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Development of a fast duplex real-time PCR assay for simultaneous detection of chicken and pigeon in raw and heat-treated meats

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ABSTRACT

We developed a fast duplex real-time polymerase chain reaction assay based on TaqMan® probes for the simultaneous identification of chicken and pigeon meat in under 40 min. The primer sets used for both pigeon and chicken targeted the mitochondrial cytochrome *b* gene. To obtain simultaneous fluorescent signals, FAM and HEX probes were used to label the pigeon- and chicken-specific probes, respectively. This method specifically amplified chicken and pigeon DNA without any cross-reactivity to 20 animal species. The absolute detection limit was 0.1 pg of DNA extracted from both chicken and pigeon meat. Using DNA extracted from either raw or heat-treated meat, as little as 0.01% pigeon DNA was detectible in chicken DNA, as well as the reverse. This assay might serve as a rapid, sensitive, and specific detection method for the identification of pigeon and chicken meats in both raw- and cooked-meat products.

1. Introduction

The adulteration of meat products frequently takes place through replacement with cheaper meats for economic gain and causes not only economic issues, but also health and ethical problems (Dooley, Paine, Garrett, & Brown, 2004; Lee, Kim, Hong, & Kim, 2016; Li et al., 2015). Pigeon meat is popular among European consumers, and many pigeon farms have been extended to produce meat and meat products (Rojas, González, García, Hernández, & Martín, 2012). On the other hand, the intake of pigeon meat is undesirable for many people due to ethical reasons or preference (Haunshi et al., 2009). The consumption of chicken meat has been rapidly increasing worldwide (Li et al., 2015); however, the likelihood of the adulteration of other meat products with chicken is very high due to its low price. In addition, since chicken meat can cause an allergic reaction for certain individuals, products containing chicken meat should be clearly indicated on the label (Tanabe et al., 2007). The identification of the animal species contained in meat products has been a major issue for consumers, producers, and regulators due to regulations on accurate labeling information on food composition (Danezis, Tsagkaris, Camin, Brusic, & Georgiou, 2016; Rojas et al., 2012). Therefore, rapid, sensitive, and reliable detection methods are required to identify the animal of origin in meat products.

Among the methodologies for species determination, molecular techniques based on PCR assays have been used extensively (Danezis et al., 2016; Kim & Kim, 2017). In the past decade, the real-time PCR assay, with its high sensitivity and specificity, has been a useful tool for qualitative and quantitative detection of various species in meat (Kesmen, Celebi, Güllüce, & Yetim, 2013; Kim, Yoo, Lee, Hong, & Kim, 2016; Okuma & Hellberg, 2015; Rojas et al., 2012; Rojas et al., 2010). The use of TaqMan® probes in real-time PCR provides specific fluorescent signals for the target species, which is essential for the multiplex reactions needed for detection of several species in meat mixtures in a single reaction (Cheng, He, Huang, Huang, & Zhou, 2014; Iwobi et al., 2015; Köppel, Zimmerli, & Breitenmoser, 2009). Furthermore, the fast real-time PCR assay is used for the more rapid and efficient identification of animal species using special chemistry and equipment than conventional real-time PCR method (Cammà, Di Domenico, & Monaco, 2012; F. Herrero, Royo, Lago, Vieites, & Espineira, 2013).

The aim of this study was to develop a simultaneous detection method for pigeon and chicken meats using fast multiplexed real-time PCR. No previous reports have estimated the effects of heat- and pressure-treatments on duplex real-time PCR assays for the authentication of animal species. Our optimized assay was successfully applied to both heat-treated and raw meat mixtures. Therefore, this method should be sufficient to monitor for chicken and pigeon meats in processed meat products.

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2. Materials and methods

2.1. Sample preparation

A total of 20 raw muscle samples of pigeon (*Columba livia*), chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), turkey (*Meleagris gallopavo*), ostrich (*Struthio camelus*), goose (*Anser anser*), pheasant (*Phasianus colchicus*), rufous turtle dove (*Streptopelia orientalis*), tree sparrow (*Passer montanus*), Japanese quail (*Coturnix japonica*), house swallow (*Hirundo rustica*), black-tailed gull (*Larus crassirostris*), white-fronted goose (*Anser albifrons*), meadow bunting (Emberiza cioides), crow (*Corvus macrorhynchos*), beef (*Bos taurus*), pork (*Sus scrofa domestica*), horse (*Equus caballus*), lamb (*Ovis aries*), and goat (*Capra hircus*) were collected from the National Institute of Animal Science (NIAS), the Conservation Genome Resource Bank (CGRB), and local markets in Korea.

The raw chicken and pigeon meats were heated in an autoclave at 121 °C and 150 kPa for 15 min to verify the applicability of this assay for cooked meat products. The raw and heat-treated samples were cut into small pieces and immediately stored at −20 °C until DNA extraction.

Reference binary mixtures containing 10% (w/w) of pigeon in chicken, or vice versa, were prepared to determine the detection limit of the fast duplex real-time assay. Meat samples for binary meat mixtures were freeze-dried and ground. Then, each reference sample was measured to a final weight of 100 mg and extracted immediately for analysis. Binary mixtures of heat-treated meat were also prepared.

