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Simultaneous Identification of Pork and Poultry Origins in Pet Foods by a Quick Multiplex Real-Time PCR Assay Using EvaGreen Florescence Dye

M. Safdar · M. F. Abasıyanık

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Abstract EvaGreen multiplex real-time polymerase chain reaction (EMRT-PCR) was designed for an assay that can join the advantages of multiplex PCR and real-time PCR to recognize animal genes more quickly in pet foods. EMRT-PCR based on melting temperatures discrimination by using EvaGreen fluorescence dye was developed for the analysis of pork and poultry in pet food. The method combines the use of poultry- and pork-specific primers that amplify small fragments of 12S rRNA and mitochondrial DNA genes. Appropriate mixtures of poultry and pork meat in reference samples were used to develop the assay. Gene yields of poultry and pork were represented in two melting peaks generated simultaneously at temperatures of 80.5 and 87.2 °C, respectively. Based upon the assay results, it has been concluded that EMRT-PCR assay might be an efficient tool for the verification of species origin in pet foods.

Keywords Real-time PCR · EvaGreen fluorescence dye · Multiplex analysis · Pet food

Abbreviations

EMRT-PCR	EvaGreen multiplex real-time polymerase chain reaction
Tm	Melting temperature
GC	The number of G's and C's in the primer

The paper highlights the following points.
• The first report of development of multiplex real-time PCR based on EvaGreen florescence dye for pork and poultry species detection simultaneously in pet foods.
• We report the assay which saves cost, time, chemicals, and materials because EvaGreen dye is cheaper than probe-based methods.
• We report the specificity and sensitivity of the assay has been evaluated very high as before reported.
• We report the developed system was successfully applied to commercial pet foods for detection of low quality meat species (poultry and pork).

M. Safdar (✉) · M. F. Abasıyanık
Department of Genetics and Bioengineering, Faculty of Engineering, Fatih University, Büyükçekmece, 34500 Istanbul, Turkey
e-mail: drmuhammadsafdar8@gmail.com

Introduction

Most recent food crisis and poor practice on part of some producers have clearly emphasized public awareness concerning the origin of species in pet food products. Based on the legislation, all the ingredients used for the preparation of pet food products must be indicated on the label. With regard to pet food, labels must specify either all the ingredients present in the product or the categories to which the ingredients belong to [1]. In this perspective, the identification of animal species has been achieving a high importance to avoid unfair competition and to promise correct labeling. The ability to verify pet food products is also a major concern to the pet food industry as manufacturers must be able to assure the authenticity of components of their products to obey with government legislation. The pet food industry has consistently grown all over the world with a massive offer of various types of pet foods available in the market [2] and the most common proteins in the pet foods belong to beef, mutton, and poultry. However, the high price of beef and mutton than poultry species could lead to fraudulent practices involving the substitution of beef and mutton with other less valuable species (poultry and pork) [3].

Every cell in an organism has a different protein profile and, in addition, proteins begin to denature and modify their epitopes, which are vital for immunological techniques, after an animal's death, and heavy processing [4]. Several researchers have used conventional gel electrophoresis-based PCR-detection of meat species in food products [5–7]. In contrast to conventional PCR techniques, real-time PCR-approaches allow discrimination and measurement of even minute traces of animal species in foods. Real-time PCR has been used to identify feed and food products [8, 9]. The fluorescent-based methods used in real-time PCR can be classified into two categories: probe-based [10, 11] and DNA intercalating dyes [8, 12]. Although probe-based methods are sequence specific, they are more expensive [12], time and labor intensive, and are much more difficult to design and optimize. Alternatively, EvaGreen binds in the minor groove of double-stranded DNA in a sequence-independent way. Moreover, DNA intercalating dyes, such as SYBER Green and EvaGreen provide a flexible method without the need for individual probe design and complex optimization steps [13]. Economically, EvaGreen is cost-efficient compared with other probe-based methods [14] and DNA intercalating dyes which are more effective [15].

