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Detection of shiga-like toxigenic *Escherichia coli* from raw milk cheeses produced in Kerman-Iran

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ABSTRACT

The Shiga-like toxin-producing *Escherichia coli* (STEC) is the most important group of food-borne pathogens that emerged recently. These bacteria can cause severe health problems in humans like diarrhea, hemorrhagic colitis and hemolytic uraemic syndrome which have become a serious health problem in various countries. Cattle are thought to be a reservoir for *E. coli* STEC, and many foodborne diseases have been associated with the consumption of minced beef, beefburgers and raw milk. Although some data suggest that STEC are not prevalent within dairy products, the aim of this work was to assess the prevalence of *E. coli* O157 and non-O157 STEC in raw milk cheeses produced in the Southern part of Iran (Kerman province). For this purpose, 125 samples of soft and semi-soft cheeses made with raw cow milk were analysed with multiplex-PCR method for the presence of STEC. The use of consensus primers detected *stx* genes in 6.4% of the samples, but STEC strains could be isolated in only five of them (4%). Just one sample was found to be contaminated with *E. coli* O157. Our results suggest that in our area study raw milk cheeses could be considered a risk for food born STEC contamination.

Key words: raw milk cheese, *Escherichia coli*, shiga-like toxin

Introduction

Shiga-like toxin-producing *Escherichia coli* (STEC), also called verotoxin-producing *E. coli* (VTEC), is the most important recently emerged group of food-borne pathogens. These bacteria can cause severe disease in humans, such as hemorrhagic colitis (HC),

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nonimmune hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). Cattle, especially young animals, have been implicated as a principal reservoir of STEC. Foods implicated are of bovine origin, in particular minced beef, beef burgers and raw milk (BEUTIN, 1999; BLANCO et al., 2003; KOBAYASHI et al., 2001). Human and bovine STEC elaborate two potent phageencoded cytotoxins, known as Shiga-toxins (Stx1 and Stx2) or verotoxins (VT1 and VT2) (PATON and PATON, 1998). SLT produced by STEC are very similar to those produced by *Shigella dysenteriae* type 1 and those also known as Shiga-like toxins (SLTs). SLT1 and SLT2 are different proteins and encoded by different sets of genes, but their active molecular structure and biological functions are similar. Verotoxins inactivate ribosomal RNA, inhibit protein synthesis and eventually result in the host cell's death (STROCKBINE et al., 1988). It therefore seems reasonable to assume that any food contaminated with Shiga-like toxin *E. coli*, which possess accessory virulent factors, could be at risk for public health. In addition to toxin production, another virulence-associated factor expressed by STEC is a protein called intimin, which is responsible for intimate attachment of STEC to the intestinal epithelial cells, causing attaching and effacing (A/E) lesions in the intestinal mucosa (BOERLIN et al.,1999). STEC strains that cause human infections belong to a large number of O:H serotypes (a total of 472 serotypes). Most outbreaks of HC and HUS have been attributed to strains of the enterohemorrhagic serovar O157:H7. However, as the STEC non-O157 is more prevalent in animals and as a contaminant in foods, humans are probably more exposed to these strains. (BLANCO et al., 2003).

Several techniques such as immunoassay, verocell assay and PCR have been used to detect verotoxins (HOLLAND et al., 2000). Amongst these, PCR has been widely used, and a number of studies have targeted stx genes and one or more STEC-specific genetic markers (CHAPMAN et al., 2001; HOLLAND et al., 2000; OMISAKIN et al., 2003).

There is little information on the incidence of infection with non-O157 STEC and O157 in Middle East including Iran (ASLANI and BOUZARI, 2003).

The aim of this study has been two-fold: to detect STEC bacteria in raw milk cheeses produced in Kerman-Iran, and to assess the prevalence of *E. coli* O157 and non-O157 STEC serovars in their samples.

Materials and methods

Samples collection. One hundred twenty five samples of soft and semi-soft cheeses made from raw cow milk were purchased from local retail stores in south-East Iran (Kerman province).

Preparation of oligonucleotide primers. Nucleotide sequence of primer pairs specific for VT1, VT2 were the same as in the study published by HOLLAND et al. (2000), and the

primers were purchased from Bioneer (Korea). The nucleotid sequences and predicted product sizes of the primers are shown in Table 1.

