REAL-TIME PCR ASSAY FOR HIGHLY SPECIFIC DETERMINATION OF PORK IN RAW AND HEAT TREATED MEAT MIXTURES

Hasan YETIM[*], Zulal KESMEN[1], Fikrettin SAHIN[2] and Herbert W. OCKERMAN[3]

[*1] Erciyes University, Coll. of Eng., Food Science Dept., 38039, Kayseri, Turkey
[2] Yeditepe University, Coll. of Eng., Bioengineering Dept., 34755, Istanbul, Turkey
[3] The Ohio State University, Dept of Animal Science, 43210, Columbus, OH, USA

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Introduction

Numerous analytical methods have been developed for species identification of animal tissues in meat products to protect the consumer from the illegal and undesirable adulteration for economical, religious and health reasons. The methods based on the detection of species-specific proteins such as electrophoresis, isoelectric focusing (IEF), enzyme-linked immunosorbent assays (ELISA) are proved to be inadequate (less sensitive) and often not suitable for the species identification of meat products which had been previously exposed to very high temperatures causing denaturation of the proteins (Meyer et al., 1994). However, methods of DNA analysis based on the polymerase chain reaction (PCR) offer a potential for the doubtless detection of the animal species used, even for the products that have been subject to intensive processing with complex composition (Meyer et al., 1993). Conventional PCR techniques allow the qualitative detection of different animal species in a mixture, but they are not appropriate to achieve the quantification of the species tissue in a product (Rodriguez et al., 2005). Real time PCR is widely accepted as a robust assay for the species identification and quantification of nucleic acid molecules due to its high sensitivity and specifity, large dynamic range of detection and a low carry-over contamination risk (Mackay et al., 2002). In this technique, amplification of the target gene is monitored by an increased fluorescence signal which enables direct assesment of the results after the PCR application without additional detection steps. In this paper, a TagMan real-time PCR assay was studied to optimize the determination of pork in heat treated meat mixtures, added fraudulently to the products and consequently verify the concordance of the labels.

Materials and Methods

Preparation of meat samples: Binary meat mixtures were prepared from raw minced beef by adding porcine meats at 7 different levels (0.0, 0.01, 0.05, 0.1, 0.5, 1.0 and 5.0%, w/w) to a final weight of 2 g and then each samples were subjected to heating for 20 min at 100°C and 121°C.

DNA extraction: DNA was extracted from raw and heat treated mixtures following the method of Chisholm et al. (2005).

Design of TaqMan primer and probe sets: The primer and probe set was designed using porcine mitochondrial NADH dehydrogenase subunit 2 gene (Gen Bank Accession No. NC000845) and Primer 3 software. The probe (5'-AATGTCCGGAACCATACTAGTAATAATC-3') was 5' labelled with 6-carboxyflourescein (FAM) and 3' labelled with the flourescent quencher dye 6-carboxytetramethylrhodamine (TAMRA). One set of primer (full lenght sense 5'- TACACTACCCTTATCATAACAG -3', full lenght antisense 5'- ATACTGGGATTATTGCTAATAG -3') was designed to amplify a 115 bp fragment of the NADH2 gene. (for the specific detection of pork)

Real-time PCR assay conditions: Amplification reactions (50 µl) were performed with QuantitecTec Probe PCR Master Mix (Qiagen) and optimized 800nM sense and antisense primer 100 nM probe. TaqMan probe reactions were run on the Line-Gene II Real-time PCR Detection System (Bioer) by following the thermal cycling protocol; 50 °C for 2 min, 95 °C for 15 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

PCR primers specificity and sensitivity test: The specificity of each species specific primers was confirmed by performing amplification of 100 ng DNA/µl water of bovine, ovine, horse, donkey and porcine meat genomic DNA and a negative control was without DNA. To determination detection limit of specific primers, serial 1:10 (100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng DNA/µl water) dilutions of porcine DNAs were made, and each dilution was added separately to reaction mixtures for the sensitivity test.