A fast and simple assay to use DNA based on commercial analysis and surveillance of food is required. Several attempts have been made for simultaneous detection of genes in the multiplex PCR with DNA intercalating dyes, but they deal with microorganism detection [16, 17]. Herein, the assay of EMRT-PCR have been reported to specifically detect pork and poultry fragment DNA in simultaneous reactions by using the 12S rRNA gene region of poultry and the mitochondrial DNA gene region of pork sequences in pet foods. EMRT-PCR combines both reactions within a single PCR tube, hence, saving time, cost, and materials. The aim of the present study was to develop EMRT-PCR assay, which appears to be a promising tool for rapid, sensitive, specific, and accurate detection of fragments of pork and poultry mitochondrial DNA origins in pet foods.

Materials and Methods

Selection and Preparation of Reference Samples

To validate the commercial pet food samples, the reference samples were prepared from beef, poultry, mutton, pork meats, and additive materials such as vitamin A and brains (wheat and rice) by Vahdet Meat and Meat Products Endustri (Turkey). Reference pet foods were prepared by

homogenized mixing in appropriate ratios of 0.001 to 100 % (Table 1) of poultry and pork meats in beef and mutton meats to test the sensitivity of the assay for commercial pet food products. Fifteen commercial pet foods were obtained from supermarkets in Turkey. They all were directly transported to the Genetic Research Laboratory of Fatih University and stored at −20 °C until autoclaved and used for the extraction of the DNA to prevent the enzymatic degradation of DNA.

DNA Extraction

DNA was extracted from 200 mg of reference and commercial pet food samples following manufacturer's instructions using the Nucleospin Tissue Kit (Macherey-Nagel). After the concentration of DNA (100 ng) was measured, the DNA solutions were stored at 4 °C until usage. DNA concentration was measured with a NanoDrop2000 spectrophotometer (NanoDrop2000, UV–vis spectrophotometer, USA). The samples were exposed to ultraviolet light at 260 and 280 nm. The ratio (260:280) was used to calculate the quantification of nucleic acids based on the following formula: DNA concentration=OD260×extinction coefficient (50 μg/mL)×dilution factor.

Sensitivity Test

To determine the detection limit of pork- and poultry-specific primers, EMRT-PCR, a mixture of pork and poultry DNA was performed in serial 1:10 (10, 5, 1, 0.1, 0.01, 0.0001 ng DNA/μL) dilutions of DNAs of each species in beef and mutton DNA. For the determination of the relative detection limit, DNA was extracted from each reference pet food samples (Table 1) and the concentration of DNA was measured 50 ng/μL in pork and poultry multiple DNA solution by NanoDrop2000 spectrophotometer (NanoDrop2000, UV–vis spectrophotometer, USA).

Primers

Pork and poultry primers published respectively by Lahiff et al. [18] and Dalmasso et al. [3] were used (Table 2). All primers used in this study were synthesized by the Metabion Company (Germany). To check the specificity of each primer, an instrument called BLAST (Basic Local Alignment Search Tool) of NCBI (National Center for Biotechnology Information) was used.

Optimization of the Primer Concentration for EMRT-PCR

The optimization of the primer quantity in the master mix was performed by adding primers together at several quantities between 1 and 34 pmol. We began with equal concentrations of poultry and pork primers. However, the pork amplicons were produced at a higher efficiency

Table 1 Samples preparation for sensitivity test

No.	Pork (%)	Poultry (%)	Others (%)[a]
1	60	20	20
2	30	10	60
3	3	1	96
4	0.3	0.1	99.6
5	0.03	0.01	99.96
6	0.003	0.001	99.99

[a]Include beef, mutton, vitamin A, wheat, and rice

Table 2 Design of Oligonucleotides primers for poultry and pork species

Primes	Species	Genes	Position	Oligonucleotides primers	Sources	
Pork	*Sus scrofa*	Mt. DNA	AF039170	5′ GCCTAAATCTCCCC TCAATGGTA 3′	212	Lahiff et al. [18]
				5′ ATGAAAGAGGCAA ATAGATTTTCG 3′		
Poultry	*Gallus gallus*	12S rRNA	*Gallus gallus* NC 001323 1799–1981	5′ TGA GAA CTA CGA GCA CAA AC 3′	183	Dalmasso et al. [3]
				5′ GGG CTA TTG AGC TCA CTG TT 3′		

The primer sequences for pork and poultry were published earlier

Mt. DNA mitochondrial DNA

than the poultry amplicons. The ratio of primer concentrations was, therefore, adjusted so that both amplicons were produced with high efficiency in the same reaction.

