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Analytical Methods

# Multiplex PCR assay for the detection of five meat species forbidden in Islamic foods

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ABSTRACT

Food falsification has direct impact on public health, religious faith, fair-trades and wildlife. For the first time, here we described a multiplex polymerase chain reaction assay for the accurate identification of five meat species forbidden in Islamic foods in a single assay platform. Five pairs of species-specific primers were designed targeting mitochondrial *ND5*, *ATPase 6*, and *cytochrome b* genes to amplify 172, 163, 141, 129 and 108 bp DNA fragments from cat, dog, pig, monkey and rat meats, respectively. All PCR products were identified in gel-images and electrochromatograms obtained from Experion Bioanalyzer. Species-specificity checking against 15 important meat and fish and 5 plant species detected no cross-species amplification. Screening of target species in model and commercial meatballs reflected its application to detect target species in process foods. The assay was tested to detect 0.01–0.02 ng DNA under raw states and 1% suspected meats in meatball formulation.



## 1. Introduction

Authentication of declared components in foods is an ever increasing public demand and a key priority in policy making and regulatory bodies. It helps to safeguard public health, consumers' lifestyle, food choice, religious faith, fair-trade economy and wildlife in natural habitats (Fajardo, González, Rojas, García, & Martín, 2010; Hou et al., 2014; Karabasanavar, Singh, Kumar, & Shebannavar, 2014; Kesmen, Celebi, Güllüce, & Yetim, 2013). The turnover of halal food items has crossed USD 661 billion (Ali et al., 2012) and has been expanding rapidly even among the non-Muslim consumers because of its perceived quality attributes and significantly reduced risk to be a carrier of zoonotic diseases (Gregory, 2008; Mohamad, El Sheikha, Mustafa, & Mokhtar, 2013). Perceiving the huge opportunities of the halal food markets, even the European food industries have been investing in the production of halal foods (van der Spiegel et al., 2012). For the Muslims, choices of foods depend on the "Halal" (means allowed) attributes enshrined by the Qur'an and holy prophet Mohammed (peace be upon him) and Islamic fiqah (opinions of a team of Islamic scholars) (Nakyinsige, Man, & Sazili, 2012). It is more hygienic than the conventional foods since it must meet both the religious and health requirements. Consequently, the prices of halal foods are significantly higher than the conventional ones and thus it offers a significant scope of adulteration (illegal mixing of low-priced items).

The Islamic law prohibits Muslims from eating pork and ingredients derived from animals having canine teeth or fangs such as dog, cat, monkey and rat. However, in certain countries such as Vietnam, Switzerland, Tahiti, Mexico, South Korea, Taiwan and some parts of the United States, these animals have been consumed for ages (Ali et al., 2013). Additionally, these animals could be obtained without any offered prices and hence there is a significant chance of mixing them in halal foods (Rahman et al., 2014). The recent horse meat scandal in Europe and pig and rat meat scandal in China (Ali, Razzak, & Hamid, 2014) have put Muslim consumers in red alert in determining the presence of prohibited ingredients in marketed foods. Moreover, some of the species such as rat, cat, dog and monkey are potential carrier of several zoonotic diseases such as plague, ringworm, hydrophobia (rabies), and herpes virus (*Herpevirus simiae*), simian virus 40 (SV40) (Conly & Johnston, 2008; Fajardo et al., 2010) and thus they are not safe and hygienic for public consumption.

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The recent innovations in packaging technologies have made it difficult or impossible to identify food components based on physical attributes (Ali, Kashif, et al., 2011; Bottero & Dalmasso, 2011; McMillin, 2008). Currently available meat authentication schemes depend on lipid, protein and nucleic acid biomarkers (Rahman et al., 2014 ; Rohman, Sismindari, Erwanto, & Che Man, 2011). Protein-biomarkers are fragile under physio-chemical shocks and both the type and amount of fats (lipid biomarkers) could be extensively modified during food processing (Ali et al., 2012; Karabasanavar et al., 2014). On the other hand, DNA biomarkers, especially the shorter ones, are extraordinarily stable under compromised conditions (Hou et al., 2014; Kitpipit, Sittichan, & Thanakiatkrai, 2014). A myriad of DNA-based assays including species-specific PCR (Karabasanavar et al., 2014), PCR-RFLP (Ali, Hashim, Mustafa, & Che Man, 2011), PCR product sequencing (Ali et al., 2013), real-time PCR (Kesmen et al., 2013) and DNA barcoding (Di Pinto et al., 2013) have been documented for meat species authentication. Species-specific PCR seems to be the best and is considered as a robust method in comparison with other methods such as single nucleotide polymorphism (SNP) analysis, PCR-RFLP, PCR-RAPD and DNA barcoding (Ali et al., 2014; Ballin, 2010; Bottero & Dalmasso, 2011; Karabasanavar et al., 2014).

