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Araştırma Makalesi

Escherichia coli O157 and O157:H7 in Raw Cow Milk

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A B S T R A C T

Background/Aim: Infection with *Escherichia coli* (*E. coli*) O157 and O157:H7 is a significant health concern in a growing number of regions around the world. Dairy cattle have been regarded as primary host of the bacteria and raw cow milk or milk products carry a potential risk for the bacteria.

Material and Methods: Therefore, this study was conducted in a Turkish province to investigate the presence of *E. coli* O157 and O157:H7 strains and to detect the presence of the *stx1, stx2* genes and present of *intimin* (*eaeA* gene) in the isolates obtained from 150 raw cow milk samples using enrichment-based immunomagnetic separation (IMS) technique and multiplex PCR assay, respectively.

Results and Conclusion: As a result, *E. coli* O157 was detected in one (0.66%) out of 150 raw cow milk samples, and the number of isolates obtained from one raw milk sample was two. Then, these two isolates were analyzed for being *E. coli* O157:H7. According to analyses result, the two isolates were identified as *E. coli* O157:H7. While the *eaeA* gene alone was detected in the one isolates, none of the *stx1* and *stx2* genes were detected in the isolates. Due to the higher possibility for contamination of milk at dairy farms with *E. coli* O157 or O157:H7, consumption of raw milk should be avoided.

Keywords: *E. coli* O157, *intimin (eaeA*)**,** O157:H7, raw milk, *stx1, stx2,*

Çiğ İnek Sütlerinde *Escherichia coli* O157 ve O157:H7 Varlığı

Ö Z E T

Özbilgi/Amaç:*Escherichia coli* (*E. coli*) O157 ve O157:H7 dünyanın hemen her bölgesinde görülen ve özellikle çocuklar olmak üzere, her yaştan insan için önemli sağlık problemlerine yol açan en önemli gıda patojenlerindendir. Ruminantlar, özellikle de süt sığırları bu bakterinin ana konakçısıdır ve bağırsak gaitaları ile çevreyi kontamine edebilmektedir. Bu nedenle çiğ inek sütü ve süt ürünleri bu bakteri için potansiyel bir risk taşımaktadır.

Materyal ve Metod : Bu çalışma Samsun ili ve çevre köylerinde yetiştirilen ineklerden alınan 150 çiğ süt örneğinde, zenginleştirme-temelli immunomanyetik seperasyon (IMS) tekniği ve multipleks PZR yöntemlerini kullanarak sırasıyla *E. coli* O157 ve O157:H7 varlıklarının belirlenmesi ve elde edilen izolatlarda *stx1, stx2* ve *intimin* (*eaeA*) gen varlıklarını saptamak için yapıldı.

Bulgular ve Sonuç: Sonuç olarak, analiz edilen 150 çiğ süt örneğinin sadece 1'inde (% 0.66) *E. coli* O157 izole edildi. Elde edilen izolat sayısı ise iki idi. Daha sonra bu iki izolat O157:H7 yönünden analiz edildi ve analiz sonucunda bu iki izolat *E. coli* O157:H7 olarak identifiye edildi. İzolatlarda *stx1, stx2* ve *intimin* (*eaeA*) gen varlığı belirleme çalışmaları sonucunda; intimin (*eaeA*) varlığı sadece bir izolatta saptanırken, *stx1*ve *stx2* gen hiçbir izolatta saptanamadı. Sütçü sığır çiftliklerinde sütün *E. coli* O157 ve O157:H7 ile kontamine olabilme olasılığının diğer çiftliklere göre daha yüksek oluşu nedeniyle, çiğ süt tüketiminden kaçınılmalıdır.

Anahtar kelimeler: Çiğ süt, *E. coli* O157, O157:H7, intimin (*eaeA*), *stx1, stx2*

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Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) strains are a subset of the Shiga toxin *E. coli* (STEC) that cause diseases in humans and pose a threat to public health, worldwide (Griffin, 1995). Serogroup O157:H7 in particular, include significant food-borne pathogens which can cause severe infection. Their zoonotic relevance is related to the severity of caused diseases and the low dose of ten to 100 bacteria (Ho et al., 2103). EHEC can produce asymptomatic infections, such as non-bloody diarrhea, bloody diarrhea or hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombocytic thrombocytopenic purpura (TTP) (Griffin, 1995; Lim et al., 2010).