EMRT-PCR Protocol

The PCR-amplification was performed in a final volume of 20 μL contained 4 μL of 5× HOT FIREPol EvaGreen® qPCR Mix Plus (ROX), Solis Bio Dyne, 14 pmol of pork primers, 20 pmol of poultry primers, and 100 ng of DNA template. The amplification was performed in a Corbet Rotor-Gene 6000 rotary analyzer (Corbett, Australia). After an initial heat denaturation step at 95 °C for 15 min, 40 cycles were programmed as follows: 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Subsequently, a melting curve analysis was programmed in such a way that its slope formed from 72 to 95 °C by raising 1 °C each step. Program waits for 118 s of pre-melt conditioning on first step and for 5 s for each step afterwards.

Melting Curve Analysis

At the end of each reaction melting curve analysis tools of the Rotor Gene Software Program (Corbett, Australia) were used to identify species-specific melting temperature (Tm) values of the amplified region of the template DNA that belong to reference samples.

Results and Discussion

Development of EMRT-PCR

The primers for poultry and pork were mixed for the multiplex reaction. The templates were amplified in the real-time PCR followed by a melting curve analysis using EvaGreen and the Corbett Rotor Gene Analyzer System. The addition of amplicons in the same reaction was verified in a graph representing the changes in fluorescence as a function of time (dF/dT) versus the temperature of the reaction products. Pork and poultry amplicons were easily distinguished through specific Tm values due to the different length and base compositions of two amplicons. The EMRT-PCR resulted in a single curve with two peaks as shown in Fig. 1a, b. These peaks formed at a specific location on the temperature axis at 80.5 °C for pork and 87.2 °C for poultry (Fig. 2).

