Detection of shiga toxin producing *Escherichia coli* isolated from mastitis milk of sheep by qPCR technique

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Abstract

This study was conducted to determine the Shiga toxin producing *Escherichia coli* isolated from mastitis milk samples of sheep that collected from Diwanyia city by qPCR technique. A total of (60) clinical investigated mastitis milk samples from sheep were subjected to bacterial DNA extraction by using (Presto^{TM} Mini gDNA Bacteria Kit). The extracted DNA subjected to Real-Time PCR technique (qPCR) for detection of shiga toxin 1 (stx1) gene. Results were revealed that the mastitis sheep are more prevalence for shedding shiga toxin 1 producing *Escherichia coli* (13.3%) of (8/60) positive samples. In conclusion, the Real-Time PCR technique was shown high specific and rapid method in detection of shiga toxin gene and the sheep which infected by mastitis can be carried the shiga toxin producing *Escherichia coli*, that may be considered to be risk factor of human infections.

Key words: *Escherichia coli* , Shiga toxin, Real-Time PCR.

Introduction

Escherichia coli is classified according to their virulence factors such as enterohemorrhagic *Escherichia coli* (EHEC), Enterotoxigenic *Escherichia coli* (ETEC), enteropathogenic *Escherichia coli* (EPEC), attaching and effacing *Escherichia coli* (AEEC), and Shiga toxin-producing *Escherichia coli* (STEC) **(1).** Mastitis is believed that the mainly costly disease in dairy herds due to lowered milk production for approximately 80% of costs associated with mastitis, treatment costs, and death **(2).** Several studies showed that Shiga toxin-producing *Escherichia coli* (STEC) isolates which are important group that causes mastitis **(3,4).** E. coli is a bacteria which is often found in raw milk, especially in poor hygiene cases during milking and primary milk process **(5).** STEC bacteria causes mastitis in lactating

cows and sheep **(6).** The major reservoirs for STEC bacteria is cattle and other ruminants **(7).** STEC it demonstrated in shedding in small ruminants and that may represent an equally serious risk for people as cattle infections **(8,9).** Shiga toxins are major virulence factors of STEC it is encoded by Shiga toxins (Stx1) and (Stx2) genes, these genes located in the genome of temperate bacteriophages. The Stx1 and Stx2 toxins are have similar biological activities, they are immunologically different **(10).** The STEC isolates produce one or both of major types of Shiga toxins, and these toxins that are characterized by the production of cytotoxins that disrupt protein synthesis within host cells **(11).** STEC isolates are constitute serologically and biochemically heterogeneous group of organisms and are most reliably detected by methods that target the toxins they produce or the genes encoding these toxins **(12).** PCR assays have been used widely to detect small numbers of STEC present in milk specimens **(13).** The introduction of real-time PCR provides the opportunity for the rapid detection as apart from saving time, real-time PCR is highly specific, sensitive and offers the potential for detection and quantification of target pathogen **(14).** The present study aimed to use highly sensitive real-time PCR based TaqMan probe technique for detection of STEC bacteria from milk of sheep suffering from mastitis in Al-Diwanyia city.

Materials and Methods

Samples collection: Sixty milk samples were collected from sheep infected by mastitis that investigated by California mastitis test (CMT) from different sheep field in Al-Diwanyia city. The milk samples were collected in 25ml sterile containers after clean and washing the quarters of udder by disinfectant solution, then the milk samples transported into laboratory and stored in a refrigerator until use for bacterial isolation.

Bacterial isolation: *Escherichia coli* was isolated from milk samples by inoculation on Brain Heart Infusion Broth media at 37°C overnight for primary enrichment culture and then the bacterial growth were inoculated on Eosin methylene blue agar and sheep blood agar at 37°C overnight for selective isolation of pure culture *Escherichia coli* isolates.

Bacterial genomic DNA extraction: Bacterial genomic DNA was extracted from *Escherichia coli* isolates by using **(PrestoTM Mini gDNA Bacteria Kit. Geneaid. USA).** 1ml of overnight bacterial growth on BHI broth were placed in 1.5ml microcentrifuge tubes and than transferred in centrifuge at 10000rpm for 1 minute. After that, the supernatant was discarded and the bacterial cells pellets were used in genomic DNA extraction and the extraction was done according to company instruction. Then, the extracted gDNA was checked by Nanodrop spectrophotometer, and store in -20C at freezer until perform Real-Time PCR assay.

