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Research Article

Molecular Identification of *Listeria monocytogenes* and *Escherichia coli* O157: H7 Isolated from Fresh Kashar Cheese and Milk Creme [1]

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Abstract

In this study, it is aimed to analyze the risk factors related to *Listeria monocytogenes* and *Esherichia coli* O157:H7 monitoring in fresh kashar cheese and milk creme in the province of Istanbul. It has 100 pieces of fresh kashar cheese and 100 pieces of milk creme material. Samples were confirmed by PCR methods and genetic basis with conventional microbiological sowing methods and analysis methods. Another case for the study is to obtain significant epidemiological environment for the field strains in the country in terms of *L. monocytogenes* and *E. coli* OH157: H7, where high polymorphic protein structures and rapid genetic variation exist. In the study, 4 (2%) of *E. coli* OH157: H7 and 6 (3%) of *L. monocytogenes* were found among 200 milk slices and fresh kashar cheese samples. Those microorganisms can easily reproduce in natural environment such as soil and silage and with primer/secondary contamination, they appear to be health threatening food related pathogens. It is suggested to have studies for optimal food safety and this can be provided in accordance with appropriate legislation.

Keywords: *PCR, Food safety, L. monocytogenes, E. coli*

Taze Kaşar Peyniri ve Süt Kaymağından İzole Edilen *Listeria monocytogenes* ve *Esherichia coli* O157:H7'nin Moleküler Tanımlaması

Öz

Bu çalışma ile İstanbul ilinde satışa sunulan, taze kaşar peyniri ve süt kaymağı örneklerinde *Listeria monocytogenes* ve *Esherichia coli* O157:H7 varlığı ile halk sağlığı açısından oluşturabileceği risklerin değerlendirilmesi amaçlanmıştır. Araştırma kapsamında rastgele örneklem yöntemi ile toplanmış olan 100 adet taze kaşar peyniri ve 100 adet süt kaymağı materyal olarak kullanılmıştır. Örnekler, konvansiyonel mikrobiyolojik ekim teknikleri kullanılmasının yanısıra PCR yöntemi ile moleküler ve genetik bazda doğrulanmıştır. Çalışmanın farklı bir amacı da, yüksek polimorfik protein yapılarına sahip ve hızlı genetik varyasyon yeteneği bulunan *L. monocytogenes* ve *E. coli* OH157:H7 açısından ülkemizdeki saha suşları hakkında önemli epidemiyolojik verilerin elde edilmesidir. Çalışma sonucunda, analiz edilen toplamda 200 adet olan süt kaymağı ve taze kaşar peyniri örneklerinden 4 adedi (%2) *E. coli* OH157:H7 ve 6 adedi (%3) ise *L. monocytogenes* açısından pozitif olarak tespit edilmiştir. Söz konusu mikroorganizmalar, toprak ve silaj gibi doğal çevre ortamlarında rahatça üreyebilmesiyle ve primer/sekonder kontaminasyonlar nedeniyle halk sağlığını ciddi şekilde tehdit eden gıda kaynaklı patojenler arasında yer almaktadır. Optimal gıda güvenliğinin sağlanması ve gıdanın mevzuata uygun şekilde tüketiciye ulaşması yönünde çalışmalar yapılması önerilmektedir.

Anahtar sözcükler: *PCR, Gıda güvenliği, L. monocytogenes, E. coli*

INTRODUCTION

Foodborne pathogens are considered to be a significant risk factor for public health in developed and developing countries [1] due to their ability to spread throughout the world For almost all of the foodborne infections, the surface of the staff and the surfaces in the facilities, the tools and equipment used in the food production and processing and the sources of contamination from the end consumers, the points where the food prepared in

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bulk and/or the food prepared in the institutions such as hotels, schools, workplaces and hospitals are consumed is defined as a serious risk factor [2]. Inadequate hygiene conditions, and/or microbiologically charged hands can be the weakest chain of food safety chains and seriously threaten public health [3].

