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ORIGINAL ARTICLE

Comparison of two methods for detection of *E. coli* O157H7 in unpasteurized milk

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ABSTRACT

Background and Objectives: The most common serotype of enterohaemorrhagic *Esherichia coli* group or Shiga-toxin-producing *E. coli* is O157:H7. Domestic and wild ruminants are regarded as the main natural reservoirs. O157:H7 serotype is the major cause of gastrointestinal infections in developed countries. In this study was conducted to survey on the toxigenic *E. coli* O157: H7 strains in milk of industrial dairy farms.

Materials and Methods: A total number of 150 milk samples were collected from dairy industry in Khuzestan, over a period of 6 months and were evaluated by cultivation in selective media (CT-SMAC) and multiplex PCR.

Results: Two isolates were identified as *E. coli* using biochemical tests, none of them were toxigenic *E. coli* O157:H7 as determined by multiplex PCR. Using direct PCR on milk samples, 45 samples contained at least one gene of the studied genes in this investigation (*rfb, flic, stx1*, *stx2*). With direct PCR, 2 milk samples were positive for toxigenic O157:H7.

Conclusion: *E. coli* O157:H7 is present in this region and so the necessity for strict compliance of health standards is recommended. This is the first study on O157: H7 *E. coli* milk contamination in Khuzestan province. Based on these results, direct PCR is more accurate than indirect PCR.

Keywords: Milk, *Escherichia coli* O157:H7, PCR

INTRODUCTION

Serotype of enterohaemorrhagic *E. coli* is one of the most harmful food borne pathogenic bacteria and is responsible for many cases of infection and deaths worldwide (1). Infection with serotype is usually self-limiting, but the bacterium can cause hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura in children and the immunocompromised patients, which are life-threatening complications (2).

Shiga toxin 1 and 2, which respectively encoded

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by the *stx1* and *stx2* genes (two phage-encoded cytotoxins), the intimin protein (encoded by the chromosomal gene eae), and enterohaemolysin (encoded by the *ehxA* gene), are the most commonly assayed virulence factors of Shiga-toxin-producing *E. coli* (3). The bacterium has a locus of enterocyte effacement (LEE), a pathogenicity island (4), encodes a type III secretion system which includes a translocated intimin receptor (Tir) (5) three enterohaemorrhagic *E. coli* secreted proteins A, B and D (EspA, EspB, and EspD) which are important in signal transduction of mammalian host cells as well as attaching and effacing lesion formation (6). Cattle are the major reservoir of this bacterium for human, although *E. coli* O157:H7 has also been isolated from sheep, goat, horses, dogs, deer, birds and flies (7). Consumption of unpasteurized contaminated milk and under-cooked contaminated meat with fecal material are the main transmission routes in human. Different methods such as culture in special media, serological and molecular assays have been used for detection of this serotype in food, environmental and clinical samples. Sorbitol-MacConkey agar (SMAC) supplemented with cefixime and potassium tellurite, is one of the most sensitive media and improves selection of *E. coli* O157:H7 from other serotypes of *E. coli* and non sorbitol fermenter bacteria such as *Morganella, Aeromonas, Providencia,* and *Plesiomonas* (8, 9).

Since the conventional methods take time, many studies have been designed based on molecular techniques such as PCR (10). Primers in many PCR assays are used for investigation of specific genes, but combining these primers in a single reaction would be a fast and reliable method for detection of the O157:H7 *E. coli*. The presence of these organisms in milk has been studied in different countries (11) but in Iran most studies have been done on the meat and another dairy products in shopping center or bulk milk of farms.

This study was designed to investigate the presence of *E. coli* O157:H7 in raw cattle milk in Khuzestan province, south west of Iran, using both bacterial culture method and PCR.

MATERIALS AND METHODS

Sample collection. In total, 150 fresh milk samples were obtained from six dairy farms in different parts of Khuzestan Province (Ahvaz, Izeh, Baghmalek, Behbahan, Dezful and Shadegan) from March to September, 2013. The clinically healthy Holstein cows with normal physical characteristics of milk were selected for this study. The California Mastitis Test (CMT) was used to detect mastitic milks. Samples were in normal distribution and 10% of dairy farms in each sample were assayed. All samples were collected in sterile 50 ml containers, in total volume of 30-40 ml, aseptically, and were transported to the laboratory at 4ºC within a < 6 h after sampling. For the isolation of *E. coli* O157:H7 from milk, the samples were centrifuged and the sediments were plated on selective media (referred to as the ''milk pellet enrichment-direct plating method'', or MPE-DP method).