Real-Time PCR

Real-Time PCR technique was performed by using TaqMan qPCR amplification kit for detection (shiga toxin 1 gene) .The primes and probe were designed in this study by using NCBI-GenBank recorded sequence for *Escherichia coli* partial stx1 gene for shiga toxin 1, strain EHEC FE94084 **(GenBank: FR875151.1)** and by using primer3 plus design online. The primers were provided by (Bioneer company . Korea) as show in the following (table 1).

Table (1): Shiga toxin 1 gene (stx1) primers and probe.

Primer	**Sequence (5'-3')**		**Amplicon**
Stx1 primer	**F**	TGCGGTTACATTGTCTGGTG	**87bp**
	R	TGGCGATTTATCTGCATCCC	
Stx1 probe	FAM-CGTGTTGCGGGGATCAGTCGT-BHQ1		

The Real-Time PCR amplification reaction was done by using **(AccuPower® DualStar™ qPCR PreMix kit, Bioneer. Korea)** and the qPCR master mix were prepared for each sample according to company instruction as following (table 2).

Table (2): Real-Time PCR master mix preparation.

qPCR master mix	**Volume**
Genomic DNA template	2µL
Stx1 forward primer (20pmol)	1µL
Stx1 reverse primer (20pmol)	1µL
Stx1 probe (50pmol)	1µL
DEPC water	15µL
Total volume	**20µL**

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 thermocycler conditions as the following (table 3).

Table (3): Real-Time PCR thermocycler conditions.

qPCR step	**Temperature**	**Time**	**Repeat cycle**
Initial Denaturation	95 °C	3 minute	1
Denaturation	95 °C	15 sec	45
Annealing\ Extension	60 °C	30 sec	
Detection(scan)			

Results

The Real-Time PCR results were show that 8 out of 60 *Escherichia coli* isolated from milk in this study were positive for Shiga toxin-producing *Escherichia coli* (STEC). The positive samples in Real-Time PCR which appeared in threshold cycle (CT value) ranged from CT: 15 to CT: 38 as in (Figure 1).

The efficiency Real-Time PCR amplification based TaqMan probe (FAM) was checked by prepared standard curve from four serial DNA dilution of positive control STEC isolates. where, the results show high qPCR efficiency up to (99.9%) as in (figure 2 and figure 3).

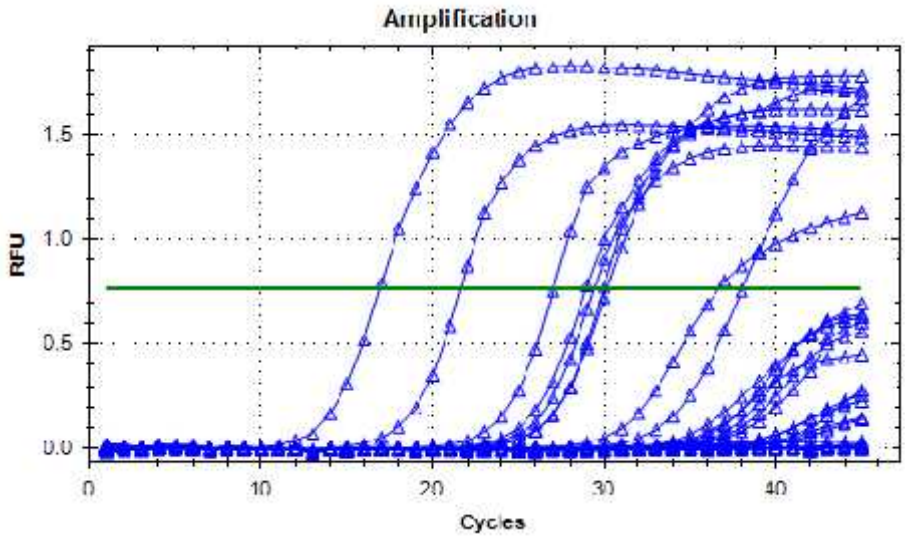

Figure (1): Real-Time PCR amplification plots that show the positive amplification of stx1 gene in shiga toxin-producing *Escherichia coli* (STEC) isolates. The isolates which appeared amplification at 15 cycles was contained very large amount of DNA *Escherichia coli* (STEC). The amplification were appeared later, samples were negative which appeared under threshold line.