The pathogenicity of *E. coli* O157 and O157:H7, including STEC, is associated with several virulence factors. The main factor contributing to their pathogenicity is their capacity to produce two potent phage-encoded cytotoxins called Shiga-toxins (namely, Stx1 and Stx2). They act like the plant toxin ricin by inhibiting protein synthesis in endothelial and other cells. These toxins cause diseases, such as HC and HUS, through cytopathic effects on the vascular endothelial cells of the kidneys, intestines, the central nervous system, and other organs. Another virulence-associated factor expressed by STEC is a protein called intimin, which is encoded by the *eae* gene and is responsible for the intimate attachment of the STEC to the intestinal epithelial cells. This further causes attaching and effacing lesions in the intestinal mucosa (Garrido et al., 2006).

Infection with *E. coli* O157:H7 is a significant health concern in a growing number of regions around the world, particularly in areas of Europe, North and South America, South Africa, and Japan (CDC 2005; CDC 2008). It is reported that of the 73,000 illnesses annually in the United States, 2% to 15% of those infected with *E. coli* O157:H7 develop HUS, and 25% are thought to develop long-term renal sequelae (Denny et al.,2008). In Canada, there were 4 reported cases in 2005 of illness due to *E. coli* O157:H7 infections that were associated with raw-milk consumption in Ontario (Weir et al., 2007). In 2007-2009, a total 211 food borne outbreaks by pathogenic *E. coli*, including STEC/VTEC were reported by the EU Member States. The implicated food vehicle was dairy product in nine outbreaks (ECDPC/EFSA 2011). Recently, in September 2014, five children in California were infected with *E. coli* O157:H7 after drinking raw/unpasteurized milk. Four of the 5 children developed hemolytic uremic syndrome reported by Marler Clark Team (2015).

Epidemiological investigations demonstrate that dairy and beef cattle are primary reservoirs of *E. coli* O157 or O157:H7 (Cobbaut et al., 2008; De boer and Heuvelink, 2001), carrying it asymptomatically and shedding it intermittently and seasonally in their feces (Caprioli et al., 2005; Sheng et al., 2006) and feces have a great vehicle for the contamination of carcass or milk. Therefore, many outbreaks of the disease have also been linked, in particular to the consumption of beef (De boer and Heuvelink, 2001) or consumed raw milk or products (Allerberger et al.,2001; Denny et al., 2008; Espie et al., 2006; Upton and Coia, 1994). Likewise, there have been many studies on the prevalence of *E. coli* O157 or *E. coli* O157:H7 in dairy cattle feces in the parts of the world. In these studies, *E. coli* O157 or *E. coli* O157:H7 prevalence in dairy cattle feces was reported between 0.0% and 24.7% (Byrne et al., 2003; Lejeune and Wetzel 2007; Liptakova et al.,2004; Mcdonough et al., 2000; Vicente et al., 2005). There are also a number of studies from different countries around the world concerning the incidence of *E. coli* O157 and O157:H7 in the milk samples. In these studies, the prevalence of the organism in the milk are wide range, between 0.0%-33.5% (Ansay and Kaspar 1997; Chye et al., 2004; Coia et al., 2001; Öksüz et al., 2004; Seker and Yardimci, 2008, Vicente et al., 2005).

Total world and Turkey milk production reached average 636 and 18 million tones, respectively in 2013 (AHDB Dairy 2015; TUIK 2014), resulting in an enormous consumption of and trade in dairy products. In this regard, safety and prevention of milk borne pathogens are of primary importance to public health. Among the pathogenic *E. coli* of greatest relevance to milk is *E. coli* O157:H7, an STEC serotype, which, because of its high virulence, is of major concern to the dairy industry (Bandyopadhyay et al., 2012; Garrido et al., 2006). According to study results reported from various parts of the world mentioned above, it seems that dairy cattle have been regarded as primary host of *E. coli* O157 / O157:H7 and raw cow milk or milk products carry a potential risk for the bacteria. In addition, these isolates also carry some virulence factors. Therefore, the aim of this study was to investigate the presence of *E. coli* O157 and *E. coli* O157:H7 strains and to detect the presence of the *stx1*, *stx2*, and *eaeA* genes in the isolates derived from raw cow milk (unpasteurized) samples obtained from Samsun region, Turkey.