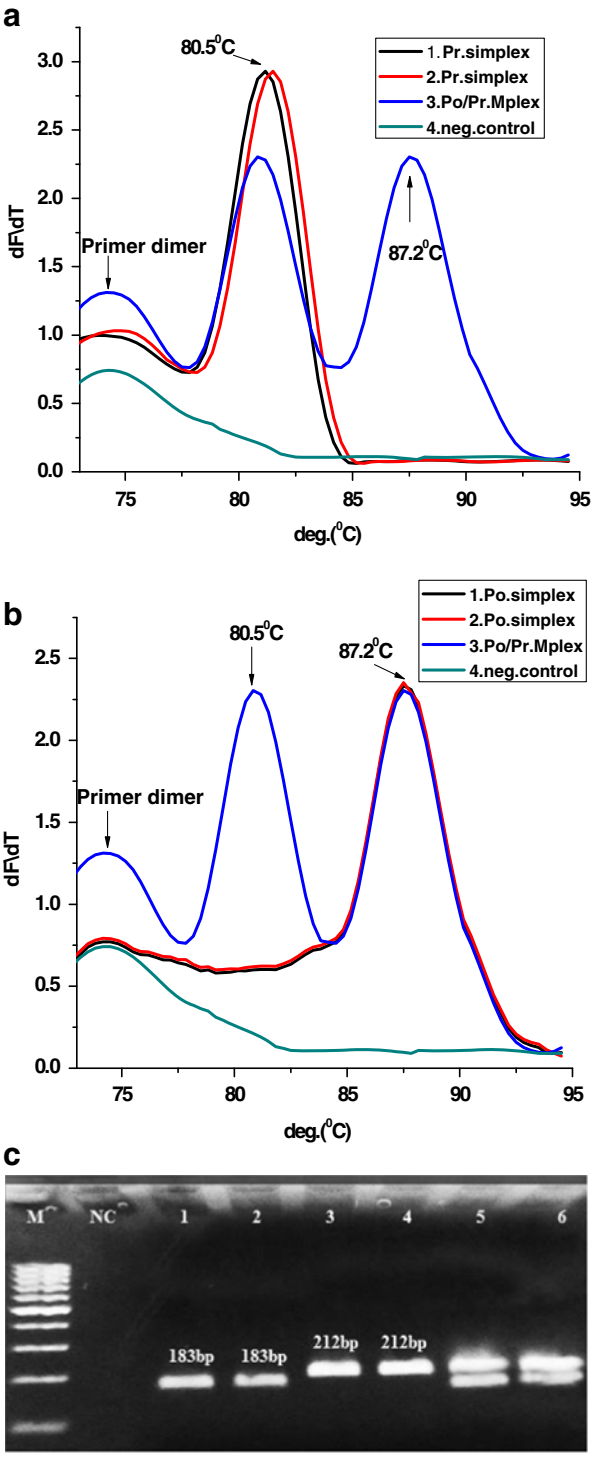

Fig. 1 **a** Specificity of the EMRT-PCR assay with fluorescence melting curve for pork and poultry in EvaGreen multiplex real-time PCR performed with Corbett Rotor Gene Analyzer. Tm profiles of pork and poultry; pork simplex-positive control: Tm, 80.5 °C; poultry/pork multiplex-positive control (multiplex amplification): Tm, 80.5 and 87.2 °C. **b** Specificity of the EMRT-PCR assay with fluorescence melting curve for pork and poultry in EvaGreen multiplex real-time PCR performed with Corbett Rotor Gene Analyzer. Tm profiles of pork and poultry; poultry simplex-positive control: Tm, 87.2 °C; poultry/pork multiplex-positive control: Tm, 80.5 and 87.2 °C. **c** Agarose gel electrophoresis (*cross check*) of EMRT-PCR products. *Lanes M*, 100-bp ladder; *1* and *2*, poultry simplex-positive control; *3* and *4*, pork simplex-positive control; *5* and *6*, pork/poultry multiplex positive control. *NC* negative control

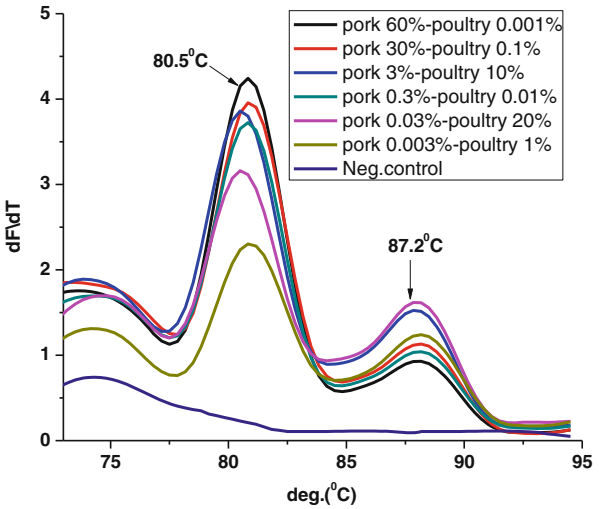

Fig. 2 Determination of the relative sensitivity by using multiple DNA of pork, poultry, and beef in different ratios. Peaks at Tm of 80.5 °C correspond to amplicons obtained with 60, 30, 3, 0.3, 0.03, and 0.003 % pork DNA against a background of 100 ng pork, poultry, and beef DNAs. Similarly, peaks at Tm of 87.2 °C correspond to amplicons obtained with 20, 10, 1, 0.1, 0.01, and 0.001 % poultry DNA against a background of 100 ng pork, poultry, and beef DNAs. *NC* negative control

Specificity of EMRT-PCR System

For the multiplex determination of pork and poultry tissues in pet foods, pork- and poultry-specific primers respectively designed by Lahiff et al. [18] and Dalmasso et al. [3] were used. Both primers were used to develop amplification conditions. The specificity of the primers was tested in seven livestock species: pork, horse, soybean, bovine, sheep, chicken, and turkey. It was verified that primers specific to the species of bovine and poultry showed no cross-reaction with any of the nontarget species. Specificity of the multiplex assay was developed by performing melting curve analysis. This multiplex real-time PCR assay was specific for each species investigated with a little changeable Tm. The Tm values of pork and poultry genes were recorded as 80.5±0.3 and 87.2±0.4 °C. EMRT-PCR yields were run on 2 % agarose gel stained with ethidium bromide to crosscheck. An agarose gel electrophoresis of the PCR products showed that pork and poultry samples produced clear bands of the expected size of 212 and 183 bp, respectively.