A total of eight practical food samples labeled as chicken or pigeon were collected from local markets. All processed food samples were ground with liquid nitrogen and stored at −20 °C until use.

2.2. DNA extraction

Each DNA of animal samples, binary mixtures, and practical food samples was extracted from 25 mg of raw or heat-treated meat in duplicate using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The purity and concentration of the extracted DNA were measured using a UV spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan), and DNA with a 260/280 nm ratio between 1.8 and 2.0 was used as template DNA.

2.3. Primer and probe design

The sequences of the mitochondrial cytochrome *b* gene for pigeons and various other animal species were obtained from the GenBank database and aligned using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Fig. 1). The pigeon-specific primer set and probe were designed using Primer Designer, version 3.0 (Scientific and Educational Software, Durham, NC, USA), and the chicken-specific primer set and probe were previously reported by Dooley et al. (2004) (Table 1). All primer sets and probes were synthesized by Bionics (Seoul, Korea) and Bioneer (Daejeon, Korea), respectively.

2.4. Fast duplex real-time PCR assay conditions

The fast duplex real-time PCR was performed with TaqMan® probes in a ViiA 7 real-time PCR system (Applied Biosystems, Foster City, CA, USA) and was analyzed using the enclosed software. To optimize the primers and probes concentration, the following primer/probe combinations were estimated: 0.125/0.125, 0.25/0.125, 0.5/0.125, 0.75/0.125, 0.125/0.25, 0.25/0.25, 0.5/0.25, and 0.75/0.25 μM. Each DNA of pigeon and chicken was used for optimization test. The real-time PCR was carried out with optimized concentration of primers and probes. The 20-μL PCR reaction mixtures contained 10 μL of TaqMan® Fast Universal Master Mix (Applied Biosystems) and 10 ng of DNA template, with a final concentration of 0.5 μM for the primer pairs and 0.25 μM for the probes (both pigeon and chicken). The fast duplex real-time PCR assay was performed under the following conditions: 1 cycle at 95 °C for 20 s, followed by 40 cycles at 95 °C for 1 s and 60 °C for 20 s. Each PCR was performed in triplicate, and the no-template control (NTC) was used as a negative control for the assays.

2.5. Specificity and sensitivity of the fast duplex real-time PCR assay

The specificity of the fast duplex real-time PCR assay was tested in triplicate by amplifying 10 ng of genomic DNA from each of the species tested using both the pigeon and chicken primer/probe mixtures. The sensitivity of the fast duplex real-time PCR was first measured using 10-fold serially diluted DNA (from 10 ng to 0.01 pg per reaction) isolated from raw chicken and pigeon meats. Each of serially diluted chicken and pigeon DNA was performed in triplicate on three times. The detection limit was further tested using binary DNA mixtures from the raw and heat-treated meats. To determine the detection limit of pigeon-specific primer/probe in the fast duplex real-time PCR, DNA was extracted from a binary mixture containing 10% (w/w) of pigeon in chicken, and 10 ng/μL of DNA extract was diluted 1:10, 1:100, 1:1000, and 1:10000 with 10 ng/μL of chicken DNA. The concentration of pigeon DNA ranged from 10% to 0.001%. The detection limit of chicken-specific primer/probe was performed in the same manner, using serially diluted DNA extracts from a binary mixture containing 10% (w/w) of chicken in pigeon. To test the effects of heat treatment on this assay, the spiking tests were repeated using DNA extracts of heat-treated chicken and pigeon meats.

2.6. Statistical analysis

Statistical analyses were conducted by Microsoft Excel® Data Analysis Tools and IBM© SPSS™ Statistics 22.0. Student's *t*-test was employed to determine whether Ct values between raw and heat-treated meat mixtures were different. One-way ANOVA test was used to test whether Ct values across various ratios of binary mixtures and practical food samples were different. When there were significant differences, Duncan's multiple-range tests were also done. A probability level of $p < 0.05$ was used in testing statistical significance.

3. Results and discussion

3.1. Specificity of fast duplex real-time PCR assay

A fast duplex real-time PCR method using the TaqMan® probe system was developed for the simultaneous detection of chicken and pigeon meats in raw and heat-treated meat mixtures. The chicken-specific primers/probe and pigeon-specific primers/probe were selected to target the mitochondrial cytochrome *b* gene. Because the mitochondrial DNA has a circular shape as well as multi-copies per cell, the use of this gene for the detection of meat species improves specificity and sensitivity compared to nuclear DNA. Thus, some studies have been developed targeting mitochondrial DNA to identify pigeon and chicken in meat products (Haunshi et al., 2009; Rojas et al., 2012). The pigeon-specific primer set was designed to have a small amplicon size (106 bp) to detect pigeon meat in processed meat products. To simultaneously obtain signals for the two targets, different fluorescent dyes were used to label each probe. The sequences of the primers and probes are provided in Table 1. The optimization test for real-time PCR assay