Cheese is the mostly consumed variety of food in the world. At the same time cheese is a milk product, which is easily degradable by reducing the rate of humidity, moisture, converting it to a product that can be kept undisturbed for a long time (up to 10 years from 4-5 days depending on the type of cheese and storage conditions) [4]. Chemical and microbiological quality of raw poultry to be used in cheese making is of great importance for consumer health. Otherwise, some of the saprophytes and pathogens in the raw milk can pass to cheese, which can threaten consumer health. In addition, it should be kept in mind that, in the case of toxigenic pathogens such as *Listeria monocytogenes* and *Escherichia coli* in the raw milk, the toxins produced by the agents are not denatured at such temperatures and the important risk factors for consumer health continue even when the pasteurisation procedure is applied [5]. In addition, kashar cheese amongst the cheese varieties constitute the primary risk group for consumers due to short-term maturation procedures [6].

L. monocytogenes is a foodborne pathogen causing gastro-enteritis, septicemia, central nervous system infections, maternal-fetal infections and abortions in humans. Listeriosis caused by this microorganism has the highest mortality rate (reaching 40%) even though it has a lower incidence than other foodborne pathogens, leading to the fact that microorganism is among the foodborne pathogens most seriously threatening public health in medical literature [1]. *E. coli* is an important display of fecal contamination and hygienic applications in milk, dairy products. *E. coli O157:H7* pathogenity is related to Shiga toxins, and intimin. It is known that Shiga toxins are associated with symptoms such as Hemorrhagic Colitis (HC), Hemolytic Uremic Syndrome (HUS) and bloody diarrhea [7,8].

The fact that the agent can be easily grown in natural environments such as soil and silage and isolating it from raw and processed foods, such as milk and its products, meat and its products, vegetables and seafood, seriously raises the risk to consumer's health [9]. Six large listeriosis outbreaks have been reported in the United States and Canada between 1979-1999. According to the results of the investigations, it was determined that the hospitalized cases had been contaminated from green leaf salad, carrot, potato, pasteurized milk, pork products, raw milk, hot dog sandwich, chocolate and various cheeses [10].

The aim of this study is to investigate the presence of *L. monocytogenes* and *E. coli O157: H7* in fresh kashar cheese and milk creme, which have extremely high potential risks for consumer health in milk and products. In addition, in the study, it is aimed to type isolates obtained from samples by molecular methods. Another aim of the study is to demonstrate the advantages/disadvantages of methods used, which are, confirming the isolates on the molecular/genetic basis by PCR procedures in addition to the use of conventional microbiological seeding techniques. However, it is also aimed to obtain important epidemiological data on the field strains in the country in terms of *L. monocytogenes* and *E. coli O157: H7*, which have high polymorphic protein structures and rapid genetic variation ability.

MATERIAL and METHODS

In the scope of the research, 100 fresh kashar cheese and 100 milk creme collected by random sampling method were examined. In the kashar cheese samples, specimens subjected to short-term maturation such as primary risk group were collected. From the point of milk cremes, samples which are offered for sale in the open are preferred. In order to provide sample homogenization, an equal number of sales points were visited for each category at Asian and European sides of Istanbul. The collected samples were delivered to the laboratories of the Faculty of Veterinary Medicine of Istanbul University by observing the antisepsis rules and preserving the cold chain and the samples were started to be analyzed in the laboratory on the same day. Detailed breakdown data of the samples collected during the study are shown in *Table 1*.

Table 1. *Detail breakdown table of fresh kashar and milk samples collected*

Province	Region	Sample Name	Sample Number	Sales Point Type	Explanation
Istanbul	Europe	Fresh kashar cheese	50	Sales points selling on the market or dairy products (cheeses being the point of selling breakfast products)	Products that have been subjected to short-term maturation, preferably openly offered for sale
Istanbul	Asia	Fresh kashar cheese	50	Fix/weekly market	
Istanbul	Europe	Milk creme	50	Sales points selling on the market or dairy products (cheeses being the point of selling breakfast products)	Preferably products that are offered for sale in open form
Istanbul	Asia	Milk creme	50	Fix/weekly market	
Total	200 samples				

