Check for updates

Article

FOOD SCIENCE AND TECHNOLOGY INTERNATIONAL

A quantitative polymerase chain reaction assay for the detection of equine (horse and donkey)-originated meat in processed bovine meat products

Aysun Türkanoğlu Özçelik[1], Semiramis Yılmaz[2], Sevda Gökbora[2] and Mehmet İnan[1,2]

Abstract

Meat is one of the most important basic foodstuffs in human nutrition. Nowadays, adulteration and authenticity are common problems for meat products. Identification of meat species is important in terms of consumer protection and prevention of adulteration. There are different methods to determine adulteration of meat and meat products. These methods are histological controls, serological tests, and quantitative polymerase chain reaction. In this study, species identification and quantification analysis of meat and meat products were done by using horse-, donkey-, and bovine-specific primers with quantitative polymerase chain reaction method. Triple meat mixtures containing horse and donkey meat ranging from 0.1 to 50% levels were prepared within a bovine mixture for using species identification and quantification analysis. The method specificity was confirmed by melting curve analysis. In conclusion, quantitative polymerase chain reaction is an easy, rapid, and reliable method for meat species identification, and with this study an applicable method was developed for the detection and quantification of equine-originated meat in bovine meat products.

Keywords

Equine, horse meat, donkey meat, species identification, quantitative polymerase chain reaction, authentication

Date received: 3 April 2018; accepted: 25 July 2018

INTRODUCTION

Nowadays, the most common problems in meat industry are adulteration and originality. Pork, horse, and donkey meats can be mixed with the bovine and sheep meats because of their organic similarity. When the meat species that have similar pigmentation are used in processed meat products, consumer cannot detect them by organoleptic evaluation. For this reason, identification of meat species in processed meat products such as soujouk, salami, sausage, and meatball has become important.

Some methods have been developed based on protein and DNA analysis to detect meat species. Protein-based methods such as isoelectric focusing (King, 1984; Skarpeid et al., 1998) and enzyme-linked immunosorbent assay (Chen and Hsieh, 2000) have high sensitivity for unprocessed meats but not for processed meats. Researchers cannot take same results due to protein degradation under high pressure, temperature, and process technology conditions. Thus, protein-based methods are not commonly used to detect meat species for processed meat products. When compared to proteins, DNA molecule can withstand high temperature and pressure.

Food Science and Technology International 25(1) 38–46
© The Author(s) 2018 Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/1082013218794849
journals.sagepub.com/home/fst
SAGE

[1]Food Safety and Agricultural Research Center, Akdeniz University, Antalya, Turkey
[2]Department of Food Engineering, Faculty of Engineering, Akdeniz University, Antalya, Turkey

Corresponding author:
Aysun Türkanoğlu Özçelik, Food Safety and Agricultural Research Center, Akdeniz University, Antalya, Turkey.
Email: aysunozcelik@akdeniz.edu.tr

In recent years, the usage of DNA-based methods has become crucial. Species-specific primers used in polymerase chain reaction (PCR) are simple, rapid, specific, and sensitive analysis for the identification of meat species. Techniques based on PCR include mitochondrial 12 S rRNA restriction fragment length polymorphism (Girish et al., 2007), specific PCR (Mane et al., 2009; Rodriguez et al., 2003), randomly amplified polymorphic DNA fingerprint (Calvo et al., 2001; Saez et al., 2004), and actin gene-related PCR (Hopwood et al., 1999). There are lots of studies to identify meat species of unprocessed and processed meats in literature. The meat species can be detected in the binary meat mixtures by using conventional PCR (Kesmen et al., 2007, 2010). Although conventional PCR method is reliable and sensitive method for identification of meat species, it cannot be used for the quantification analysis in meat mixtures. Thus, quantitative PCR (q-PCR) method can be used for both detection and quantification of species and it has been used commonly (Dooley et al., 2004; Eugster et al., 2008; Fajardo et al., 2008; Soares et al., 2013). q-PCR technique allows obtaining result without post-PCR detection step. Amplification of target gene is followed by an increased fluorescence signal coming from fluorescent dye or fluorescence-labeled probe. Probe-based systems are more accurate and reliable for detection and quantification but they are very expensive systems (Chisholm et al., 2005; Dooley et al., 2004; Kesmen et al., 2009, 2012; Laube et al., 2003).