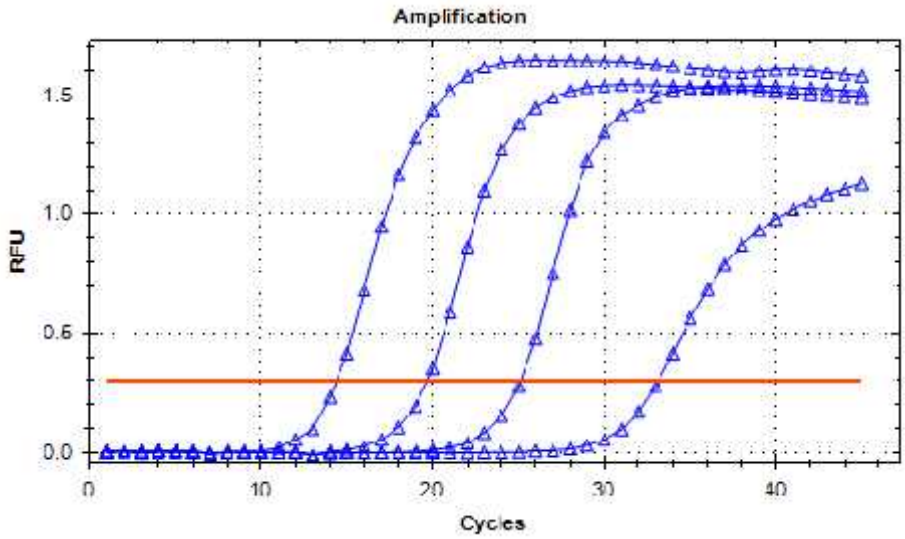

Figure (2): Real-Time PCR amplification plots that show the amplification of positive control STEC isolate for standard curve of stx1 gene that show specifity of Real-Time PCR based TaqMan probe (FAM) in four serial DNA dilution.

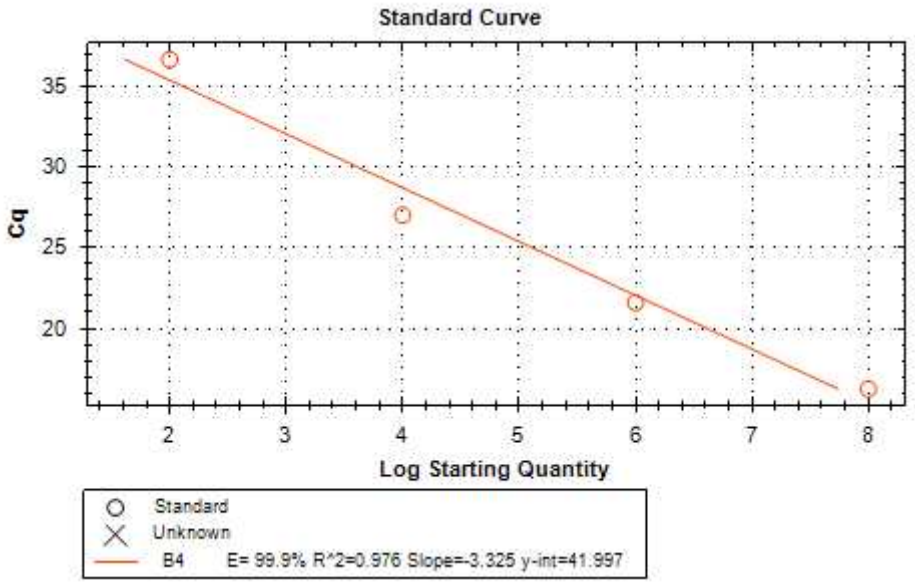

Figure (3): Real-Time PCR standard curve that show the log amplification stx1 gene in positive control four serial DNA dilution of STEC isolate that show qPCR efficiency (99.9%) .