Material and Methods

*Sample collection and isolation procedure***:** In the present study, a total of 150 raw cow milk samples were randomly collected in Samsun region in Turkey between 2008 and 2009 were analyzed for the determination of *E. coli* O157 and O157:H7. In addition, the presence of the *stx1*, *stx2*, and *eaeA* genes in the same isolates were detected using PCR technique.

*Isolation procedure***:** For the isolation, 25 ml each of milk samples was transferred to sterile polyethylene bag and 225 ml modified tryptone soy broth (mTSB-Oxoid-CM 989, Basingstoke, England) supplemented with novobiocine (20 mg/l, Sigma) was added to the samples for the enrichment step. Then, they were homogenized using a stomacher (Interscience bag mixer 400, Saint Nom, France) and then the bags were incubated at 41.5 °C for 24 h (Sheng *et al.* 2006). After that, the IMS technique was applied using immune-magnetic beads coated with an anti-*E.coli* O157 antibody (Dynabeads- anti-*E. coli* O157, Dynal A.S., Oslo, Norway) according to the manufacturer's instructions. At the end of the IMS procedure, 100 mL of wash buffer was added to resuspend the beads-bacteria complex, and finally 50 µL of resuspended complex was spread onto Tellurite Cefixime-Sorbitol MacConkey (TC-SMAC) agar plates and the plates incubated for 24 h at 37. After the incubation, about 5 colonies suspected of being *E. coli* O157 colonies from each plate were selected and subcultured onto Yeast Extract-Tripticase Soy Agar (YE-TSA) plates. The plates were incubated for 24 h at 37 °C, and then confirmatory test described below was done.

*Identification procedure***:** First, the pint point indole test was carried out on the colonies. Then, indole positive colonies were selected and then the colonies were streaked onto methylumbelliferyl glucuronide (MUG)-supplemented-SMAC agar (MUG-SMAC-Suppl. Oxoid-BR 071 E, Basingstoke, England) plates and incubated overnight at 37 °C. Colonies displaying no fluorescence on the MUG-SMAC agar when illuminated with UV light (under 366 nm wavelength) were identified as showing the typical characteristics of *E. coli* O157 species. After that, the cellobiose fermenting test was applied. For this purpose, purple-broth base (Difco-0227-01-6) broth was used. The colonies showing no cellobiose-fermenting were chosen. At the last step, motility test were applied using Motility Test Medium (BBL Motility Test Medium, No 211436, Difco). All sorbitol non-fermenting, indole-positive, MUG-negative, and cel-

lobiose fermentation-negative colonies were cultured on TSA plates and incubated at 37 °C for 24 h. Confirmation was based on agglutination with *E. coli* O157 (Denka- Sheiken, 210753, Tokyo, Japan) and O157:H7 antisera (Denka-Sheiken, 211057, Tokyo, Japan). Colonies that showed a positive precipitation reaction with O157 antiserum were identified as *E. coli* O157, whereas the colonies showing positive precipitation reaction with the O157:H7 antiserum was identified as *E. coli* O157:H7.

Multiplex PCR assay for the determination of the stx1, sxt2 and eaeA virulence genes: DNA templates used for PCR were prepared by boiling bacterial cultures. For the detection of *stx1*, *stx2* (Shiga-toxin genes) and the *eaeA* genes of both *E. coli* O157 and O157:H7 isolates, the multiplex PCR assay described by Paton and Paton (1998) and further modified by Fitzmaurice (2003) was used. *E. coli* O157:H7 (ATCC 43895) was used as a positive control. During the procedures, a gradient thermocycler (Bio Rad-MJ Mini-PTC-1148, Singapore) was used. The nucleotide sequences (*stx1*, *stx2* and *eaeA*) of the primers and the predicted product sizes are depicted in Table 1.

Results

In the present study, two *E. coli* O157 strains were detected in one (0.66%) raw cow milk sample. Then, these two isolates were evaluated as *E. coli* O157:H7 because of non-sorbitol fermenters, MUG negative, indole and motility positive, non-cellobiose fermenters, and O157 and O157:H7 agglutinating. For the detection of some virulent genes, multiplex PCR assay were applied. As a result, the *eaeA* gene alone was detected in the one *E. coli* O157:H7 strain, *stx1* and *stx2* genes were not detected (Fig. 1).

