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Comparison of real-time PCR and conventional cultural methods to detect *Escherichia coli* O157:H7 in spices in Turkey

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Abstract

In this study, 66 different spice samples were analyzed in order to compare Real-Time PCR and conventional cultural methods for the detection of *E. coli* O157:H7 in spices. Thus, 66 different spice samples (raw materials) were collected from various places in Izmir, Turkey. Conventional cultural methods included pre-enrichment step, selection step and confirmation step. The molecular method included two steps: pre-enrichment and Real-Time PCR step. Beside these methods, total aerobic mesophilic bacteria, coliform, fecal coliform in samples, were also counted and the presence of *Salmonella sp.* was investigated in order to determine total microbial load in samples. The results showed that aerobic mesophilic bacteria counts were $5.1x10^3$ -$2.0x10^8$ CFU/g, coliform counts were 8.0 x 10^2 CFU/g for the average values of all samples. *Salmonella sp.* was not found in any of the samples.In four samples *E. coli* O157:H7 positive by Real-Time PCR were found while all of the samples were negative by cultural methods. We observed that the results of Real-Time PCR were more reliable than conventional methods. Furthermore, the results were obtained in only 20 hours by Real-Time PCR method whereas conventional cultural method was completed in 4 days.

Keywords: Cultural method, *E. coli* O157:H7, real-time PCR, spice
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Türkiye'de baharatlarda *Escherichia coli* O157:H7'nin belirlenmesinde real-time PCR ve konvansiyonel kültürel yöntemin karşılaştırılması

Özet

Bu çalışmada, baharatlarda *E. coli* O157:H7'nin belirlenmesinde Real-Time PCR ve kültürel yöntemlerinin karşılaştırılması için 66 farklı baharat örneği analiz edilmiştir. Bunun için 66 farklı baharat örneği (ham madde) İzmir'in çeşitli yerlerinden temin edilmiştir. Örneklerdeki toplam mikrobiyal yükün belirlenmesi için *E. coli* O157:H7 yanında toplam aerobik mezofilik bakteri, koliform, fekal koliform sayımları yapılmıştır ve *Salmonella sp.* varlığına bakılmıştır. Bütün örneklerde ortalama değerler aerobik mezofilik bakteri sayımı $5.1x10^3$ -$2.0x10^8$ CFU/g ve koliform sayımı 8.0 x 10^2 CFU/g olarak bulunmuştur. Hiçbir örnekte *Salmonella sp*. bulunmamıştır. Kültürel yöntemle bütün örnekler *E. coli* O157:H7 bakımından negatif bulunurken Real-Time PCR yöntemiyle 4 tane örnek pozitif bulunmuştur. Bu çalışmada Real-Time PCR yönteminin kültürel yönteme göre daha güvenilir olduğu gözlenmiştir. Bunun yanında kültürel yöntemle sonuç 4 günde alınırken Real-Time PCR yöntemiyle sadece 20 saatte sonuç alınmıştır.

Anahtar kelimeler: Kültürel yöntem, *E. coli* O157:H7, real-time PCR, baharat

Introduction

Escherichia coli that belongs to the Enterobacteriaceae family is a gram negative, non-spore forming, rod, facultative anaerobic, mobile bacteria and lives in the native intestine flora of human and hot blooded animals. *E. coli* is important as an indicator of fecal contamination in waters and various foods in food microbiology. Pathogen *E. coli* strains can cause various infectious diseases that bring about diarrhea, menengitis and sepsis. According to the type of disease and serological differences, the strains of *E. coli* that cause diarrhea were separated into groups as enteropathogenic *E. coli*, enteroinvasive *E. coli*, enterotoxigenic *E. coli*, enterohemorrhagic *E. coli* and enteroaggregative *E. coli* (Ünlütürk et al. 2003).

E. coli O157:H7 was reported in the United States for the first time in 1983. It was related to hemorrhagic colitis which occurs from the consumption of unbaked ground meat. After this event, beef was investigated as a source of *E. coli* O157:H7 and 28% of beef were found to be *E. coli* O157:H7 positive (Chui 2004).

Infections of *E. coli* O157:H7 are diarrhea, hemorrhagic colitis, hemolitic uremic sendrom, thrombotic thrombocytopenic purpura (Chui 2004). The incubation period of the agent varies from 3 to 8 days after the agent is taken into the body and it causes stomach ache besides cramps, emesis, nausea, gastroenteritis, diarrhea, bloody diarrhea, and hemorrhagic colit (Alişarlı and Akman 2004). EHEC can contaminate from food, animals and humans to humans. Consumption of contaminated food originating from beef causes various diseases (Nataro and Kaper 1998).