Fluorescent dyes such as SYBR Green I and EvaGreen are also employed for q-PCR method due to the high cost associated with probe-based q-PCR. These dyes are essentially nonfluorescent by themselves, but become highly fluorescent upon binding to double-stranded DNA. The primary disadvantage of these dyes is that they detect accumulation of both specific- and non-specific PCR products. In order to overcome this problem, melting curve analysis is used after q-PCR. In the literature there are many studies employing SYBR Green I to determine the meat species both in the processed and unprocessed meat products (Fajardo et al., 2008; Lopez-Andreo et al., 2006; Soares et al., 2013). Recently in q-PCR, EvaGreen has been used as an intercalating dye, which is more stable and sensitive than SYBR Green I (Chen et al., 2009; Santos et al., 2012). EvaGreen can be used at higher concentrations without causing PCR inhibition and resulting in better fluorescence signal and higher sensitivity in the assay. Santos et al. (2012) reported that q-PCR with EvaGreen dye identifies hare meat DNA at the level of 1 pg compared to 10 pg detection limit in conventional PCR.

The aim of the present study was to develop simple, rapid, cheap, and reliable q-PCR method for identifying and quantifying equine-originated meat species (horse, donkey) in processed bovine products.

MATERIALS AND METHODS

Raw meat and processed meat samples

In this study, bovine meat was obtained from local breeder, horse meat from Ankara Directorate of Provincial Agriculture Control, and donkey meat from a farm in Bala, Ankara. The samples were stored at −20 °C until the analysis was performed. Chicken, sheep, goat, pork, and corn DNAs were used as negative controls to check primer specificity of bovine, horse, and donkey meats. Twenty-one processed bovine meat samples such as soujouk, sausage, meatball, and doner were collected from local markets in Antalya. In addition, 12 horse- and/or donkey-positive samples were obtained from an accredited laboratory in Antalya.

DNA isolation

Genomic DNA was isolated from individual samples using the DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. The concentration of DNA in the sample was determined by using Qubit fluorometer. Intactness of DNA samples was verified by agarose gel electrophoresis. Agarose gel (1%) was run under 120 V for 40 min and then visualized under UV light by Vilber Lourmat EBox-VX2 (France) gel imaging system. Isolated DNA samples were stored at −20 °C until used for the PCR reactions.

Preparation of reference DNA and meat mixtures

Binary DNA mixtures were prepared by mixing horse and donkey genomic DNAs separately with bovine genomic DNA at six different levels (0.1, 0.5, 1, 5, 10, and 25% v/v). First, genomic DNAs were isolated from bovine, horse, and donkey tissues independently and then all DNA concentrations were adjusted by dilution to 5 ng/μl with nuclease-free water. DNA isolated from donkey and horse was mixed with DNA isolated from bovine to obtain two sets of binary DNA mixture containing 0.1, 0.5, 1, 5, 10, and 25% v/v horse or donkey DNA with a final concentration of 5 ng/μl. These DNA series were used as reference DNA mixture in q-PCR analysis.

Two independent binary meat mixtures (horse in bovine and donkey in bovine) were prepared using raw meats from the target species. For each meat mixture, six different mixtures containing 0.1, 0.5, 1, 5, 10, and 25% (w/w) of the target species in bovine meat

were prepared to a final weight of 50 g. These binary meat mixtures were homogenized with a grinder (Retsch GM200, Germany) and then DNA was extracted to calculate reaction efficiencies of each primer set.

Triple meat mixtures (horse, donkey, and bovine) were prepared for using validation experiments of method and construction of quantification curves. Equal amounts of donkey and horse meats in bovine meat were mixed in different percentages (50, 25, 10, 5, 2.5, 1, 0.1% (w/w) donkey or horse meat).

Selection of target gene and primers

A target gene for horse, donkey, and bovine mitochondrial cytochrome b (cytb) gene was selected. And as a positive amplification control for PCR, the conserved region of nuclear 18S rRNA fragment which can be amplified in all eukaryotes was preferred. Species-specific primers were adapted from literature and are given in Table 1. Primer sequences were validated by NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) data bank to ensure specificity for the target species and gene.

Conventional PCR for specificity test of specific primers

Mitochondrial cytb gene of bovine, horse, and donkey DNAs was amplified with conventional PCR by using the specific primers given in Table 1. In this way, cross-reactivity of the specific primers was tested. The PCR mixture was prepared by using HotStarTaq Master Mix (Qiagen, Germany). The PCR mixture for each sample was prepared as follows: 2.5 U HotStarTaq DNA polymerase, 1X PCR buffer, 200 M dNTP mixture, 0.2 M forward and reverse primers, and 5 ng DNA template in a 25 µl PCR volume. The PCR conditions consisted of an activation step at 95 °C for 15 min, followed by 35 cycles of melting at 94 °C for 1 min, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min. A final extension step at 72 °C for 10 min terminates the process. The PCR products were analyzed electrophoretically on an ethidium bromide-stained 3% agarose gel.

q-PCR

The q-PCR analysis was performed according to EpiTect HRM PCR kit (Qiagen, Germany) protocol. The 25 µl PCR mixture contained 1X EpiTect HRM Master Mix, 0.75 M forward and reverse primers, and 5 ng template DNA. All reactions were prepared as triplicate and analyzed with RotorGene Q 5plex HRM (Qiagen, Germany). Thermal cycling protocol of 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 65 °C for 30 s, and 72 °C for 10 s was applied. And then for melting curve analysis temperature was raised from 70 to 99 °C with 0.1 °C temperature increase in every 2 s. Results were analyzed by using software of RotorGene Q 5plex HRM machine (version 2.0.3).