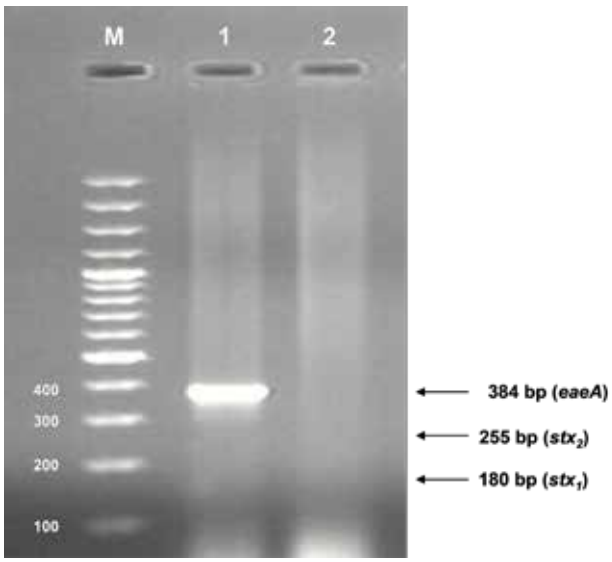

Figure 1. The determination of the presence of *stx1, stx2,* and *eaeA* genes in the *E. coli* O157:H7 strain isolated from raw cow milk samples using the multiplex PCR technique. M: Marker; Lanes 1 and 2: *E. coli* O157:H7 strains isolated from one raw cow milk samples; Lane 1: strain positive for *eaeA* gen, negative for *stx2* and *stx1* genes, lane 2: strain negative for *stx1*, *stx2* and *eaeA* genes.

Şekil 1. Çiğ inek süt örneğinden izole edilen *E. coli* O157:H7 izolatında *stx1, stx2* ve *eaeA* genlerinin multipleks PZR tekniği kullanılarak .belirlenmesi. M: Marker; Sütun 1 ve 2: çiğ inek sütünden izole edilen *E. coli* O157:H7 izolatı; Sütun 1: *eaeA* gen varlığı yönünden pozitif ama *stx1* ve *stx2* yönünden negatif izolat, sütun 2: *stx1*, *stx2* ve *eaeA* gen varlıkları yönünden negatif izolat.

Discussion and Conclusion

For the microbiologic quality of milk and dairy products, the initial microbiologic quality of the milk and dairy cattle health are very important. The farm and dairy cattle condition are also very important for the presence of *E. coli* O157 and O157:H7 and the other pathogenic microorganism in milk and dairy products as well as milk and dairy process conditions. For instance, the feces have a great vehicle for the contamination of carcass or milk. Likewise, there have been many studies on the prevalence of *E. coli* O157 or O157:H7 in dairy cattle feces in the parts of the world. In these studies, *E. coli* O157 or O157:H7 prevalence in dairy cattle feces was reported between 0.0% and 24.7% (Byrne et al., 2003; Mcdonough et al., 2000; Vicente et al., 2005). In all these studies mentioned above, some virulence factors such as *stx1*, *stx2* or *eae* genes were not studies in the all isolates except a few. One of them, Lejeune et al. (2006) found at least one *stx*-positive faecal sample was identified on all Norwegian farms but only on 70% of Ohio farms. In Menrath et al. (2010)'s study showed that *stx* gene was detected in 24.7% of all feacal samples of dairy cattle. Within the herds, the mean *stx* prevalence varied between 11.1% and 32.3%. Another study, reported from Baazize-Ammi et al. (2015), the faecal samples from 51 adult dairy cows were positive for the *stx1* gene alone (83.6%) and those from 10 other dairy cows were positive both *stx1* and *stx2* genes (16.4%). According to these study results, dairy cattle and their feces appear to be a major reservoir for this pathogen, and the contamination of milk at dairy farms with *E. coli* O157 and O157:H7 could be possible. Also, epidemiological studies indicate that the horizontal transmission of *E. coli* O157:H7 occurs in groups of cattle may facilitate its spread and persistence of within the herds (Faith et al., 1996; Jacob et al., 2009). In addition, during the particularly manual milking, cross contamination may have occurred between manure and milk. There are also a number of studies from different countries around the world concerning the incidence of *E. coli* O157 and O157:H7 in the milk samples. The reported results are up to 33.3%. The present study results is between these ratios and relatively low like the other EU countries. From the world, for instance, Egypt, Abdul-Raouf et al. (1996) reported that *E. coli* O157:H7 was isolated with 6% ratio from raw cows' milk. Another study reported from Austria, *E. coli* O157:H7 was isolated with 3% from the milk samples (Allerberger and Dierich, 1997). From Germany, the strain was of 0.3% of the milk samples. However, from the USA (42 samples) (Ansay and Kaspar, 1997; the Netherlands (Heuvelink et al, 1998), China (209 samples) (Chao et al., 2007), Argentina (n=150) (Roldan et al., 2007), reported that *E. coli* O157:H7 was not detected in the milk samples analyzed. In the same way, Coia et al. (2001) from UK (500 milk samples), Dontorou et al. (2003) from Greece (100 cows' milk) and Vicente et al. (2005) from Brazil, *E. coli* O157:H7 was not isolated from the analyzed samples. Apart from these studies, EFSA reported that in 2008, in the EU countries, *E. coli* O157/STEC contamination ratio of raw milk was 1.0% (n=1300) (30). In Slovakia, *E. coli* O157 was detected in 0.4% (n=269) in raw cow's milk (2011). Recently report from Qatar, Mohammed et al. (2015) reported that *E. coli* O157:H7 were detected in 1% in the milk and 2% the udder swabs samples. Another recent study reported from Saudi-Arabia, Al-Zogibi et al. (2015) found the bacteria at 4.81% ratio (26 out of 540 samples) in the milk. In addition, *stx2* and *eaeA* genes were detected in all samples, too. In contrast these low contamination ratio, from Malaysia, Chye et al. (2004) reported that *E. coli* O157:H7 was detected in 33.5% (n=312 of 930) milk samples tested. This result is very high reported other studies and the present study results. In the same way, Bandyopadhyay et al. (2012) reported that STEC was detected in 22.2 % ratio in the milk samples. In addition these, there is a recent