Sample Isolation and Identification Procedure

***Listeria monocytogenes*:** 25 g samples were transferred into 225 mL BLEB (Buffered Listeria Enrichment Broth Base) (Merck, Germany), incubated at 30°C for 4 hours and then supplemented with selective agents and 25 mg/L natamycin in media at 48°C for 48 h. At the 24th h of incubation, Oxford agar (Merck, Germany) and Palcam agar (Merck, Germany) were passaged and incubated for 48 h at 35°C. At the end of the 48th h of incubation, passage was made to the Chromogenic Listeria Agar Base (Merck, Germany), one of the *L. monocytogenes/ivanovii* differential selective agar. *Listeria* spp. cultures were purified by passage of susceptible Trypticase anagen (TSA) (Merck, Germany) agar containing yeast extract [11]. Identification of suspicious isolates was performed by Gram staining, catalase, motility, dextrose, maltose, rhamnose, mannitol, xylose fermentation, esculin hydrolysis, nitrate reduction. In addition, CAMP test with *S. aureus* was performed and it was determined whether the isolates had CAMP factor [12].

***Escherichia coli/E. coli O157: H7*:** Asepsy conditions were followed by dilution and homogenization procedures first in sterile containers and then transferred to the laboratory. Afterwards, Tryptone Bile X-glucuronide (TBX) (Merck, Germany) by the spread method into Petri dishes. This Petri dishes were incubated at 44°C for 24 h and typical colonies were counted at the end of the incubation period. For *E. coli O157*: *H7*, homogenization was followed by Modified Tryptic Soy Broth (MTSB) (Merck, Germany) and incubation at 37°C for 24 h. Afterwards, 10 mcL of Sorbitol Mac Conkey Agar (SMAC) (Merck, Germany) was passed from each medium and incubated at 37°C for 24 h. At the end of the incubation period, "straw" color columns in the SMAC Agar were trasnferred to *E. coli* O157:H7 medium (EOH). After an incubation period of 18-24 h, typical pink colonies on EOH were as "suspected". Then, suspected colonies were transferred to cefixime-tellurite (Merck, Germany) (CT-SMAC) to determine CT resistance and the purity of the suspected colonies and the colonies were incubatedat 42°C for 18-24 h. CT resistant colonies were tested for the utility of sorbitol and methyumbelliferyl-β-glucuronide in modified Haemorrhagic Coli (Merck, Germany) broth. Suspected colonies were analyzed to determine of fermentation lactose and sucrose in Triple Sugar Iron agar (Merck, Germany) slants, indol production, methyl red and Woges Proscauer reactions, citrate utilisation (the IMVIC tests) and typical colony morphology on Levine Eosine Methylene Blue agar (L-EMB Merck, Germany) Presence of the O157 antigen was investigated by the latex agglutination test using the *E. coli* O157 test kit (Oxoid). Antisera contained in a commercially available O:H serotyping kit Escherichia coli antisera (SEIKEN, Denka Seiken Co., Ltd., Tokyo, Japan) was utilised for O:H serotyping following the manufacturer's manual [12].

PCR

The presence of *L. monocytogenes* and *E. coli O157: H7* in all samples collected as planned by the PCR procedure will be sought. For this purpose, the PCR procedure defined for each microbiological parameter was applied to the isolates evaluated as suspect or positive after the sowing procedure with conventional methods for microbiological parameters *(Table 2)* [12].

Electrophoresis

The PCR products were 2% (wt/vol) electrophoresed in agarose containing ethidium bromide, and the source of specific bands is searched with the help of UV trans-illuminator. Positive control groups containing DNA-free negative control group and phenol extracted specific active DNA for each parameter were also used during the procedure.

RESULTS

The collected samples were analyzed for *L. monocytogenes* and *E. coli O157:H7* using both conventional microbiological sowing methods and PCR. According to the results obtained in the study four samples of fresh kashar cheese subjected to short time maturation were found to be positive for *E. coli O157:H7* and six samples for *L. monocytogenes* out of two hundred milk samples analyzed. The results of our study are shown at *Table 3*.

Products obtained after PCR are shown in *Fig. 1* and *Fig. 2*.

DISCUSSION

According to the results obtained in the study, 4 (4%) of 100 milk creme analyzed were contaminated with *E. coli O157: H7*, 3 (3%) were contaminated with *L. monocytogenes* and 100 fresh short-term maturated 3 (3%) of the samples of kashar cheese were positive for *L. monocytogenes*.