Table 1. Oligonucleotide primers sequences

Primer	Sequence (5′-3′)	Reference	Amplification product size (bp)
VT1F VT1R	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	Paton and Paton (1998) modified by Fitzmaurice J. (2003)	180
VT2F VT2R	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	Paton and Paton (1998) modified by Fitzmaurice J (2003)	255

Extraction of DNA and detection of STEC strains by (M-PCR). The protocol used was described by PIERARD et al. (1997). Briefly, the enrichment medium (1:10 dilution from 10 g sample) was Modified MacConckey broth (Oxoïd). After blending the sample in a stomacher, the incubation was conducted at 37 °C during 24 h. One mL of bacterial material was suspended in sterile water and heated at 100 °C during 10 min to release DNA. PCR was directly performed using consensus primers amplifying the Shiga-like toxin *stx* genes. For each PCR-positive sample, a maximum of 20 colonies obtained on the MacConckey agar plate was tested separately in order to isolate STEC strains. Positive *stx* consensus PCR isolated colonies were subsequently identified through biochemical tests (indole production, Klieger test, ß-glucuronidase activity).The *stx*1 and *stx*2 genes were finally detected in sample isolates by the Multiplex-PCR procedure. Primers specific for *stx*1, *stx*2 genes are shown in Table 1. Each of the primers was used at 20 pM, with 200 mM each deoxynucleotide triphosphate (Boehringer Mannheim, Meyher, France), 2.5 10X PCR buffer, 2.5 mM $MgCl_2$, and 2 U of *Taq* DNA polymerase (Denmark). Then, 3 min in 95 °C, 35 cycles including, denaturation for 45s at 94 °C, primer annealing for 90s at 58 °C, and extension for 90s at 72 °C and, finally, 7 min incubation at 72 °C in a Corbett Research DNA thermal cycler (Corbett, Australia) were applied. The reaction products were then analyzed by electrophoresis on 1.2% agarose gels with 1% ethidium bromide (Fermentas, Germany). The expected product sizes are given in Table 1. DNA from the reference strain, *E. coli* reference VTEC *E. coli* O157:H7 (ATCC; 43895), and a reagent blank, which contained all components except the template DNA(NTC), were included as positive and negative controls, respectively (Fig.1.).

Serotyping. Anitbody monoclonal monospecific anti O157 (Mast, UK) were used for detection of O157 *E. coli*.

Results

By using direct M-PCR on cheese samples following perenrichment step, 8 samples (6.4%) were contaminated with STEC. From eight direct positive samples only 5 STEC were isolated, out of just one colony was positive with monospecific monoclonal antibody anti O157. In other words, 75% of isolates were non-O157.

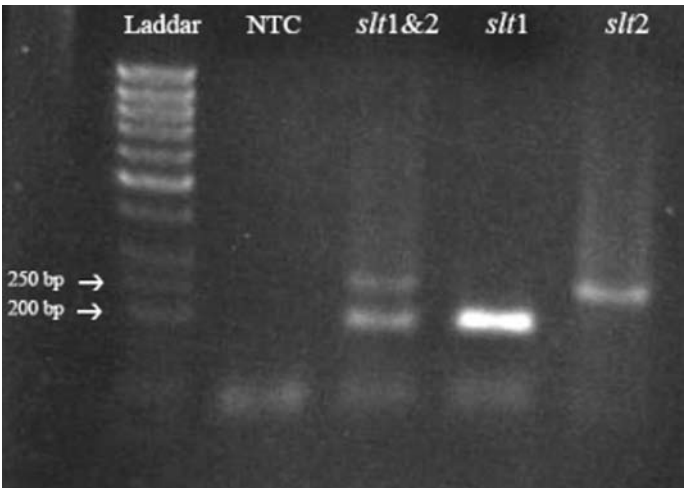

Fig. 1. Products seen on gel electrophoresis after multiplex PCR with a reference VTEC *E. coli* O157:H7 (ATCC; 43895). Marker is 50 bp GeneRuler.