Result & Discussion

In this study, the specificity of TaqMan PCR system was tested by the reaction of bovine, ovine, horse, donkey and porcine genomic DNA (100 ng) with porcine specific primer-probe system, and application of standard 40 cycle system resulted no cross reactivity with the DNA of the other non-target species. The dynamic range and detection limit of specific primer probe set was determined using 10 fold dilutions in water of porcine DNA starting with 100 ng. In the results, 17.04 Ct value was observed with 100 ng pork DNA and the detection of pork extended to a 1.000.000-fold (100 fg DNA) dilution for the pork DNA in water (average Ct 36.58)

(Fig.1). Additionally, range of the dilution responses was linear, and the slope of the ct values plotted against log of the DNA dilutions was -3.28 indicating a closeness to the theoretical value (-3.32), that can be achieved with a PCR efficiency of 100%. The mitochondrial encoded gene NAHD2 was selected in the study, and these results confirmed the appropriateness of the mt-DNA for meat species detection. Because the mt-DNA accepts the mutations fast enough to allow differentiation between the closely related species, and the mitochondrial based DNA analyses derived from the fact that each cell contains several copies of mt-DNA. This improved the possibility of amplifying template molecules of adequate size among the DNA fragments brought about by extreme processing conditions such as sterilization (Montiel-Sosa et al., 2000).

Indeed, the results of the PCR reactions for the raw and heat treated binary meat mixtures showed that detection of the meat species was successful in all meat mixtures from the 0.01 to the 5.0 % level. The ct value of the samples heated at 100 ºC for 20 min were 0.86-1.63 Ct values which are lower than that of the ct value (0.99-1.18) of the samples heated at 120 ºC for 20 min, and they were higher than that of the raw meat mixtures (Table 1). Also, in this study, small size of amplicon (<115 bp) was used for the specific detection of pork, that is recommended for the development of TaqMan type real-time assays. However, the small target size is also advantageous for the detection of material in processed products like cooked meat products because heat treatments affect the quality of DNA causing its degradation into small size fragments.

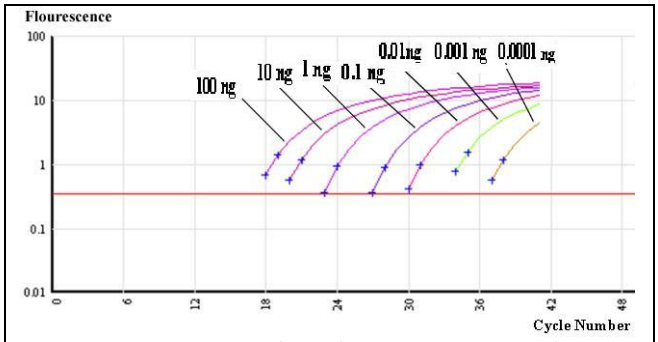

Figure 1. Flourescent profiles of PCR products amplified pork specific PCR system from 10 fold dilutions of pork DNA in water.

Table 1. Ct values of the binary meat mixtures.

Porcine (%) in beef	Ct value		
	Raw Mix.	100ºC 20 min.	121ºC 20 min
0	n	n	n
0.01	29.65	30.72	32.01
0.05	26.90	27.89	28.72
0.1	25.87	26.95	27.81
0.5	23.82	25.00	26.15
1	23.18	24.52	25.41
5	20.58	21.76	23.18

n: indicates no amplification signal detected after 40 cycles.

Conclusions

In this study, a TagMan real-time PCR assay was developed for the species identification of porcine tissue in the raw and heat treated meat mixtures with a single reaction step PCR. By means of the optimized assay, porcine tissue could be distinguished from the phylogenetically close species and/or other livestock animals, and the presence of a 100 fg template pork DNA in water might be detected. Also, the detection of pork was successful in all binary meat mixtures studied in this research even in the harshly heated samples which was expected to contain a large amount of fragmented DNA. Consequently, the real-time based assay can be recommended for the detection of porcine tissue in food control agency or laboratories, this might be a reliable and practical method for the determination of mislabeled and/or fraudulent species substitution in the heat treated meat products.

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