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Research Note

Rapid and Simple Identification of Pork in Meat Products by Using the Isothermal Target and Probe Amplification Assay

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Abstract

Species identification of animal tissues in meat products is an important issue to protect the consumer from illegal and/or undesirable adulteration, for economic, religious and health reasons. For example, pork DNA was found in halal chicken sausages served in a primary school in Westminster, central London, the local authority had stated on 14 March 2015. PCR is one of the most widely used techniques in diagnostic applications because it allows a sensitive and rapid diagnosis. However, this technique is not suitable for routine identification of pork in meat products because of the need for expensive thermal cycler equipment and complex operations. The isothermal target and probe amplification (iTPA) analysis has been developed for identifying pork species in meat samples. In this study, the isothermal target and probe amplification assay was developed for rapid, simple and highly specific identification of pork DNA. For the assay, the mitochondrial 16S ribosomal RNA gene and the FRET-based signal probe were amplified at 61°C, followed by measurement of the fluorescent signal. As little as 1 pg of template DNA could be detected without any cross-reactivity with non-target species. Meat mixtures prepared by mixing pork meat with other meats at different ratios (0.01% - 10%) were tested, and the iTPA assay allowed detection of as little as 0.01% pork in meat mixtures. Thus, this work showed that the iTPA assay could be used for specific identification of pork species. This assay only requires a heat block and a fluorescence reader for amplification and detection, respectively, and it has great potential for use as a hand-held device or point-of-care-testing system. The iTPA assay is sensitive and specific for rapid screening of fraudulent adulteration/substitution of meat products.

INTRODUCTION

Species identification of animal tissues in meat products is an important issue to protect the consumer from illegal and/or undesirable adulteration, for economic, religious and health reasons [1]. For this purpose, numerous analytical methods have been developed based on protein and DNA analysis. Methods based on protein fractions, including physico-chemical, electrophoretic, and immunological techniques, have been introduced [2]. Unfortunately, these methods are often not suitable for complex food products because they are not sensitive in processed materials to differentiate closely-related species, are time-consuming, inadequate, and/or expensive. DNA-based methods such as PCR are the most specific and sensitive techniques for food component authentication and they are relatively quick. DNA-based techniques have many potential advantages over protein-based techniques such as ELISA, which depends primarily on protein epitopes that are detectable by the antibodies to detect analytes [3]. In DNA-based techniques for meat speciation, genetic markers are used. They may be nuclear gene or mitochondrial gene markers. Among nuclear markers, leptin gene [4], actin gene [5] and melanocortin receptor 1 (MC1R) gene [6] are common, while mitochondrial genes used for this purpose include cytochrome-b [7-10], 12S and 16S ribosomal RNA subunits [11-13], displacement loop region (D- loop) [14,15], and cytochrome oxidase I (COI) [16,17]. On comparison of both these genes, it can be said that mitochondrial genes are more convenient and applicable because mt-DNA isolation is easier due to the presence of multiple copies in a cell; mt-DNA copies range from 100-10,000 per cell, and hence, a very small number of samples can be tested. Other reasons for its preference include more stability of mt-DNA and higher strength in comparison to nuclear DNA. mt-DNA is protected from degradation, even when it is exposed to prolonged environmental conditions.

PCR is a rapid and specific nucleic acid amplification method for the identification of pork species, and a number of PCR assays have been described for food identification. In spite of this, the PCR-based method continues to have some disadvantages, such as complicated operation and requirement of special heating cycle equipment. The iTPA technology was developed [18], and it proved to be a good DNA detection method that amplifies the

target gene and the signal probe simultaneously under isothermal conditions, with a high specificity and sensitivity for foods [19-21] and clinical specimens [22,23]. The iTPA technology relies on the strand displacement activity of DNA polymerase and the RNA degrading activity of RNase H, which specifically hydrolyzes the targeted DNA-RNA hybrid only.

In this study, a species-specific iTPA of mt-DNA method was developed for porcine species identification in meat products. The objective of this study was to develop a technique that could successfully be used in routine control assays to detect fraudulent adulteration/substitution of meat.