Upon arrival at the laboratory, the sampling containers (containing 25 ml of milk) were centrifuged (Eppendorf, Germany) at 3000 rpm for 20 min at 4°C. The supernatant was discarded and the pellets were dissolved in1 ml of milk. One milliliter was added in 14 ml of Tryptone Soya Broth containing 20 mg/l novobiocin (mTSB-n) in 50 ml tubes. After 18h incubated at 37°C, the enriched cultures were centrifuged same before and loopful of pellet cells were used to streak on supplemented sorbitol MacConkey agar supplemented with cefixime (2.5 mg/l) and tellurite (0.05 mg/l) (CT-SMAC, Merck, Germany) and incubated for 24h at 37 ℃. Three to five colonies that had the characteristic of *E. coli* morphology (clear and colorless) were chosen from each plates and cultured on blood Agar (BA). At the first step, every selected suspicious colony were streaked onto plates containing eosin methylene blue agar (EMB,Merck, Germany) and incubated at 37°C. After 24 h, biochemical tests including conventional indole reduction, methyl red, voges proskauer, citrate utilization and lysine decarboxylase tests was done for isolates with typical *E. coli* metallic sheen on EMB. Certain *E. coli* isolates were stored at -70°C in TSB with glycerol (20%).

Multiplex-PCR assay. Every isolates of *E. coli* were screened by PCR for the presence of shiga-like toxins (*stx1* and *stx2*), O157:H7 serotype (O157 and H7 genes). Standard strain of *E. coli* O157:H7 (ATCC 43894) and sterile distilled water were used as a positive and negative control, respectively.

After prepared of suspension of every *E. coli* colony in sterile TE (Tris-EDTA) buffer with 2% 2-mer-

captoethanol, by heating the bacterial suspension for 10 min in boiling water temperature the bacteria was lysed. The lysate was spun at 13000 rpm for 3 min to pellet the cellular debris.

Supernatant was stored at -20°C as template for amplification by m-PCR. The primer sequences used were: *flic* H7 and *rfb* O157 which encoded the flagellar and somatic antigens respectively (12). Certain isolates as *E. coli* O157:H7, were examined by second m-PCR assay and using *stx1* and *stx2* genes specific primers (13) (Table 1). The amplification conditions and reagents for the m-PCR assays were those described by Berenjchi et al. (2010) with total volume of 25 µl in each m-PCR reaction and amplification mixture consisting of 12.5 µl 2X mastermix (Sinagen, Iran), 1µl of each primer (0.5 µM), and 5 µl of template. The thermocycler (Eppendorf, Germany) PCR program was started with initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 52°C for 30 sec and elongation at 72°C for 60 sec, and final extention at 72°C for 10 min(14). The PCR products were electrophoresed in TAE (Tris- Acetic acid- EDTA) buffer containing 1% agarose, visualized by safe- staining (Sinagen, Iran), illuminated by UV-transilluminator apparatus. As a DNA marker, 100 bp DNA ladder was used.

Direct multiplex-PCR. Direct PCR was done on enriched milk samples in N-TSB. For this purpose 1ml enriched milk sample in N-TSB from the first stage was defrosted and centrifuged with 13000 rpm for 3 min. Sediment was resolved in 1ml TE buffer and diluted 1:20 in TE buffer again. Bacterial suspension was used in DNA extraction like the previous protocol and was stored at -20ºC. The second stage of PCR was done similar to first stage.

RESULTS

Although 14 colonies of non-sorbitol fermenting (NSF) were isolated from 150 milk samples, after enrichment and selective plating only two isolates were identification as *E. coli* by biochemical tests. In m-PCR assay, using specific primers for *rfb* and *flic* genes,

the isolate confirmed serotypes other than *E. coli* O157:H7 and the second m-PCR assay, using specific primers for *stx1* and *stx2* genes, showed that the isolates were harboring none of the toxin genes (*stx1*, *stx2*). In the second stage of research by direct PCR on 150 enriched milk samples, different patterns of studied genes were observed in 45 samples. Column chart of primary and secondary PCR results is shown below. Twenty (33.3%) of 150 samples were positive for *E. coli* O157 (carrier for *rfb* gene), eighteen isolates of which (90%) were non carrier for H7 gene (O157) and only two (10%) were motile (O157:H7). *E. coli* O157 was found with the highest level in milk samples of Behbahan and Eizeh counties. In this research the number of *stx2* producing bacteria was more than *stx1* producing bacteria (Fig. 1).

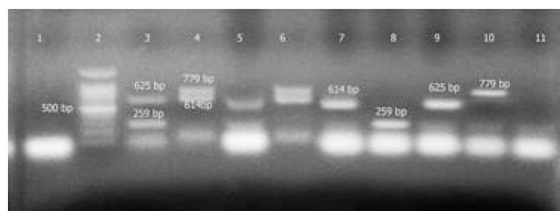

Fig. 1. Results of direct mPCR; line1: negative control, line2: marker 100 bp, line3: positive control for *rfb* (259bp) and *flic* (625bp), line4: positive control for *stx1* (614bp) and *stx2* (779bp), lines 5 and 6:positive samples for all genes studied, lines 7, 8, 9, 10: four positive samples for every one of the genes studied, line 11: negative sample.