Table 2. *Primer sets and properties to be designed according to different serotypes used in our work*

Primer No	Sequence (5'–3')	Target Gene/Amp (bp)	Target Microorganisms
1	GCTGATTTAAGAGATAGAGGAACA	*actA* 827	*L. monocytogenes*
2	TTTATGTGGTTATTTGCTGTC	*actA* 827	*L. monocytogenes*
3	GATAGACTTTTCGACCCAACAAAG	*shigaliketoxin*/208	*E. coli O157:H7*
4	TTGCTCAATAATCAGACGAAGATG	*shigaliketoxin*/208	*E. coli O157:H7*

Specifically designed primers were replicated and used in this study (GenBank accession no. NC-003210 for L. monocytogenes and strain: Sakai, substrain: RIMD 0509952, serovar: O157:H7 E. coli O157:H7)

Table 3. *Detail breakdown in terms of L. monocytogenes and E. coli O157:H7 analyzed in fresh kashar cheese and milk creme samples*

Microorganism Information	Sample Name	Example Province/Region	The Total Number of Samples Analyzed	Positive Sample Number
E. coli 0157:H7	Fresh kashar cheese	İstanbul/Europe	50	0
E. coli 0157:H7	Fresh kashar cheese	İstanbul/Asia	50	0
E. coli 0157:H7	Milk creme	İstanbul/Europe	50	3 (6%)
E. coli 0157:H7	Milk creme	İstanbul/Asia	50	1 (2%)
L. monocytogenes	Fresh kashar cheese	İstanbul/Europe	50	2 (4%)
L. monocytogenes	Fresh kashar cheese	İstanbul/Asia	50	1 (2%)
L. monocytogenes	Milk creme	İstanbul/Europe	50	2 (4%)
L. monocytogenes	Milk creme	İstanbul/Asia	50	1 (2%)

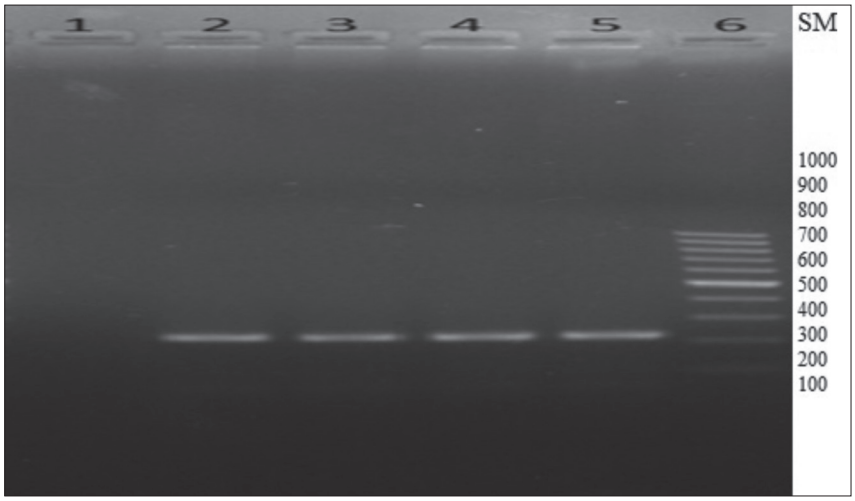
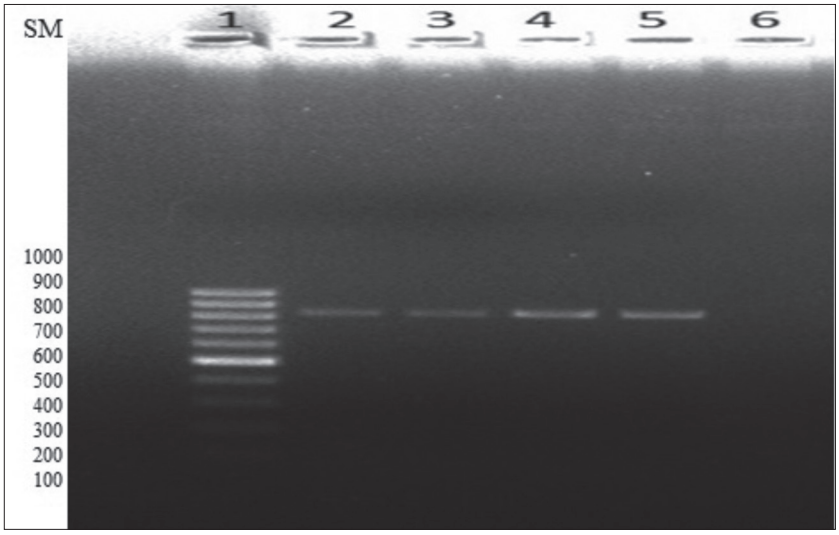