The microbiological quality of the spices has been a research subject for decades. To our knowledge this study is the first investigation in which two methods (conventional cultural and Real-Time PCR methods) were compared for the detection of *E. coli* O157:H7 in spices as its detection has not been reported in Turkey before. Since the nucleic acid amplification and detection steps are performed in the same closed vessel, the risk for release of amplified nucleic acids into the environment, and contamination of subsequent analyses, is more suitable when compared with the conventional PCR method. Application of Real-Time PCR requires considerably less hands-on time and testing and is much simpler to perform compared to the conventional PCR method (Espy et al. 2006).

In this study, the aim was to compare two methods (cultural and Real-Time PCR methods) for the analysis of spices to determine whether these new, fast and developing molecular methods are more reliable for routine laboratory analysis than cultural methods.

Materials and methods

Samples

Raw spice samples were collected from various spice sterilization companies in Izmir, Turkey**.** These samples consist of 5 bays samples (500 g), 5 rosemary samples (500 g), 10 thyme samples (500 g), 9 coriander samples (500 g), 8 sages samples (500 g), 6 fennels samples (500 g), 15 cummin samples (500 g), 8 aniseeds samples (500 g). Totally 66 different spice samples were analyzed. Sterilized samples were used as negative controls. *E. coli* O157:H7 (approximately 10^6 CFU/g) was inoculated into one spice sample as a positive control. Positive and negative controls were prepared for each kind of sample.

Conventional cultural method

E. coli O157:H7 detection

25 g spice samples were added into 225 ml modified Tryptic Soy Broth (m-TSB, MERCK) containing Erlenmeyer flasks. These flasks were incubated at 37 ˚C for 24h (Bhagwat 2002; Fitzmaurice 2008). After incubation, samples were inoculated onto Cefixime Tellurite-Sorbitol McConkey Agar (CT-SMAC, MERCK)

and incubated at 37 ˚C for 24h (Bhagwat 2002; Islam et al. 2006; Temelli et al. 2004). After incubation, suspicious colonies (colorless colonies) were taken and inoculated onto 4-methylumbelliferyl-β-D-glucuronide Agar (MUG Agar, MERCK). Plates were incubated at 37 ˚C for 24h. After incubation, plates were analyzed under UV (approximately 360nm). Suspicious colonies (the ones that were not shining) were tested by latex agglutination test. The agglutinated colonies were tested by H7 antiserum according to the manufacturer's instructions (WELLCOLEX) (Islam et al. 2006; Temelli et al. 2004).

Other microbiological tests

Pour plate method was used for the quantification of total aerobic mesophilic bacteria. Plate Count Agar (PCA, Merck) was used as indicated before (Rosmini et al. 2004; Yıldırım et al. 2005).

Most Probable Number (MPN) method was used for total coliform analysis. Decimal dilutions were prepared after the samples were homogenized. The samples from these dilutions were inoculated into the tubes containing Lauryl Tryptose Broth (LTB medium, MERCK) for the presumptive test. The tubes were incubated at 35 °C for 24-48h. Confirmed test was applied on all the presumptive positive tubes (gas producing). A loopful of suspension from each gassing LTB tube was transferred to the tubes containing BGLB broth for the confirmation step. The tubes were incubated at 35 °C for 24-48h. The results were calculated by using the MPN table (Geissler et al. 2000; Feng et al. 2002).

One loopful from gas positive LTB tubes was transferred to the tubes containing EC broth for the fecal coliform confirmation. The tubes were incubated in a covered circulating water bath at 44.5° for 24 -48h. Gas production in EC was accepted as positive confirmed test for fecal coliforms. The results were calculated according to the MPN table as described above for coliform (Feng et al. 2002)

25 g spice samples were added into 225 ml buffered pepton water (BPW, MERCK) and incubated at 37 ˚C for 24h for the pre-enrichment step of *Salmonella sp.* (Catarame et al. 2006; Kök et al. 2007; Akkaya and Alişarlı 2006; Schönenbrücher et al. 2007; Ates et al. 2011). After incubation 0.1 ml samples from BPW were inoculated into the tubes containing 10 ml Rapaport Vassiliadis Enrichment Broth (RVEB, MERCK) and Selenite Sistein Broth (SSB, MERCK) for selective- enrichment step (Kök et al. 2007; Akkaya and Alişarlı 2006; Ateş et al. 2011). After 24-48h incubation period, samples from these broths were inoculated onto Brillant Green Phenol Lactose Agar (BGPLA, MERCK) and Bismuth sulphite Agar (BSA, MERCK) mediums for selective step. After incubation, red colonies in BGPLA medium and black colonies in BSA medium were analyzed by latex agglutination test. Observation of agglutination was accepted as positive result (Kök et al. 2007; Ates et al. 2011).