METHODS

The 16S ribosomal RNA gene (GenBank AY920913) was used as the target for iTPA primers and probe design. Four primers, two outer and two inner, and one fluorescence resonance energy transfer (FRET) signal probe that recognized five regions of the target sequence were designed using DNASTAR software (Madison, WI) and synthesized by IDT (San Diego, CA). Oligonucleotide sequences and locations of the primers and the probe are presented in (Table 1). The basic local alignment search tool indicated a high level of specificity (no similarity to any other species). The 20 ul iTPA reaction mix consisted of 15 mM Tris-HCl (pH 8.5), 22 mM $MgSO_4$, 15 mM KCl, 15 mM $(NH_4)_2SO_4$, 0.1 mg/ml acetylated bovine serum albumin, 6 mM dithiothreitol, 1.4 mM concentration of the deoxynucleoside triphosphates (dNTPs), 0.11 mM concentration of each outer primer, 1.1 mM concentration of each inner primer, 75 nM FRET signal probe, 4 units of Bst polymerase (NEB, Ipswich, MA), 5 units of RNase H (Epicentre, Madison, WI), 6 units of RNase inhibitor (Solgent, Daejeon, South Korea), and 10 ul of DNA template (replaced by 10 of sterilized water for the negative control). The iTPA reaction mix was incubated at 61 for 60 min in a heat block and then cooled to room temperature. After a quick spin-down, the reaction tube (0.2 ml PCR tube) was inserted into a Fluo-100 fluorometer (Allsheng, Hangzhou, China) to read the fluorescence. The F-score was calculated by the following equation:

F-score = [(fluorescence of the sample – fluorescence of the negative control) / fluorescence of the negative control] x 100

RESULTS AND CONCLUSION

In this study, we designed a set of DNA–RNA–DNA chimeric primers and a FRET probe to specifically target the mitochondrial 16S ribosomal RNA gene for porcine species. Thus, a novel and rapid identification system has been developed. By simultaneously utilizing the dual amplification powers of the target DNA and the FRET probe, we have demonstrated that iTPA can be used to rapidly detect 0.01% of pork in meat samples. Four chimeric primers and one FRET probe were designed from five regions of the 16S ribosomal RNA gene for porcine species that are highly specific for pork meat (Table 1). The number of meat samples was tested on a rapid fluorescent detection platform and as a result, 100% inclusivity and 100% exclusivity for porcine species were detected (Figure 1). The iTPA assay is suitable for the identification of pork meat both in laboratories and in field situations because iTPA produces a fluorescence signal in the amplification microtube which is detected directly by any conventional portable fluorescent reader. As no post-amplification handling is required for detection, it significantly reduces any cross-contamination risk caused by amplicon-carryover. Thus, the iTPA assay is more cost-effective and practical than both conventional PCR and real-time PCR assays.

Table 1: Oligonucleotides used in this study.

Name	Sequence (5'-3')	Position#
Outer forward	CAA CCT TGA CTA GAG AGT AAA ACC	466-489
Outer reverse	GGT ATT GGG CTA GGA GTT TGT T	580-601
Inner forward	ACC TCA GAC ATC CAG rArArArA AAA TAC TAC	492-512
Inner reverse	CAT AGT AGG CCT A	542-567
FRET probe	TAG AAG CGA TGG CAT GAC T rArGrArG GAT TAT GTT GGT GAA TTT GTT GAG CT	520-545
	FAM- CCA TCA ATT rGrArGrArArA GCA TTA AAG CT -BHQ1##	

GenBank : AY920913
Black hole quencher 1

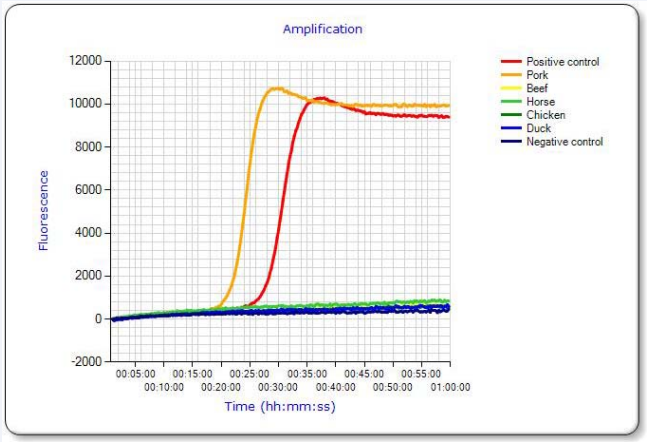

Figure 1 Specificity of the primers used in this study.
Results of accurate measurements in different meat products. Only porcine meat was detected.

In conclusion, we have developed a DNA detection system that can be conveniently used as it only requires a heat block and a fluorescence reader and has great potential in applications for hand-held or point of testing diagnostics. The 16S ribosomal RNA gene-based iTPA assay developed in this study is a specific, sensitive, and rapid method for the detection of porcine species in meat products. This simple method is expected to enable rapid screening for fraudulent adulteration and substitution of foods at a low cost.

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