RESULTS AND DISCUSSIONS

Genomic DNAs were isolated from bovine, horse, donkey, pork, and chicken meats and then concentration of extractions was measured. Confirmation of quality and quantity of extracted DNAs was achieved with 1% agarose gel electrophoresis by loading 1 µl of DNA in each well and capturing gel images. DNAs were extracted from all samples with the same efficiency and no degradation was observed in all samples. Concentration values of DNAs and agarose gel band results were consistent when compared to each other.

In order to detect cross-reactions, mitochondrial cytb fragments were amplified with each set of primers in bovine, horse, and donkey DNAs separately by conventional PCR. A mixture of DNA samples from

Table 1. Sequences and amplicon size of primers used in this study

Primers	Sequence (5′–3′)	Amplicon size (bp)	Reference
BCytb-F (Bovine)	CCCGATTCTTCGCTTTCCAT	119	Tanabe et al. (2007)
BCytb-R (Bovine)	CTACGTCTGAGGAAATTCCTGTTG		
HCytb-F (Horse)	CCA GAA TGG TAC TTC CTG TTT GC	85	Köppel et al. (2009)
HCytb-R (Horse)	TAG AGA GGA TTA GGG CTA ATA CGC		
DCytb-F (Donkey)	GGTGTATTAGCCCTTATCCTTTCCATCT	80	This study
DCytb-R (Donkey)	CATGTTAGTGTTAGTAAGTCTGCTACTAAGAGTCAGAAC		
18S rRNA-F	TGGTGCCAGCAGCCGC	77	Pegels et al. (2012)
18S rRNA-R	TCCAACTACGAGCTTTTTAACTGCA		

chicken, sheep, goat, pork, and corn was used as negative control to check cross-reactivity with nontarget species. An endogenous control 18S rRNA gene region in all samples was amplified–with relevant primers to confirm the PCR-amplifiable DNA content of each reaction. As a result of this experiment, no cross-reaction was observed in any case and primers generated species-specific fragments of 119, 84, and 80 bp for bovine, horse, and donkey, respectively (Figure 1).

The q-PCR amplification efficiency and sensitivity of primers were evaluated by obtaining standard curve for each primer set with DNA dilution series of the bovine, horse, and donkey (Table 1). DNA extract of each species was fourfold serially diluted with nuclease-free water at six different levels (1:1, 1:4, 1:16, 1:64, 1:256, and 1:1024). All q-PCR reactions were run in triplicate. q-PCR standard curves were generated by plotting Ct values versus logarithm of DNA concentrations. The following equation was used to calculate the reaction efficiency

$$E = \left(10^{-1/\text{slope}} - 1\right) \times 100$$

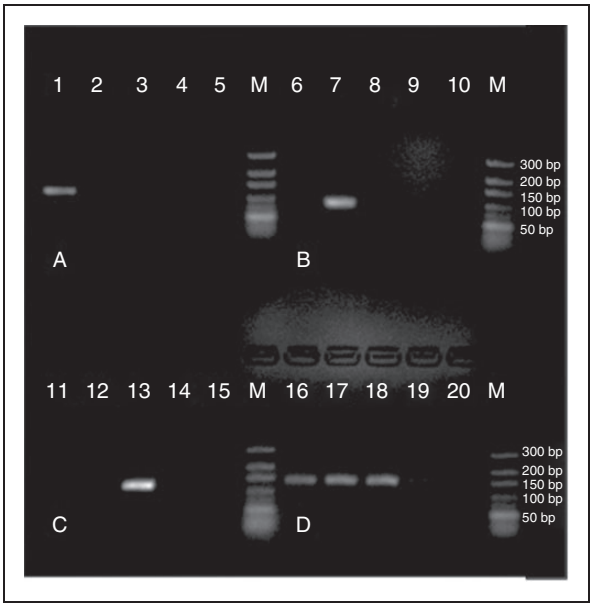


Figure 1. The result of primer. A—PCR specificity of specific bovines primers: 1: bovine DNA, 2: horse DNA, 3: donkey DNA, 4: negative control, 5: no template control. B—PCR specificity of specific horse primers: 6: bovine DNA, 7: horse DNA, 8: donkey DNA, 9: negative control, 10: no template control. C—PCR specificity of specific donkey primers: 11: bovine DNA, 12: horse DNA, 13: donkey DNA, 14: negative control, 15: no template control. D—PCR specificity of 18S rRNA primers: 16: bovine DNA, 17: horse DNA, 18: donkey DNA, 19: negative control, 20: no template control. M: ultralow range DNA marker.

