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Multiplex TaqMan locked nucleic acid real-time PCR for the differential identification of various meat and meat products

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ABSTRACT

Meat adulteration incidents have been reported frequently over the past few years, and the corresponding traceability issues attracted much more attention due to the customer's demands and administration's responsibility. Therefore, it is important to develop high-throughput and rapid detection methods to identify the specific sources from meat samples. In this study, a multiplex TaqMan locked nucleic acid real-time polymerase chain reaction assay (MLNA-RT-PCR) was developed to simultaneously detect multiple meat sources (duck, pork, beef and chicken). PCR primers and TaqMan-LNA probes were designed based on species-specific mitochondrial gene sequences, and the MLNA-RT-PCR was developed and optimized for better performance. The specificity of this assay was verified through identifying unrelated (sheep, horse, deer, donkey, rabbit, goose, goat, shrimp, salmon and maize) mitochondrial DNA as species-specific targets. The detection limit for MLNA-RT-PCR reached to the level of 0.01% of each species. The assay was then used to identify the meat sources of commercial meat and meat-derived products that were obtained from markets in Shantou, and the results were 98% consistent with that obtained from detection based on the national standard. In conclusion, this MLNA-RT-PCR is a high-throughput, sensitive and specific method that can be used to identify multiple meat sources in meat and meat-derived products.

1. Introduction

As one of the most consumed protein and oil sources, meat is most prone to suffer adulteration for economic gain among the foods. Cheaper meat that has been sourced from “non-traditional” sources can be used as partial or total replacement for highly valued meats for economic benefit. Frauds in the meat industry and retail markets have become a widespread problem, especially after a European horse meat scandal that spread throughout many countries in 2013, including Sweden, Britain, France, Ireland and Romania (O'mahony, 2013). In a recent investigation performed on one thousand types of meat products, it was found that nearly 20% of the products identified were not of consistent quality (Ballin, Vogensen, & Karlsson, 2009). Otherwise, Adulteration of meat and meat products because of species substitution is also of major importance for traditional and religious foods (Costa, Mafra, Carrapatoso, & Oliveira, 2016). Therefore, the presence of certain animal species is perhaps the most concern of consumers and the responsibility of administrators, which highlighting the necessity of determining method to track meat and derived products.

Various detection methods were developed to identify specific meat sources, some of which based on species-specific proteins have been developed, such as electrophoresis, isoelectric focusing (IEF) and enzyme-linked immunosorbent assays (ELISA) (Zhang et al., 2014). However, these methods are laborious, and sometimes less sensitive for denatured proteins in products during heating, dehydration, chemical processing and pressurization. Some protein-based assays are also not sensitive to effectively differentiate closely related species (Karabasanavar, Singh, Kumar, & Shebannavar, 2014). The application of the polymerase chain reaction (PCR) is an ideal way for target identification because of its sensitivity, reliability and convenience. Various PCR-based identification methods have been developed, such as PCR-RFLP (Rahman et al., 2015), RAPD (Rastogi et al., 2007) and SSCP (Tisza et al., 2016). However, difficulties in obtaining reproducible patterns and the inappropriateness of analyzing admixed meat species make the applicability of these methods of limited scope (Fajardo, González, Rojas, García, & Martín, 2010). Recently meat

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Table 1
The primers and TaqMan-LNA probes.

Meat species	Primer	Primes and probes sequence (5′-3′)	Final concentration (μM)	Product size (bp)	GenBank ID
Pig	F	CAGCAACCGTATTCACAGGAT	0.6	137	KP301137.1
	R	GGCTACTGGTTGAATAAATAGGC	0.6		
	P	Cy3-TTTCTACCACAA + GGAACACCCGCC-BHQ2	0.1		
Cows	F	TTGAATTAGGCCATGAAGCA	0.6	108	KF926377.1
	R	CGACTTGTCTCCTCTCATGTAGC	0.6		
	P	FAM-CGCCCGTCA + CCCTCCTCAAATA-BHQ1	0.3		
Chicken	F	TAGCCCTAAATCTAGATACCTCCC	0.3	88	KM096864.1
	R	ACCGCCAAGTCCTTAGAGTTT	0.3		
	P	HEX-CACATGTATCCGC + CTGAGAACTACGA-BHQ1	0.1		
Duck	F	ACCTTCCCGCACCCTCTAAT	0.8	63	KC466567.1
	R	GGCAGATGGCGAGCAGAGAT	0.8		
	P	Cy5-ATCTCYG + CYTGATGAAACTTCGG-BHQ1	0.4		

detection through sensors and biosensors were developed for sensitive and fast, or even visible identification of meat sources (Ali et al., 2014; Kamruzzaman, Sun, ElMasry, & Allen, 2013; Wang, Zhu, Chen, Xu, & Zhou, 2015).