Fig 1. Agarose gel electrophoresis of amplified PCR products of *E. coli O157: H7* strains detected (1 - NC, 2/5 - *O157:H7* shigaliketoxin 208 bp, 6 - sm)

Fig 2. Agarose gel electrophoresis of PCR products of amplified *L. monocytogenes* strains (1 - sm, , 2/5 - *L. monocytogenes* - actA, 827 bp, shigaliketoxin, 6 - NC)



Although there are few cases of listeriosis in Brazil, it has been reported that pathogenic bacteria are isolated repeatedly from the dairy farms. For example, in a survey on 437 products in Brazil, 3 (0.7%) were detected in retail products and 1 was in dairy farm products (0.2%) *L. monocytogenes* [13]. Tümbay et al.[14] in a study conducted over feta cheese in Turkey, collected from different points of sale, 323 cheeses of 5.8% contaminated with Listeria spp and they found that it was 3.4% were *L. monocytogenes*. Sağun et al.[15], found 3.93% of *L. monocytogenes* in herbage cheese samples in their study on raw milk and herb cheeses from Van and the region. Çetinkaya et al.[4] detected *L. monocytogenes* in one of 51 samples of Shavak type white cheese. Loncarevic et al.[10] found a *L. monocytogenes* level of 6% in a study on 333 imported cheese in Sweden. Kevenk et al.[9] found *L. monocytogenes* in 5 milk samples

(5%) in 100 milk samples and 9 milk samples out of 110 (8.2%) in a study of 210 total dairy products. Aksoy et al.[16] found *L. monocytogenes* in 16 (5.3%) dairy products in 300 food samples . Şanlıbaba et al.[17] raported that *Listeria* spp. the prevalence were found in homemade cheese (9.09%), raw milk (8.19%) and white cheese (3.64%), respectively. *L. monocytogenes* strains were isolated from raw milk and homemade cheeses.

In a study by Ombarak et al.[18] in Egypt, it was found that 109 (55 raw milk, 41 local cheese, Karish, 13 local cheese Ras) samples were contaminated with *E. coli* out of 187 regional milk products examined. *E. coli* bacteria have been reported to be potentially harmful to consumers if they carry virulence genes and 69 of the 187 strains tested (36.9%) were found to have one or more virulence genes. Can and Elmalı [3] studied 71 traditional cheese products and 60 mincing samples and found that 17 (13%) of *E. coli O157* and 16 (16.2%) of 131 samples had *E. Coli O157:H7*. Sağlam and Şeker [19] they have isolated 3 (3%) *E. coli O157* from 100 samples in their study using conventional culture methods and serological confirmation tests on slip samples sold in Afyonkarahisar at local public markets. Ioanna et al.[8] found that *E. coli O157:H7* survived throughout the production process in the local (*Cacioricotta*) cheeses with a 90-day maturation period, increased concentration on the first day of maturation, remained stable until 35 days, and decreased until the end of the hardening period. They pointed out that the 90 day maturation period for the contaminating cheese was not sufficient to completely destroy the pathogenic bacterium. They also argued that adding starter cultures during cheese construction was an important factor in reducing contamination with *E. coli O157:H7*. In another study done, Hashemi et al.[6] observed strong antibacterial activity against slipped *E. coli O157:H7* and other common pathogenic bacterial strains fermented with *Lactobacillus plantarum* strain.

Findings from our study are similar to those of the above mentioned researchers. Three microbial analyzes of *L. monocytogenes* (3%) of 100 samples of short-term maturation cheese, which were analyzed according to the findings obtained in our study, were finally determined and the positivity of the factors in the mentioned samples was confirmed by PCR procedures. As a result of this study, presence of *E. coli*, a hygiene indicator and *L. monocytogenes*, a listeriosis agent, in kashar cheese and milk creme, which can cause food poisoning requires more serious inspection for food safety applications. It is an important risk factor for public health that milk and dairy products are not given sufficient attention to hygiene conditions during production.

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