Molecular method

25 g spice samples were homogenized in 225 ml m-TSB (MERCK) containing 0.2% Novobiocin for selectivity (Ellingson et al. 2005; Perelle et al. 2004). DNA was isolated according to High Pure Foodproof I DNA Isolation Kit after 18h incubation. Real-Time PCR detection was performed according to LightCycler Foodproof *E. coli* O157 Detection Kit (Roche). LightCycler Analysis Software measurement module was used for interpretation of the results (Roche LightCycler 1.5 Instrument Operator's Manual).

Results and Discussion

The samples were analyzed by both Real-Time PCR and conventional cultural methods for *E. coli* O157:H7. Besides, total aerobic mesophilic bacteria, coliform, fecal coliform

and *Salmonella sp.* analyses were performed to determine total microbial load in samples.

The average values of the tests are shown in Table 1. The maximum value of total aerobic mesophilic bacteria was found in bay as 2.0 x 10^8 CFU/g and minimum value was found in rosemary as 5.1 x 10^3 CFU/g. However, the maximum value for total coliform was found in aniseed as 2.3 x 10^2 MPN/100g. The maximum value of fecal coliform was found in sage as 2.7 x 10^1 MPN/100g. Fecal coliform values were very low in the other samples. *Salmonella sp.* was not found in any samples.

Table 1. Average microorganism counts that belong to 66 different samples.

	Sample number	Total aerobic mesophilic bacteria (CFU/g)	Total coliform bacteria (MPN/100g)	Fecal coliform bacteria (MPN/100g)	*Salmonella sp.*	*E. coli* O157:H7
Thyme	10	$1.3x10^4$	$1.8x10^1$	< 3	-	-
Cummin	15	$1.2x10^5$	$1.8x10^2$	< 3	-	-
Aniseeds	8	$6.4x10^3$	$2.3x10^2$	< 10	-	-
Coriander	9	$7.3x10^4$	$2.0x10^2$	< 3	-	-
Rosemary	5	$5.1x10^3$	< 10	< 10	-	-
Sages	8	$5.9x10^4$	$6.8x10^1$	$2.7x10^1$	-	-
Bays	5	$2.0x10^8$	$6.3x10^1$	< 3	-	-
Fennels	6	$6.6x10^3$	< 10	< 10	-	-
Average values for 66 different samples	**66**	**$2.5x10^7$**	**$7.8x10^2$**	**< 10**	**-**	**-**

E. coli O157:H7 was detected in four samples by Real-Time PCR method while it was not detected in any of the samples by conventional cultural method. This difference between two methods might be due to observation steps in the conventional method as many uncertainties were included. Ulukanlı et al. (2006) could not confirm seven *E. coli* O157 isolates by serological test although they found the isolates O157 positive by O157 antiserum test. Moreover, Bhagwat (2002) showed that detection of *E. coli* O157:H7 was possible with produce that was washed in water contaminated at 1 cell/ml by Real-Time PCR. The possibility of detecting 1 cell/ml by the conventional method is almost impossible. Hence, these investigations suggest that the conventional method is insufficient. Besides, *E. coli* O157:H7 was found in the positive control sample and was not found in the negative control sample by both methods showing that the controls were performed correctly.

According to Figure 1 and Figure 2, four samples were positive while the other samples were negative in F2/Back F1 channel. However, all of the samples were positive in F3/Back F1 Channel. 2nd, 3rd, 6th and 7th samples were accepted as positive while all the other samples were accepted as negative according to LightCycler Foodproof *E. coli* O157 Kit Manual (Table 2).

Table 2. Interpretation of the results according to the LightCycler *E. coli* O157 Detection Kit Manual.