Real-time PCR is currently considered to be the most precise, cost-effective and applicable assay to identify meat products. Real-time PCR is performed through monitoring fluorescence signal, which allows for direct observation of results without additional steps (Chisholm, Conyers, Booth, Lawley, & Hird, 2005; Tanabe et al., 2007). Multiplex real-time PCR, which can simultaneously detect different target genes in one reaction, have been validated as an effective tool for application in a number of detection fields, including food borne pathogens (Zhang et al., 2007), genetically modified organisms (Zhao, Deng, Liu, Fang, & He, 2004), and food-induced allergies (Chen & Wang, 2011), as well as meat adulteration detection (Cheng, He, Huang, Huang, & Zhou, 2014; Fang & Zhang, 2016; Hou et al., 2015; Köppel, Daniels, Felderer, & Brünen-Nieweler, 2013; Meira et al., 2017). Of these, TaqMan probes are the most frequently used methods to monitor fluorescence. However, high melting temperature (Tm) of TaqMan probes restricts its use because of increased competition among different probes and primers (Gašparič et al., 2010). The locked nucleic acid (LNA) adds methylene bridge linking the 2′ hydroxyl group of an RNA monomer to the 4′ carbon of the ribose ring to increase the stability of the duplex when bound to the target (Braasch & Corey, 2001; Petersen & Wengel, 2003) and have previously been used in several SNP genotyping and microRNA detection assays (Várallyay, Burgyán, & Havelda, 2008; Johnson, Haupt, & Griffiths, 2004.). Recent work has shown that when LNAs are incorporated into Taqman probes they provide increased specificity and sensitivity (Reynisson, Josefsen, Krause, & Hoorfar, 2006).

In this study, a novel multiplex TaqMan-LNA real-time PCR method (MLNA-RT-PCR) was developed to simultaneously detect four meat samples. PCR primers and TaqMan-LNA probes were designed based on the sequence of mitochondrial gene in each species, which served as the target. The specificity and sensitivity were also evaluated to determine the applicability of this assay. The reliability of this method was determined using one hundred commercial meat and meat products, which were collected from local markets.

2. Materials and methods

2.1. Preparation of meat samples

Fresh samples of cows (*Bos taurus*), pigs (*Sus scrofa*), chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), sheep (*Ovis aries*), horse (*Equus caballus*), deer (Cervus), donkey (*Equus asinus*), rabbit (*Oryctolagus cuniculus*), goose (Goose calicivirus),goat (*Capra aegagrus* hircus), shrimp (Penneropeanaeus Chinensis), salmon (Oncorhynchus) and maize (*Zea mays* L.) were purchased directly from butchers or farmers in Shantou, China, and have been confirmed by national standard method to guarantee their authenticity (SN/T 2051-2008, SN/T 2978-2011, SN/T 3731. 5-2013). A total of one hundred samples, representing a variety of meat species, were obtained from local markets during October 2014 to June 2015 and were tested through MLNA-RT-PCR and standard method. SN/T 2051-2008 was used to detect beef and pork samples, SN/T 2978-2011 was used to detect chicken samples, while SN/T 3731. 5-2013 was used to detect duck samples. The results from the two different PCR detection methods (MLNA-RT-PCR and the standard method) were compared to confirm the reliability of this novel assay.

2.2. DNA extraction

DNA was extracted from the samples and purified using the Wizard kit (Promega, USA) according to the manufacturer's protocol. A reagent blank was included with each DNA extraction as a negative control. The concentration and purity of the extracted DNA were quantified using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Inc., Montchanin, DE). All DNA samples were stored at − 20 °C before use.

2.3. Primer design

The four pairs of species-specific primer and probe used for the MLNA-RT-PCR assay are designed by PrimerQuest Tool (Integrated DNA Technologies, Coralville, USA) and are listed in Table 1. The gene sequences were based on the alignments of available sequences deposited in GenBank. All primers and probes were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China).

2.4. MLNA-RT-PCR amplification

MLNA-RT-PCR procedures were performed in a final volume of 25 μL, which included the following reagent: several amounts of four types of primers and probes with final concentration of primers and probes that listed in Table 1, 12.5 μL LightCycler 480 Probes Master (Roche, Shanghai, China) and 50 ng DNA template. PCR amplification was performed on the CFX96 Touch (Bio-Rad) and the conditions were: 94 °C for 8 min, followed by 40 cycles at 94 °C for 8 s, 59 °C for 25 s, with fluorescent readings taken at the end of each cycle. Cycle threshold (Ct) values were calculated using the software at the automatic threshold setting.

2.5. Specificity and sensitivity test of MLNA-RT-PCR assays

The specificity of the MLNA-RT-PCR was evaluated using pork, beef, chicken, duck, sheep, horse, deer, donkey, rabbit, goose, goat, shrimp, salmon and maize DNA samples as the PCR templates. To test primer sensitivity, DNA from meat samples were mixed with non-target DNA (sheep) to form 100%, 10%, 1%, 0.1%, 0.01% and 0.001%,