Theoretically slope must be −3.32 for 100% reaction efficiency. We continue q-PCR reactions with the primers that provide the following three parameters:

1. Slope of standard curve must be in the range of $-3.6 \leq M \leq -3.1$
2. Reaction efficiency must be between 90 and 110%
3. $R^2 \geq 0.98$

Furthermore, optimum DNA concentration for q-PCR was defined. We continue experiments with DNA concentration corresponding to middle of linear range of the standard curve. Linear correlation was obtained for bovine DNA concentrations (reaction efficiency 103%, slope −3.25, and R^2 0.99) (Figure 2). Similar results were obtained for horse, donkey, and 18S rRNA primers. PCR products were applied in 3% agarose gel electrophoresis to check whether there is nonspecific binding of primers or not. Nonspecific amplification was not observed in any PCR.

The reaction efficiencies of primer sets were checked first with DNA dilution series and then with binary meat mixtures containing known amounts of horse and donkey meats in bovine meat (Figure 3). They were amplified with horse- and donkey-specific primers in q-PCR.

Construction of quantification curve is essential to normalize q-PCR results for obtaining reliable results. In this way, differences arising from changes in DNA extraction and amplification efficiency are controlled. The 18S rRNA was used as an internal control in order to find out whether amplification variations result from target species content or other factors like DNA degradation, amount and quality of the DNA extracted from samples. In order to control target gene amplification, variations that might have resulted from processing treatments of internal control are used. Quantification curves were constructed by plotting Ct values against the logarithm of horse or donkey meat percentages in triple validation mixtures where the following equation was used

$$\Delta Ct = Ct_{\text{horse/donkey}} - Ct_{\text{universal}}$$

$Ct_{\text{horse/donkey}}$ and $Ct_{\text{universal}}$ were obtained Ct values from q-PCR amplification of triple validation mixture DNAs with species-specific and universal 18S rRNA primers, respectively. As can be seen from the quantification curves (Figure 4(a) and (b)), this approach allows the estimation of added horse or donkey meat at concentrations of 0.1–50% that covers a linear dynamic range with a high correlation coefficient ($R^2 = 0.999$ for horse, $R^2 = 0.995$ for donkey).

Limit of detection (LOD) and limit of quantification (LOQ) are defined as the lowest amount of analyte in a

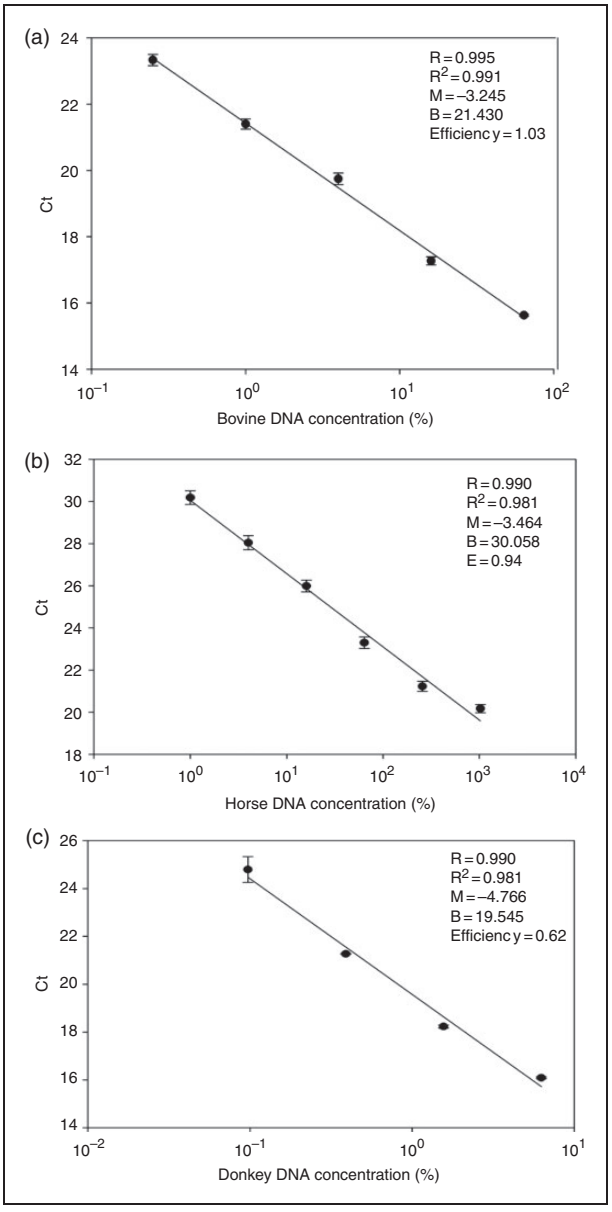


Figure 2. Reaction efficiency analysis result of bovine-specific primers (a), horse-specific primers (b), and donkey-specific primers (c) with DNA dilution series.