Channel F2/Back F1 or Channel 640/ Back 530	Channel F3/Back F1 or Channel 705/ Back 530	Interprating the results
Positive	Positive	Positive
Negative	Positive	Negative
Positive	Negative	Positive
Negative	Negative	Invalid result

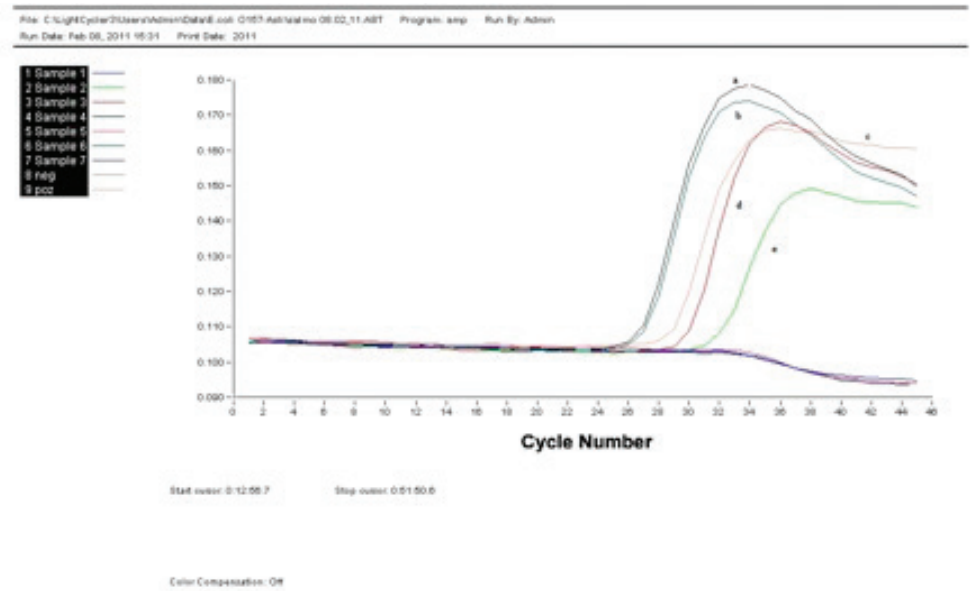

Figure 1. Real-Time PCR results in F2/BackF1 Channel. a) 7th sample. b) 6th sample c) 9th sample (positive control) d) 3rd sample e) 2nd sample. a, b, c, d and e samples were positive and the other samples were negative.

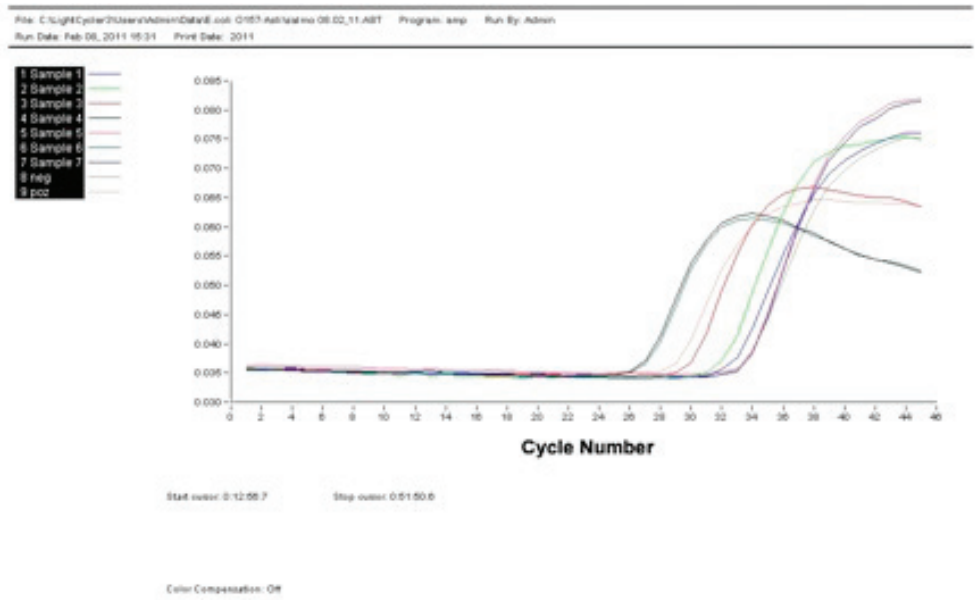

Figure 2. Real-Time PCR results in F3/Back F1 Channel. All the curves of samples were positive.