Discussion

Strains of STEC that can cause hemorrhagic colitis and HUS have been termed EHEC. *Stx* has been implicated in the pathogenesis of HUS because cases have been associated with *Stx*-producing bacteria such as *E. coli, Shigella* and *Citrobacter freundii* (KARMALI et al., 1985; KOSTER et al., 1978). In view of the increasing importance of STEC as emerging food-borne pathogens, and reports on O157:H7 and non-O157 STEC causing severe illness in human, evaluation of prevalence of these bacteria in foods is necessary. Although the incidence of *E. coli* O157:H7 with cheese-associated outbreaks seems to be very low in the United States, raw milk cheeses have been associated with some food poisoning in Europe (ALTEKRUSE et al., 1998). In the French outbreak occurring in 1992-1993 (DESCHENES et al., 1996), the serovar responsible was a non-O157 (O103:H2) and

the case control study showed that the occurrence of HUS was linked to the consumption of cheese produced from a mixture of unpasteurised cow and goat milk. In contrast to North America, United Kingdom and Germany, the epidemiological Belgian data indicate that only one fourth of STEC strains isolated in hospitals belong to the serogroup O157 (PIERARD et al., 1997). Belgian study carried out by ACHESON and KEUTSCH (1996), showed that 38% of the STEC isolates were O157:H7 but 62% were non-O157 serovars. Reported estimates on the prevalence of VTEC in raw cow's milk and cheeses range from 0 to 11.1% (COIA et al., 2001; NEAVES et al., 1994; VIVEGNIS et al., 1999).

Since there was no available data regarding the prevalence of STEC in Iran, the aim of this study was to determine the occurrence of STEC in raw milk cheeses produced in South East Iran. The DNA sequences of *stx*1 and *stx*2 have been known for more than a decade and are popular targets for diagnostic PCR assays.

We used a preenrichment phase, followed by testing by PCR, and 6.4% of the cheeses tested gave positive results. Out of 5 STEC isolated just one isolate was O157 serovar. There are different reports on prevalence STEC in raw milk products. Results are compared to the available data from literature: using PCR, *stx* genes were found in 10% cheeses samples in France, in 11.1% samples in Belgium (PRADEL et al., 2000; VIVEGNIS et al., 1999). Methods based on culture showed the STEC prevalence to be lower than in these studies. This discrepancy might be due to the high sensitivity of technique based on the direct *stx* detection.

ASLANI and BOUZARI (2003) in a study in Iran showed all of the STEC isolates from children to be non-O157, even though bovine products have been mostly implicated in food borne infections with *E. coli* serovar O157:H7. However, recent outbreaks indicate that non-O157 STEC like O91, OX3 can cause HUS. We must keep in mind that raw milk cheese may represent a hazard of enterohaemorrhagic food poisoning

In conclusion, it seems that in Iran, like in some countries, STEC are predominantly non-O157, but further epidemiological studies from other regions of Iran and elsewhere are needed to establish the exact prevalence and role for non-O157 VTEC isolates.

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SAŽETAK

Escherichia coli koja proizvodi "shiga-like" toksin (engl. shiga-toxin producing *E. coli*; STEC) pripada skupini najvažnijih bakterija trovača hranom te odnedavno ima sve veće značenje. Ta bakterija može uzrokovati teške poremećaje u ljudi, kao što su proljev, hemoragijski kolitis i sindrom hemolitične uremije, koji su postali ozbiljni zdravstveni problemi u različitim zemljama. Goveda se smatraju rezervoarom za *E. coli* STEC te su mnoge bolesti vezane uz uzimanje mljevene govedine, usitnjena oblikovana goveđeg mesa i sirova mlijeka. Premda neki podatci govore da se STEC ne nalazi u većoj mjeri u mliječnim proizvodima, svrha je ovoga rada procijeniti prevalenciju *E. coli* O157 i non-O157 STEC u svježim sirevima proizvedenima u južnom dijelu Irana, u pokrajini Kerman. U tu je svrhu 125 uzoraka mekanih i polutvrdih sireva proizvedenih od sirova kravljeg mlijeka bilo analizirano multipleks PCR-om na prisutnost STEC-a. Uporabom sukladnih početnica dokazani

su geni *stx* u 6,4% uzoraka, ali su sojevi STEC bili izdvojeni samo u pet od njih (4%). Samo jedan uzorak bio je kontaminiran bakterijom *E. coli* O157. Rezultati upućuju na zaključak da se u istraživanom području sirevi od svježeg kravljeg mlijeka mogu smatrati rizičnim za pojavu STEC-a.

Ključne riječi: svježi sir, *Escherichia coli*, shiga-like toksin