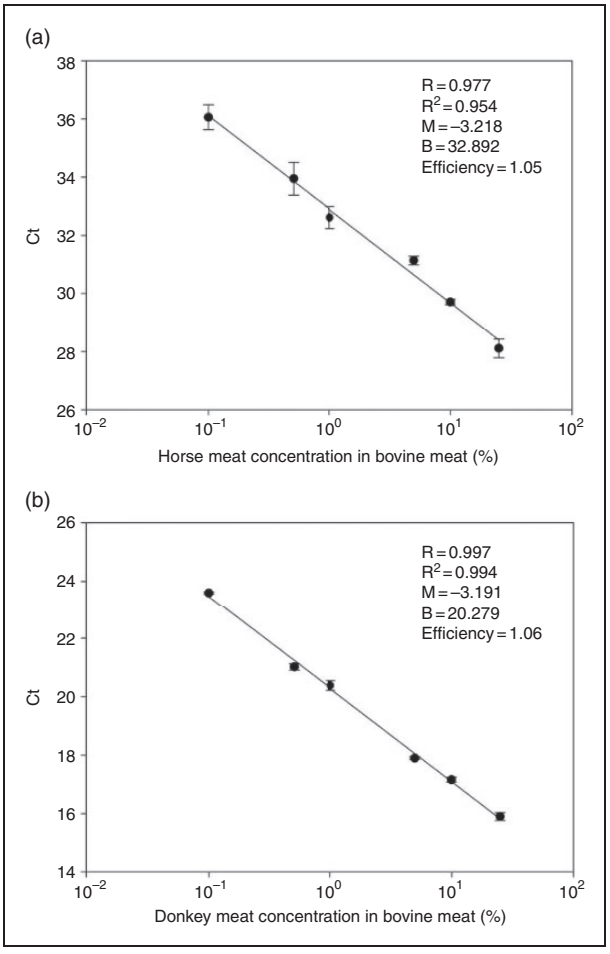


Figure 3. Reaction efficiency analysis result of horse-specific primers (a) and donkey-specific primers (b) with binary meat mixtures.

sample that can be reliably distinguished from zero and the lowest concentration of analyte that can be determined with an acceptable repeatability and trueness, respectively. The LOQ values were calculated from the slope of quantification curve and standard deviation of the all samples according to linear regression model (Shrivastava and Gupta, 2015). Subsequent melting curve analysis also showed that target cytb fragment was amplified specifically for each species (data not shown). The LOD and LOQ values were determined as 0.5 and 1.4% for donkeys, respectively. While LOD was found as 0.2% for horse species, LOQ was found as 0.6%.

Quantification estimation was done by extrapolating Ct values obtained from analysis of triple validation mixtures with species-specific and universal 18S rRNA primers on quantification curves given in Figure 4. Coefficient of variation of each reaction was calculated from the standard deviation of triplicates. Besides, accuracy of method was determined from deviation between the predicted and true values. Tables 2 and 3 show the predicted and true values of horse and donkey meat percentage of triple validation mixtures, respectively. The coefficient of variations of each reaction of both horse and donkey primers with triple validation mixtures were calculated as lower than 25% which indicates that the dynamic linear area of method is in the range of 0.1–50%.

Genomic DNAs were isolated from different processed meat market samples. DNA concentrations were measured and visualized in 1% agarose gel electrophoresis. According to the results there is a