Sensitivity of the EMRT-PCR System

To determine the sensitivity and linearity of the real-time PCR technique, the genomic DNA was obtained from each target species starting from the 50 ng/μL target DNA. Experiments with samples of decreasing concentration indicated that pork and poultry DNA concentrations as low as 0.0001 ng/μL still yielded an amplicons signal upon EMRT-PCR (figure not shown). Amplification reactions with mixtures of pork and poultry DNA samples further demonstrated that as little as 0.003 % pork and 0.001 % poultry DNA could be detected against a background of 50 ng poultry and 50 ng pork DNA, respectively.

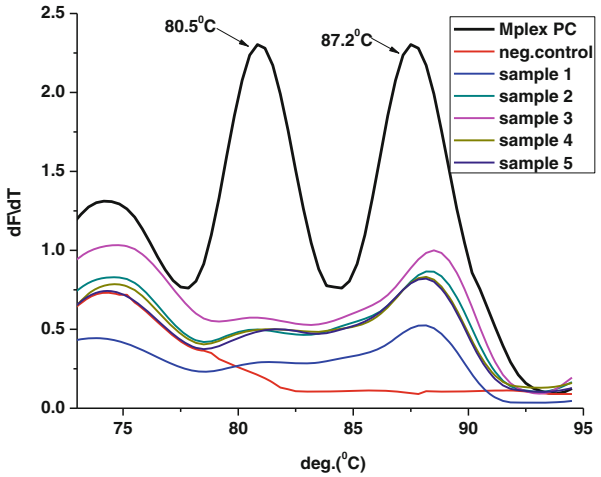

Fig. 3 EMRT-PCR with multiple DNA from pet foods contained beef and poultry using pork and poultry specific primer pairs. Peaks (Tm, 87.2 °C) correspond to amplicons obtained with poultry DNA of sample. Other peaks (Tm, 80.5 °C) correspond to amplicons obtained with pork DNA of sample. Poultry/pork multiplex-positive control. *NC* negative control

Application of the EMRT-PCR Assays to Commercial Pet Foods

The application of the EMRT-PCR assays to commercial pet foods has been verified in Figs. 3, 4, and 5 and Table 3. The pet food samples which contain poultry, claimed constituents have been confirmed by the EMRT-PCR analysis (Fig. 3). The results are shown in Fig. 4, which indicated that three fifths of the beef samples were contaminated with poultry residuals and labeled as 100 % beef. These residuals are not in accordance with

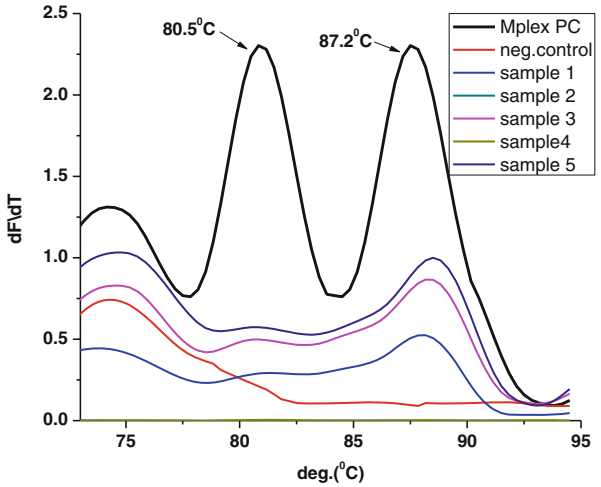

Fig. 4 EMRT-PCR with multiple DNA from pet foods contained beef using pork and poultry specific primer pairs. Peaks (Tm, 87.2 °C) correspond to amplicons obtained with poultry DNA of sample. Other peaks (Tm, 80.2 °C) correspond to amplicons obtained with pork DNA of sample. Poultry/pork multiplex-positive control. *NC* negative control