In this study,in total 66 different spice samples were analyzed for *E. coli* O157:H7 by both Real-Time PCR and conventional cultural methods. The combination of excellent susceptibility and specifity, low contamination risk, ease of performance and speed has made Real-Time PCR technology an appealing alternative to conventional culture-based or immunoassay-based testing methods used in the clinical microbiology for diagnosing many infectious diseases (Temelli et al. 2004). In this study, Real-Time PCR and conventional cultural methods were compared in terms of different parameters such as reliability and duration of procedure.

The results obtained by Real-Time PCR method took only 20 hours (18 hours pre-enrichment step, 1 hour DNA isolation, 1 hour PCR) while the cultural method was completed in 4 days. Patel et al. (2006) compared Real-Time PCR and the cultural method showing that the Real-Time PCR method was completed in 18 hours, including pre-enrichment step, while the conventional method lasted 3-5 days. Also, Takahashi et al. demonstrated that Real-Time PCR method requires less effort than the cultural method to apply. In this study *E. coli* O157:H7 was detected in four samples by Real-Time PCR method while it was not detected in any of the samples by the cultural method. According to our results,the conventional method was observed as an inadequate method to detect the microorganism in our samples due to its false negative results. Hence, Real-Time PCR method is absolutely advantageous such as time saving, low contamination risk, eliminating false negative results and requiring less effort when it is compared to the conventional method.

Traditional methods for the detection of bacteria include these main steps: pre-enrichment, selective enrichment, biochemical observation and serological confirmation steps (Chigbu et al. 2005). Identification of unknown bacteria routinely requires confirmation tests. However, interpreting the results of such tests is very difficult (Kök et al. 2007). In this study, the same method was applied in the conventional method for *E. coli* O157:H7 and subsequently latex agglutination test was performed for confirmation. Presence of agglutination led to a positive result. On the other hand, observation of agglutination might vary from person to person resulting in misconstruction. Besides, *E. coli* O157:H7-specific probes were used in Real-Time PCR method in which positive and negative controls are included and the whole procedure is completed in closed Real-Time PCR instrument leading to preclude the possibility of contamination. The results were obtained automatically through the software (LightCycler Analysis Software) suggesting Real-Time PCR as the more reliable method than the conventional method.

However, a number of methods have been developed in order to detect *E. coli* O157:H7 but they do not differentiate viable cells from dead cells. Detection of viable pathogens is important because the viable cells have the potential to cause infections (Liu et al. 2008). The differences in the results of the methods could arise from containing viable/dead cells. For this reason, further investigations using suitable dyes should be performed in order to differentiate viable cells from dead cells in the samples.

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References

Akkaya L. and Alişarlı M. (2006) Afyonkarahisar'da tüketime sunulan peynirlerde *Listeria monocytogenes* ve *Salmonella spp*. varlığının belirlenmesi. *Yüzüncü Yıl Üniversitesi Veteriner Fakültesi Dergisi*, 17: 87-91.

Alişarlı M. and Akman HN. (2004) Perakende satılan kıymaların *Escherichia coli* O157:H7 yönünden incelenmesi. *Yüzüncü Yıl Üniversitesi Veteriner Fakültesi Dergisi*, 15: 65-69.

Ateş M., Özkızılcık A. and Tabakoğlu C. (2011) Microbiological Analysis of Stuffed Mussels Sold in The Streets. *Indian Journal of Microbiology*, 51: 350-354.

Bhagwat A.A. (2002) Simultaneous detection of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* strains by real-time PCR. *International Journal of Food Microbiology,* 84: 217-224.

Catarame T.M.G., O'hanlon K.A., McDowell D.A., Blair I.S. and Duffy G. (2006) Comparison of a Real-Time Polymerase Reaction Assay with a cultural method for the detection of *Salmonella* in retail meat samples. *Journal of Food Safety*, 26: 1-15.

Chigbu P., Gordon S. and Strange R.T. (2005) Fecal coliform bacteria disapperance rates in a North-central Gulf of Mexico estuary. Estuarine, *Coastal and Shelf Science*, 65: 309-318.

Chui S. (2004) Detection and characterization of *Esherichia coli* O157:H7 and *Salmonella* in food, PhD, University of Maryland pp. 3.

Ellingson E.L.J., Koziczkowski J.J., Anderson L.J., Carlson A.S. and Sharma K.V. (2005) Rapid PCR detection of enterohemorrhagic *Escherichia coli* (EHEC) in bovine food products and feces. *Molecular Cell Probes*, 19: 213-217.