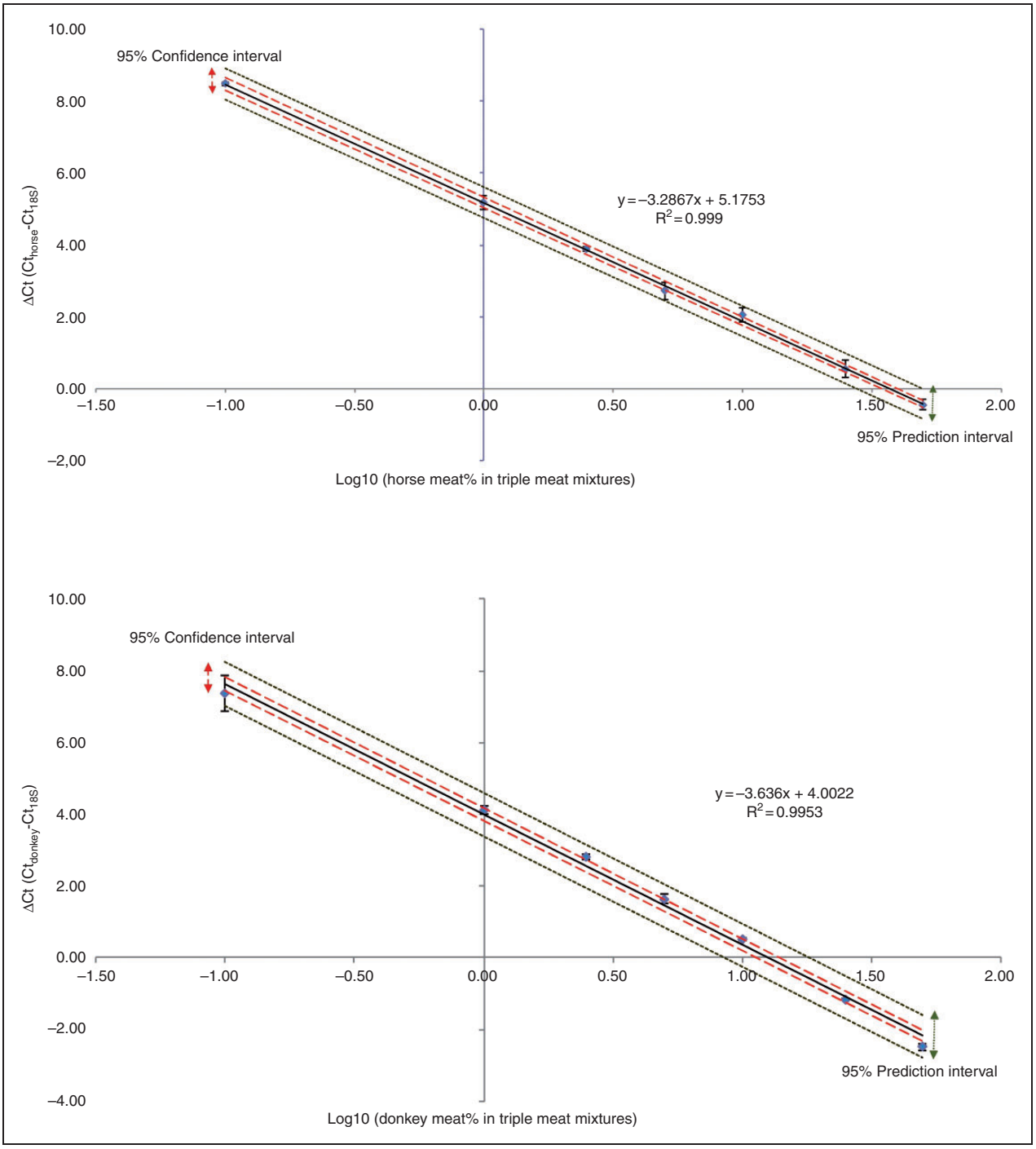


Figure 4. Quantification curves for the estimation of adulteration level of bovine meat with horse meat (a) and donkey meat (b).

correlation between gel image and measured concentration of DNAs. Genomic DNAs were successfully extracted from the processed meats for q-PCR experiments. The q-PCR was performed for 33 samples selected from different product groups such as soujouk, sausage, meatball, kebab, lahmacun, salami, pizza types food (pide and lahmacun), and doner. Twelve of these samples were already tested by accredited laboratory and reported positive for donkey and/or horse meat. Neither horse- nor donkey species-specific amplification was observed in 21 of the market samples. Quantification analysis of 12 samples known to be

Table 2. Validation results of q-PCR for detection of horse meat

	Horse meat %			
Samples	Actual	Mean predicted[a]	SD	CV (%)
1	0.1	0.10	0.03	29
2	0.5	1.01	0.10	9
3	1	2.5	0.28	11
4	5	5.6	0.40	7
5	10	8.9	0.23	3
6	25	25.6	0.10	0.4
7	50	51.0	3.96	8

CV: coefficient of variation; SD: standard deviation.
[a]Average of triplicate reaction results.

Table 3. Validation results of q-PCR for detection of donkey meat

	Donkey meat %			
Samples	Actual	Mean predicted[a]	SD	CV (%)
1	0.1	0.12	0.03	24
2	1	0.94	0.10	10
3	2.5	2.1	0.28	13
4	5	4.5	0.40	9
5	10	9.2	0.23	2.5
6	25	26.2	0.10	0.4
7	50	61.1	3.96	6.5

CV: coefficient of variation; SD: standard deviation.
[a]Average of triplicate reaction results.

positive was performed by amplifying DNA with horse, donkey, and 18S rRNA primers and obtained results given in Table 4 were consistent with the results of the accredited laboratory. As expected, horse-positive samples gave reaction with horse primers and donkey-positive samples were amplified with donkey-specific primers. The false-positive or false-negative reactions were not observed according to the results of q-PCR. As seen in Table 4, at least 10.64% and at most 65.77% of horse meat were detected in the horse-positive samples. In donkey-positive samples, donkey meat ratio was detected as 11 and 28.72%. Similarly, both horse- and donkey-positive samples were quantitatively analyzed by using this method even at lower ratios. These results were in the range of dynamic linear area of method.