Table 1. Oligonucleotide primer sequences (32, 64)
Tablo 1. Oligonukleotid primer dizilimleri (32, 64)

Sequence (5'-3')		Size of amplification product
ATA AAT CGC CAT TCG TTG ACT AC AGA ACG CCC ACT GAG ATC ATC		180 bp
GGC ACT GTC TGA AAC TGC TCC TCG CCA GTT ATC TGA CAT TCT G		255 bp
GAC CCG GCA CAA GCA TAA GC CCT GCA GCA ACA AGA GG	CCA	384 bp

study reported from Iran and in the study, STEC was detected in 17.47% (36 of 206 samples). It was also reported that STEC isolates harbored 56.41% and 43.59% *stx2* and *stx1* genes respectively (Mohammadi 2013). There have been a few studies from Turkey on the prevalence of *E. coli* O157 and O157:H7 in milk samples. Among them, Öksüz et al. (2004) reported that *E. coli* O157 was determined in 1% of total raw milk samples and in 4% of the cheese samples. In a study for the Anatolian buffalo's feces and milk to detect *E. coli* O157:H7 in Turkey. In the study, Şeker and Yardimci (2008) reported that *E. coli* O157:H7 was isolated from 11 (3.7%) of 300 faecal samples and 3 (1.4%) of 213 raw milk samples. In the present study, *E. coli* O157:H7 strains were isolated from one (0.66%) raw (unpasteurized) cow milk sample. The results slightly low compared the other two studies results reported from Turkey.