Espy M.J., Uhl J.R., Sloan L.M., Buckwalter S.P., Jones M.F., Vetter E.A., Yao J.D.C., Wengenack N.L. and Rosenblatt J.E. (2006) Real-Time PCR in clinical microbiology: Applications for routine laboratory testing. *Clinical Microbiology Reviews*, 19: 165-256.

Feng P., Weagant S.D., Grant M.A. and Burkhardt W. (2002) BAM: Enumeration of Escherichia coli and the Coliform Bacteria. *Bacteriological Analytical Manual* 8th Edition, Revision A, Chapter 4.

Fitzmaurice J. (2008) Detection of verotoxin genes VT 1 and VT 2 in *Escherichia coli* O157:H7 in minced beef using Immunocapture and Real-Time PCR. In: Louise O'Connor (Ed.) *Diagnostic Bacteriology Protocols*, New Zealand 345: 91-96.

Geissler K., Manafi M., Amoros I. and Alonso J.L. (2000) Quantitative determination of total coliforms and *Escherichia coli* in marine waters with chromogenic and fluorogenic media. *Journal of Applied Microbiology* 88: 280-285.

Islam A.M., Heuvelink A.E., Talıkder A.K. and Boer E.D. (2006) Immunoconcentration of shiga toxin-producing *Escherichia coli* O157 from animal faeces and raw meats by using dynabeads anti-*E. coli* O157 and the VIDAS System. *International Journal of Food Microbiology*, 109: 151-156.

Kök F., Özbey G. and Muz A. (2007) Aydın İlinde Satışa Sunulan Fermente Sucukların Mikrobiyolojik Kalitelerinin İncelenmesi. *Fırat Üniversitesi Sağlık Bilimleri Dergisi*, 21: 249-252.

Liu Y., Gilchrist A., Zhang J., and Li XF. (2008). Detection of Viable but Nonculturable *Escherichia coli* O157:H7 Bacteria in Drinking Water and River Water. *Applied and Environmental Microbiology* 74: 1502-1507.

Nataro J.P. and Kaper J.B. (1998) Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews,* 11: 142-201.

Patel J.R., Bhagwat A.A., Sanglay G.C. and Solomon M.B. (2006) Rapid detection of *Salmonella* from hydrodinamic pressure-treated poultry using Molecular Beacon Real-Time PCR. *Food Microbiology,* 23: 39-46.

Perelle S., Dilasser F., Grout J. and Fach P. (2004) Detection by 5'-nuclease PCR of shiga-toxin producing *Escherichia coli* O26, O55, O91, O103, O111, O113, O145 and O157:H7, associated with the world's most frequent clinical cases. *Molecular and Cellular Probes*, 18: 185-192.

Rosmini M.R., Signorini M.L., Schneider R. and Bonazza J.C. (2004) Evaluation of two alterna-

tive techniques for counting mesophilic aerobic bacteria in raw milk. *Food Control* 15: 39-44.

Schönenbrücher V., Mallinson E.T. and Bülte M. (2007) A comparison of standard cultural methods for the detection of foodborne *Salmonella* species including three new Chromogenic Plating Media. *International Journal of Food Microbiology*, 123: 1-29.

Takahashi H., Kimura B., Tanaka Y., Shinozaki J., Suda T. and Fujii T. (2009) Real-Time PCR and enrichment culture for sensitive detection and enumeration of *Escherichia coli*. *Journal of Microbiological Methods*, 79: 124-127.

Temelli S., Dokuzlu C. and Şen C.K.M. (2004) Determination of microbiological contamination sources during frozen snail meat processing stages. *Food Control*, 17: 22-29.

Ulukanlı Z., Çavlı P. and Tuzcu M. (2006) Detection of *Escherichia coli* O157:H7 from beef Doner Kebabs sold in Kars. *Gazi University Journal of Science* 19: 99-104.

Ünlütürk A. and Turantaş F. (2003) *Gıda Mikrobiyolojisi*. Meta Basım Matbaacılık Hizmetleri. İzmir 3. Basım Meta Basım Matbaacılık Hizmetleri pp. 90-91.

Yıldırım I., Uzunlu S. and Topuz A. (2005) Effect of gamma irridiation on some principle microbiological and chemical quality parameters of raw Turkish meat ball. *Food Control*, 16: 363-367.