The q-PCR has been a widely used technique on species identification in recent years. The selection of target gene and primers is important for sensitivity and specificity of q-PCR. Generally, mitochondrial genes are preferred due to their higher mutation rate and number of copies in each cell (Girish et al., 2005). In the present work, mitochondrial cytb gene was amplified as a target gene and 18S rRNA was used as an internal control gene. Primers showed higher specificity for target gene and species. Isolated DNAs were used successfully for identification of each species being tested. Differentiation of horse and donkey meat is difficult because they have a high sequence homology and they are closely related species. However, we did not observe any cross-reaction between horse and donkey primers. Similarly, there are reports in the literature

Table 4. Quantification results of market samples

q-PCR results						
			Horse primers		Donkey primers	
Sample no.	Type of food	Horse/donkey[a]	ΔCT	Amount %	ΔCT	Amount %
1	Lahmacun	+ / −	0.58	25.01	ND	ND
2	Meatball	+ / −	1.8	10.64	ND	ND
3	Doner	+ / −	−0.3	46.33	ND	ND
4	Kebab	+ / −	−0.8	65.77	ND	ND
5	Lahmacun	− / +	ND	ND	−0.4	16.25
6	Soujouk	− / +	ND	ND	−1.3	28.72
7	Kebab	− / +	ND	ND	0.2	l l.11
8	Lahmacun	− / +	ND	ND	0.1	11.84
9	Salami	+ / +	5.2	0.98	l.6	4.58
10	Soujouk	+ / +	5.4	0.85	0.6	8.62
11	Sausage	+ / +	4	2.28	2	3.55
12	Pide	+ / +	4.8	1.30	1.7	4.30

CT: cycle threshold; ND: not detected; q-PCR: quantitative polymerase chain reaction.
[a]Qualitative analysis results of the samples by an accredited laboratory.

that horse and donkey meats could be differentiated in different materials (Chisholm et al., 2005; Kesmen et al., 2009, 2007).

Identification of adulteration is very important for the protection of consumers' rights. However, not only the identification of the origin of meat in meat products but also quantification of the foreign meat is very important. In this study, designed q-PCR method allows both identification and quantification of species in meat mixtures. The detection limit of q-PCR method was found as 0.5% for donkey and 0.2% for horse meat. In addition, the quantification limit of this method was determined as 1.4 and 0.6% for donkey and horse species, respectively. This technique can be used to understand whether the presence of horse or donkey meat comes from contamination or adulteration.

The q-PCR method developed with EvaGreen dye has advantages such as high sensitivity, specificity, and high accuracy. This method was developed as a simplex assay but it can be improved and modified to a multiplex assay where many species can be detected in one PCR at the same time.

CONCLUSION

In the present study, a new q-PCR method to identify and quantify equine (horse and donkey) origin of meat in processed bovine meat products was proposed. For this aim, new generation EvaGreen dye was used to obtain more specific and accurate results of q-PCR method. The developed method is a simple and cheap technique for identification and quantification of equine (horse and donkey) originated meat. This technique does not need any post-PCR treatment and the use of expensive TaqMan probes. In this study, EvaGreen q-PCR assay has a detection and quantification capacity in the linear dynamic range of 0.1–50% for horse and donkey meat. As a conclusion, proposed EvaGreen q-PCR is a highly specific and sensitive method for identification and quantification of equine-originated meat.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship and/or publication of this article: This work was supported by Akdeniz University Scientific Research Projects Coordination Unit with the project number 2013.01.0102.015.

REFERENCES

Calvo JH, Zaragoza P and Osta R. (2001). Random amplified polymorphic DNA fingerprints for identification of species in poultry pate. *Poultry Science* 80: 522–524.

Chen FC and Hsieh YH. (2000). Detection of pork in heat-processed meat products by monoclonal antibody-based ELISA. *Journal of AOAC International* 83(1): 79–85.

Chen Y, Wu Y, Wang J, Xu B, Zhong Z and Xia J. (2009). Identification of cervidae DNA in feedstuff using real-time polymerase chain reaction method with the new fluorescence intercalating dye EvaGreen. *Journal of AOAC International* 92: 175–180.

Chisholm J, Conyers C, Booth C, Lawley W and Hird H. (2005). The detection of horse and donkey using real-time PCR. *Meat Science* 70: 727–732.

Dooley JJ, Paine KE, Garrett SD and Brown HM. (2004). Detection of meat species using TaqMan real-time PCR assays. *Meat Science* 68: 431–438.