According to all studies mentioned above, it is difficult to compare the results of field studies of *E. coli* O157 or O157:H7. Hence, all of the results of these studies indicate a wide range of isolation ratios for the organisms. One of the reasons should be season. It is reported that the excretion of STEC by ruminants seems to be sporadic (Heuvelink et al., 1998; Rahn et al., 1997), but may also be persistent over several months (Geue et al., 2009; Orden et al., 2008). Excretion varies according to the season, peaking in warmer months (Barkocy-Gallagher et al., 2003; Berry et al., 2010). Another reason is individual variations among cows (Mcevoy et al., 2003). Other factors contributing to intermittent excretion include age (Hussein and Sakuma, 2005), diet (Jacop et al., 2009), stress (Rostagno, 2009), housing (Lejeune and Wetzel, 2007), herd size (Erilsson et al., 2005), geographical variation (Lejeune et al., 2006), animal health (Byrne et al., 2003), previous contamination with STEC strains or other pathogens (La Ragine et al., 2009), study design and treatment of antimicrobial substance in the process etc. (Varela-Hernandez et al., 2007). This variation may partly be also due to sensitivity of the method or ineffective laboratory techniques. It is known that the minimal infection dose of *E. coli* O157:H7 is very low. So, several enrichment cultures and isolation methods were developed to isolation of *E. coli* O157 or O157:H7 (Tutenel et al., 2003). One of the more sensitive methods is the IMS technique (Okrend et al., 1992; Tutanel et al., 2003; Weagant et al., 1995), which is why the enrichment/IMS procedures were employed in the present study. In addition, some of the studies mentioned above, DNA based methods have been used to detect genes encoding pathogenicity and virulence factors, such as *stx1*, *stx2*, *eae* and *EhlyA*, in *E. coli* strains and have become a supplement to traditional culturing and serogrouping of STEC. For instance, at the Mohammadi et al. (2013)'s study, for the determination of STEC, they used PCR targeting *stx1* and *stx2* and then *eaeA* in other words genetic detection assay. So, the prevalence of STEC was high (17.47%). Generally, virulence gene prevalence in raw milk samples (*stx1* and/or *stx2*) is also in general significantly higher than isolate occurrence. For example again, studies from Ireland and USA showed that virulence gene prevalence in raw milk was 36% and 21%, respectively, while the occurrence of isolates was 0.8% and 3.2%, respectively (Cobbold et al., 2007, Lynch et al., 2012). The presence of *E. coli* O157:H7 in raw cows' milk is very important although the isolation ratio is low. Hence, the contamination of the milk contained *E. coli* O157 or O157:H7 to the bulk tank could be possible. Other mind, in the part of Turkey, sometimes cheese is produced from raw milk and consumed immediately as fresh cheese. So, any heat treatment or any maturing periods are not applied. Besides these, sometimes raw cows' or other kind of milk consumed directly from human particularly child. For all these reasons, the presence of *E. coli* O157 and O157:H7 pose a serious threat to consumers. It is known that the pathogenesis of *E. coli* O157 and O157:H7 depends on certain virulence genes and factors. Most isolates of *E. coli* O157 produce Stx2 only; Stx1 and Stx2 producers are occasionally found, but isolates that produce Stx1 only are rare (Griffin et al., 1995). On the contrary, intimin, also known as locus of enterocyte effacement (LEE), is a 94–97-kDa outer membrane protein encoded by *eae* (attaching and effacing) gene located in the pathogen city island (PAI). Many EHEC strains contain a PAI of LEE (Moratibo et al., 2003). It plays a significant role in the action of intimin, causing intestinal colonization and formation of lesions. In the present study, *eaeA* gene was detected whereas *stx1* and *stx2* genes were not detected in the isolate. Generally, STEC strains carrying the *eae* gene are considered more virulent for humans than strains lacking *eae* gene (Barrett et al.,1992). Nevertheless, the production of intimin is not essential to induce pathogenesis, because a number of sporadic cases of HUS have been caused by the *eae*-negative, non-O157 STEC strains (Blanco et al., 2004). In the present study, *E. coli* O157:H7 strains were isolated from one (0.66%) raw cow milk sample and *eae* gene was detected. To the authors' knowledge, in contrast to beef, no study has been carried out to *stx1*, *stx2* and *eaeA* genes in the isolates isolated from raw milk in Turkey and most of the world. However, according to outbreaks mentioned above, we can say that *E. coli* O157 or O157:H7 strains isolated from milk or dairy products was contained at least one or more virulence genes.

According to studies results, it seems that that dairy cattle, feces their environments and raw milk possess a potential risk for *E. coli* O157 and O157:H7 and these isolates also carry a virulence factor. Epidemiological studies indicate that the horizontal transmission of *E .coli* O157:H7 occurs in groups of cattle and contaminated water may facilitate its spread and persistence of within the herds (Faith et al., 1996). Therefore, controlling the spread of *E. coli* O157 and O157:H7 at farm lev-

els are very important. A contaminated environment, manual milking and insufficient basic hygiene practices suggested that cross-contamination could have occurred, either between unpasteurized milk and feces at milking time, or possibly at some point during milk products. Because of the higher possibility for contamination of milk at dairy farms with the bacteria, information on health hazards associated with contaminated raw milk should be extended to the public consumption of such raw milk should be avoided.

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