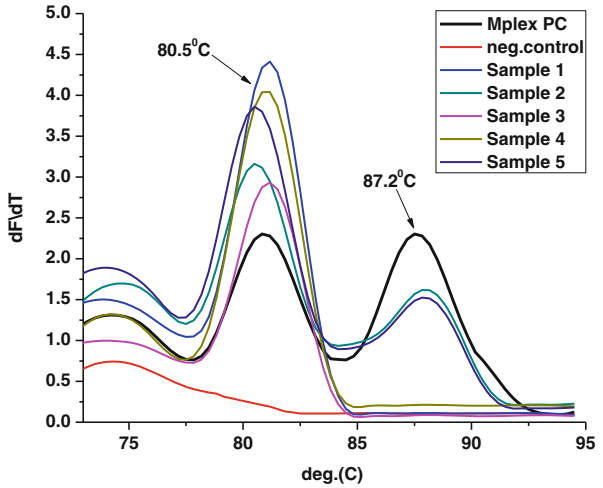

Fig. 5 EMRT-PCR with multiple DNA from pork food samples using pork and poultry specific primer pairs. Peaks (Tm, 80.5 °C) correspond to amplicons obtained with pork DNA. Other peaks (Tm, 87.2 °C) correspond to amplicons obtained with poultry DNA which showed poultry contamination. Poultry/pork multiplex-positive control. *NC* negative control

the ingredients labeled by the producer. Similarly, the results are shown in Fig. 5, which indicated that two fifths of the pork samples were contaminated with poultry residuals, and thus, were labeled as 100 % pork. The assays applied in this research have a high potential as a molecular tool that can be used in quality control laboratories for the verification of contaminated food products. This study addresses the need for development of rapid, sensitive, specific, and economical detection methods to screen mixed animal food products for label authenticity. The developed assay is new due to its ability to conduct amplification of two target genes (mitochondrial DNA and 12S RNA) simultaneously in multiplex system by using EvaGreen-based real-time PCR methods through Tm discrimination (Fig. 1). As such, this unique characteristic could serve as a reference for the development of assays for detection and differentiation of other species, such as sheep, goat, donkey, horse, and others. Some scientists [8, 12, 19] suggested that multiplex real-time PCR combined with DNA intercalating dyes are not applicable. The uniformity of each species-specific amplicons in coincidence with fluorophor-specific TaqMan probes would make these assays acquiescent to multicolor simultaneous detection, whereas DNA intercalating dye such as SYBER green-based multiplex detection is difficult [20]. On contrasting reports mentioned earlier, we performed a multiplex reaction with EvaGreen. The most critical parameters to perform EMRT-PCR successfully are the design of primers and the selection of Tm products. However, poorly designed primers can result in a PCR reaction that will not work. The primer sequence determines several things such as the length of the product and its Tm. Several parameters including the length of the PCR product and the percentage of the

Table 3 Results of EMRT-PCR performed on commercial pet foods

Products	Labeled	Results
Pet food (n®, 5)	Poultry	Poultry
Pet food (n®, 5)	Beef	Poultry
Pet food (n®, 5)	Pork	Pork and poultry

n® number of samples

number of G's and C's in the primer (GC) content need to be optimized for a successful EMRT-PCR. In EMRT-PCR experiments, the GC content of products is used to calculate their Tm value. A higher GC content level indicates a higher Tm of a product. Further advantages are enhanced sample throughput on real-time PCR machines and more economical use of limited amounts of template. Successful multiplexing requires careful, laborious optimization, and validation. To overcome multiplex-inherent impairments, checkerboard assays to determine the optimal concentration for each primer pair, as well as optimization of template concentration, magnesium, dNTP, and polymerase concentration, are often compulsory [21]. In this assay, we only optimized the primer concentration for the simultaneous amplification of pork and poultry DNAs. Further optimization of template concentration is not necessary because the DNA template can be used from 50 to 0.0001 ng/μL for the EMRT-PCR. Other PCR reagents (dNTP, polymerase, magnesium, and buffer) are provided by the EvaGreen mix (Solis Bio Dyne).

Conclusions

EMRT-PCR is a powerful tool that is accurate, simple, cost-effective, and rapid, with high sensitivity and specificity. The potential of the described method to identify minute amounts of poultry and pork DNAs in commercial pet food samples may render it a useful tool for assessment programs to enforce labeling regulations of pet foods. With the present method, two animal species in pet food could be identified more cleanly at the same time and could be extensively applied in practical detection for simultaneous identification of other animal species in different types of samples.

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