Discussion

The Real-Time PCR was appeared highly sensitive and specific assay that used in detection of Shiga toxin-producing *Escherichia coli* (STEC) that isolated from milk samples of sheep that infected by mastitis. the extracted bacterial genomic DNA by using (PrestoTM Mini gDNA Bacteria Kit. Geneaid. USA) this kit was used Spin column-based nucleic acid purification its solid phase extraction method to quickly purify nucleic acids. This method relies on the fact that nucleic acid will bind to the solid phase of silica under certain conditions. Therefore this kit was appeared rapid in one hour extraction and simple method for purification of *Escherichia coli* bacterial genomic DNA from overnight bacterial growth on enrichment BHI broth media at 37°C . Results showed that milk of sheep infected by mastitis are shedding shiga toxin 1 producing *Escherichia coli* by using Real-Time PCR technique. The application of accurate and sensitive test for detection of mastitic milks caused by shiga toxin producing *Escherichia coli* is essential, the Real-time PCR assay based on the use of fluorogenic oligonucleotide probes have been advantages, such as quantification, increased speed, prevention of carryover contamination and high throughput automated analysis **(15).** Many studies are show numerous outbreaks of diseases due to STEC bacteria **(16,17)** showed that inspection and control of food and milk is a golden solution to reducing the risk of contamination **(18).** Mastitis is known for some time as the most costly disease in dairy herds, dependent on many epidemiological studies, it has been hypothesized that cattle are infected with *Escherichia coli* from their environment, as feces and straw **(19).** 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. Our results show the milk of sheep infected by mastitis are more prevalence for shedding shiga toxin 1 producing *Escherichia coli* in (8/60) at (13.3%) positive samples. The more prevalence of Shiga toxin-producing *Escherichia coli* (STEC) in sheep is appear accepted with **(20)** 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 **(18)** which definition of Super-Shedding of (STEC) in cattle are 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. PCR assays have proved useful for detecting and characterizing (STEC) but Recent advances in molecular 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 **(21)** who appeared that real-time PCR is very rapid and sensitive assay for detection of Shiga toxin-producing *Escherichia coli* directly from clinical samples when compared to culture, enzyme immunoassay and Vero cell cytotoxicity assay. In conclusion we conclude that used the Real-Time technique was shown high specific and rapid method in direct detection of (stx1) gene and the sheep that carried the shiga toxin producing *Escherichia coli*, that may be considered one of important sources of human infections.

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تحديد جرثومة الشريكيا القالونية المنتجة لسموم الشيكا المعزولة من حليب الأغنام المصابة بالتهاب الضرع باستخدام تقنية تفاعل سلسلة البلمرة في الوقت الحقيقي الكمي

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الخلاصة

أجريت هذا الدراسة وذلك لتحديد **عزلات** جرثومة الشريكيا القالونية المنتجة لسموم الشيكا المعزولة من نماذج حليب الأغنام المصابة بالالتهاب **الضرع والتي جمعت من حقول مختلفة في** مدينة الديوانية باستخدام تقنية **تفاعل سلسلة** البلمرة في الوقت الحقيقي الكمي . **تم فحص ٦٠ نموذج** من حليب الأغنام المصابة بالتهاب الضرع **وذلك من خلال استخلاص الحمض النووي** البكتيري باستخدام **(عدة برستو** للاستخلاص الحمض النووي البكتيري) وبعدها تم إجراء فحص تفاعل سلسلة البلمرة في الوقت الحقيقي الكمي لتحديد جين الشيكا. وقد ظهرت النتائج **أن الأغنام المصابة بالتهاب** الضرع هي **قادرة على طرح جرثومة الشريكيا** القالونية المنتجة لسموم الشيكا حيث وجد **إن (٨) عزلات موجبة بنسبة (١٣,٣٪).** ومن خلال هذا الدراسة نستنتج بأن تقنية تفاعل سلسلة البلمرة في الوقت الحقيقي الكمي يعتبر طريقة سريعة **وذات** حساسة عالية في تشخيص جرثومة الشريكيا القالونية المنتجة لسموم الشيكا **وأن الأغنام المصابة بالتهاب** الضرع **تطرح تلك الجرثومة والتي قد تشكل عامل خطورة لإصابة الإنسان.**

الكلمات المفتاحية: الشريكيا القالونية، سموم الشيكا، فحص تفاعل سلسلة البلمرة في الوقت الحقيقي الكمي.