Other study by (16) which definition of Super-Shedding of (STEC) in cattle were reach to (11%). A particularly important finding was the demonstration of Shiga toxin-producing Escherichia coli (STEC) in sheep and cattle rectal swabs in a herd with commercial milk production such farms pose a real threat of milk contamination during milking, which may subsequently cause infections in people consuming raw milk or milk products. STEC were isolated more frequently from younger cattle; Shiga toxin 1 was the most common toxin type (17). PCR assays have proved useful for detecting and characterizing (STEC) but recent advances in PCR technology by have facilitated the development of real-time fluorescence PCR assays with greatly reduced amplification times and improved methods for the detection of amplified target sequences this technique used by (18) who appeared that real-time PCR is very rapid and sensitive assay for detection of Shiga toxin-producing Escherichia coli directly from stool samples when compared to culture, enzyme immunoassay and Vero cell cytotoxicity assay. We conclude that both sheep and cattle are reservoirs of STEC and our results provide the information about the involvement of STEC in diarrhea in the sheep and cattle, also direct contact with sheep and cattle or consumption of water and food contaminated with their feces may be considered to be main sources of human infections.

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