Table 1. Primers sequence and expected sizes of studied genes

Target gene	Primer sequence	Size (bp)
rfb	F: 5′- CGG ACA TCC ATG TGA TAT GG -3′	259
	R: 5′- TTG CCT ATG TAC AGC TAA TCC -3′	
flic	F: 5′- GCG CTG TCG AGT TCT ATC GAG-3′	625
	R: 5′- CAA CGG TGA CTT TAT CGC CAT TCC-3′	
stx1	F: 5′- ACA CTG GAT GAT CTC AGT GG-3′	614
	R: 5′- CTG AAT CCC CCT CCA TTA TG-3′	
stx2	F: 5′- CCA TGA CAA CGG ACA GCA GTT-3′	779
	R: 5′- CCT GTC AAC TGA GCA CTT TG-3′	

DISCUSSION

Due to the consumption of unpasteurized and traditional dairy product in Khuzestan province, we decided to survey on the presence of *E. coli* O157: H7 in the collected bovine milk from dairy farms. In this study, 2 isolates of *E. coli* O157:H7 (1.3%) were detected from 150 milk samples by direct PCR assay, but by conventional culture method and multiplex-PCR none of the samples were positive from this serotype (O157 and H7) and the virulence genes studied (*stx1*, *stx2*). Our findings do not differ greatly from those reported abroad from raw cow's milk. Studies in Egypt and Austria, were reported that 6% and 3% of raw cow's milk samples, were contaminated with *E. coli* O157:H7 respectively (15), but generally the prevalence of this serotype is various from 1 to 13% in European countries (16). Similarities and differences in the various regions may be due to similarities and differences in climate and hygiene of the area. Although according to our finding the incidence of this serotype of *E. coli* in milk is low, considering the low infective dose, the presence of this pathogen in raw cow's milk is important in this area of Iran. The same result was observed in the study conducted by Rahimi et al. in 3 provinces (Isfahan, Charmahal va Bakhtiari and Khuzestan) of Iran. From 201 traditional dairy products samples, non-O157 *E. coli* in 14 (7%), O157:NM *E. coli* in 3 (1.5%) and O157:H7.

E. coli in 1(0.49%), were recognized. All the O157:H7/NM *E. coli* isolates were positive for *eae*A and *stx1* and/or *stx2*, and one isolate was positive for *Ehly*A. In stx positive isolates, 1 and 2 isolates had *stx1* and *stx2*, respectively (17). As well as in studies carried out on ground beef hamburger and donor kabab samples in Khuzestan and parts of Iran, presence of *E. coli* O157:H7 was determined based on culture and m-PCR (18). Several studies have been conducted around the world to determine the presence of this serotype of *E. coli* in various foods such as meat (19). Comparison of various studies results is difficult because of difference in methodologies, such as isolation procedures, the type of improved enrichment, differences in sample size, and the type of sample and how and when it was collected. Culture in selective media and PCR used in this study is a conventional and available method for isolation of O157:H7 and a sensitive method for detection of virulence genes, respectively. Although using rainbow agar (a new chromogenic medium for the detection of O157: H7 *E. coli*) has been found to be more sensitive than CT-SMAC, but the difference is not significant (20). In multiplex PCR, the use combination primers for the detection of several genes eliminates the possibility of false positives, which may occur if non-O157:H7 strains were to acquire an O157:H7 specific gene. Although in some strains despite the encoding H7 flagella antigen gene within the genome, immunoreactive H7 flagella antigen is not expressed, and may lead to false negative results (21). Direct m-PCR assay was evaluated as a very suitable method for detection of toxigenic O157:H7 Taking this approach not only decreases medium used, but also increased precision in milk samples (20) because of decrease in false negative results due to viable but non-culturable bacteria (VBNC) which can be present in foods under unsuitable conditions. Also, we used primers specific for flagellar and somatic antigens genes (confirmed by serotyping), and then for virulence factors such as shiga-like toxin 1(SLT_1) and shiga-like toxin 2 (SLT_2). The ability to detect rough isolates or the masked O antigen isolates is main advantage of the employed m-PCR method (22). By direct PCR in this research, *stx1* and *stx2* were detected in two infected samples by *E. coli* O157:H7, but in another (43 samples) frequency of *stx2* (5.3%) was more than *stx1* (4%). In previous studies in USA, European countries and Japan have been reported that the *stx2* gene was more common than the *stx1* (23).

In this study all milk samples were collected in hot seasons (spring to fall), which is consistent with the findings from previous studies that showed, summer and early fall are peak prevalence of infection (24). Different distribution of *E. coli* O157:H7 as the season changes has been reported previously (25), with the highest prevalence in summer and the lowest in winter, so it is possible that the contamination rate is variable.

CONCLUSION

This is the first study on contamination of raw milk with O157:H7 *E. coli* in Khuzestan province of Iran. Comparison of the two methods results showed that direct PCR for pre-enrichment milk samples is more accurate and because of culture process deletion, is more economical and faster. The percentage of positive raw milk samples of the O157:H7 *E. coli* isolates reported herein are the same as what has been report-

ed previously from different dairy products of several provinces in Iran. Although this results, showed that a low percentage of raw milk in Khuzestan is contaminated with this serotype of *E. coli* which is potentially pathogenic for humans, but because of high consumption of raw dairy products in the province, further research is essential in this area for detection of other serotypes of O157:H7. Although finding effective pre-harvest control measures is not easy, but it is necessary to apply some approaches for preventing and reducing the incidence of STEC in animal reservoirs in primary production, finally reducing the pathogens' in foods and water, and on consumer education.

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