Eugster A, Ruf J, Rentsch J, Hübner P and Köppel R. (2008). Quantification of beef and pork fraction in sausages by real time PCR analysis: Results of an interlaboratory trial. *European Food Research and Technology* 227: 17–20.

Fajardo V, Gonzalez I, Martin I, Rojas M, Hernandez PE, Garcia T, et al. (2008). Real-time PCR for detection and quantification of red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*) in meat mixtures. *Meat Science* 79: 289–298.

Girish PS, Anjaneyulu ASR, Viswas KN, Santhosh FH, Bhilegaonkar KN, Agarwal RK, et al. (2007). Polymerase chain reaction–restriction fragment length polymorphism of mitochondrial 12S rRNA gene: A simple method for identification of poultry meat species. *Veterinary Research Communication* 31: 447–455.

Girish PS, Anjaneyulu ASR, Viswas KN, Shivakumar BM, Anand M, Patel M, et al. (2005). Meat species identification by polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) of mitochondrial 12S rRNA gene. *Meat Science* 70(1): 107–112.

Hopwood AJ, Fairbrother KS, Lockley AK and Bardsley RG. (1999). An actin gene-related polymerase chain reaction (PCR) test for identification of chicken in meat mixtures. *Meat Science* 53(4): 227–231.

Kesmen Z, Gulluce A, Sahin F and Yetim H. (2009). Identification of meat species by TaqMan-based real-time PCR assay. *Meat Science* 82: 444–449.

Kesmen Z, Sahin F and Yetim H. (2007). PCR assay for the identification of animal species in cooked sausages. *Meat Science* 77: 649–653.

Kesmen Z, Yetim H and Sahin F. (2010). Identification of different meat species used in sucuk production by PCR assay. *Gıda* 35(2): 81–87.

Kesmen Z, Yetiman AE, Sahin F and Yetim H. (2012). Detection of chicken and turkey meat in meat mixtures by using real-time PCR assays. *Journal of Food Science* 77(2): 167–173.

King NL. (1984). Species identification of cooked meats by enzyme staining of isoelectric focussing gels. *Meat Science* 11: 59–64.

Köppel R, Zimmerli F and Breitenmoser A. (2009). Heptaplex real-time PCR for the identification and quantification of DNA from beef, pork, chicken, turkey, horse meat, sheep (mutton) and goat. *European Food Research and Technology* 230: 125–133.

Laube I, Spiegelberg A, Butschke A, Zagon J, Schauzu M, Kroh L, et al. (2003). Methods for the detection of beef and pork in foods using real-time polymerase chain reaction. *International Journal of Food Science and Technology* 38: 111–118.

Lopez-Andreo M, Garrido-Pertierra A and Puyet A. (2006). Evaluation of post-polymerase chain reaction melting temperature analysis for meat species identification in mixed DNA samples. *Journal of Agricultural and Food Chemistry* 54: 7973–7978.

Mane BG, Mendiratta SK and Tiwari AK. (2009). Polymerase chain reaction assay for identification of chicken in meat and meat products. *Food Chemistry* 116: 806–810.

Pegels N, Gonzalez I, Lopez-Calleja I, Fernandez S, Garcia T and Martin R. (2012). Evaluation of a TaqMan real-time PCR assay for detection of chicken, turkey, duck, and goose material in highly processed industrial feed samples. *Poultry Science* 91: 1709–1719.

Rodriguez MA, Garcia T, Gonzalez I, Asensio L, Mayoral B, Lopez-Calleja I, et al. (2003). Identification of goose, mule duck, chicken, turkey, and swine in foie gras by species-specific polymerase chain reaction. *Journal of Agricultural and Food Chemistry* 51: 1524–1529.

Saez R, Sanz Y and Toldra F. (2004). PCR-based fingerprinting techniques for rapid detection of animal species in meat products. *Meat Science* 66: 659–665.

Santos CG, Melo VS, Amaral JS, Estevinho L, Oliveira MBPP and Mafra I. (2012). Identification of hare meat by a species-specific marker of mitochondrial origin. *Meat Science* 90: 836–841.

Shrivastava A and Gupta VB. (2015). Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles of Young Scientists* 2(1): 21–25.

Skarpeid HJ, Kvaal K and Hildrum KI. (1998). Identification of animal species in ground meat mixtures by multivariate analysis of isoelectric focusing protein profiles. *Electrophoresis* 18: 3103–3109.

Soares S, Amaral JS, Oliveira MBPP and Mafra I. (2013). A SYBR Green real-time PCR assay to detect and quantify pork meat in processed poultry meat products. *Meat Science* 94: 115–120.

Tanabe S, Hase M, Yano T, Sato M, Fujimura T and Akiyama H. (2007). A real-time quantitative PCR detection method for pork, chicken, beef, mutton, and horseflesh in foods. *Bioscience, Biotechnology, and Biochemistry* 12: 3131–3135.