Carefully designed species-specific PCR under optimized conditions is conclusive to detect and identify species, eliminating the need of restriction digestion and/or sequencing of PCR products (Karabasanavar et al., 2014; Rodriguez et al., 2004). However, compared to conventional single-species PCR systems, multiplex PCR assays with species-specific primers are greatly promising since they offer multiple target detection in a single assay platform, reducing both cost and time (Ali et al., 2014; Bottero & Dalmasso, 2011; Matsunaga et al., 1999; Zha, Xing, & Yang, 2011). Although, several multiplex PCR assays have been documented for the identification of various animal species (Dalmasso et al., 2004; Di Pinto, Forte, Conversano, & Tantillo, 2005; Matsunaga et al., 1999; Zhang, 2013), none of them has been aimed at the authentication of prohibited species in Islamic foods. For the first time, we developed here a multiplex PCR assay for the detection of five haram meat species, namely, pig, dog, cat, monkey and rat species in raw and processed meats and commercial meat products such as meatballs.

## 2. Materials and methods

### 2.1. Sample collection

Among the five target meat species (dog: *Canis lupus familiaris*, cat: *Felis catus*, rat: *Rattus rattus*, pork: *Sus scrofa*, and Monkey: *Macaca fascicularis*) dog, cat and rat meats were collected in triplicates from the Faculty of Veterinary Science, University of Putra Malaysia and Dewan Bandaraya Kuala Lumpur, Malaysia. Monkey meats from three different monkeys were obtained from Wildlife Malaysia. Pork was purchased in triplicates from three different vendors from Chinese wet market in Seri Kambangan, Selangor, Malaysia. The most commonly used commercial meat (beef (*Bos taurus*), chicken (*Gallus gallus*), goat (*Capra hiscus*), lamb (*Ovis aries*), buffalo (*Bubalus bubalis*), venison (*Odocoileus virginianus*), duck (*Anas platyrhychos*), pigeon (*Columba livia*), and quail (*Coturnix coturnix*)), expensive fish species (salmon (*Salmo salar*), cod (*Gadus morhua*), tuna (*Thunnus orientalis*), carp (*Cyprinus carpio*), rohu (*Labeo rohita*), and tilapia (*Oreochromis niloticus*)) and five different halal branded meatballs were purchased in triplicates on three different days from the various wet and supermarkets across Malaysia. All meat samples and products were transported under ice-chilled condition (4 °C) and stored frozen at −20 °C until use to prevent natural and enzymatic decompositions of meats and DNAs.

### 2.2. Meatball preparation

Three types of meatballs were prepared following Rahman et al. (2014) and Rohman et al. (2011). First category beef meatballs were prepared by spiking 1% of each target species separately and the second type included all target meat species to make a total of 5% adulterated beef meatball and the third category was made including only with five target meats. Each meatball was made using 90% emulsified ground meat/meats (target meat species and beef) and 10% of starch mixed with relevant amount of salts and spices such as pepper, garlic, onion etc. (Table 2). Prepared meatballs were subjected to autoclave at 121 °C under 45-psi pressure for 2.5 h to simulate extensive cooking and boiling effects. All samples were prepared in triplicate on three different days by three independent analysts and were stored at −20 °C prior to DNA extraction.

### 2.3. DNA extraction

Total DNA of all meat and fish samples were extracted using Yeastern Genomic DNA Mini Kit (Yeastern Biotech Co., Ltd., Taipei, Taiwan) from 20 mg of muscle tissues following manufacturer's instruction. DNA from model and commercial meat products was extracted from 50 mg specimen. Firstly, sample was grinded with Micropestle in a 1.5 ml micro-centrifuge tube to make a pulp followed by the addition of 20 µl of Proteinase K. The mixture incubated at 60 °C for 30 min to lyse the sample. After adding 400 µl of lysis buffer, sample mixture was incubated again at 60 °C for 20 min to ensure the clarity of sample lysate. Subsequent steps followed the instructions given by the kit manufacturer (Yeastern Biotech Co., Ltd., Taipei, Taiwan). DNA from plant species (wheat (*Triticum aestivum*), tomato (*Solanum lycopersicum*), garlic (*Allium sativum*), onion (*Allium cepa*), and pepper (*Capsicum annuum*)) was extracted using CTAB method according to Rahman et al. (2014). Concentration and purity of the extracted DNA was determined by UV–Vis Spectrophotometer (Libra S80, Biochrom Ltd., Cambridge, England).

### 2.4. Design of species-specific primers

Species-specific primers were designed by targeting mitochondrial genes since they are well protected by mitochondrial membrane, maternally inherited and presence in multiple copies per cell (Xin, Yue-Hui, & Hong, 2006). Among the mitochondrial genes, *NADH dehydrogenase subunit 5* (*ND5*) and *ATPase subunit 6* offer appropriate target length, sufficient degree of intra-species conserved regions and interspecies polymorphism, and available sequence database for most animals and plants (da Fonseca, Johnson, O'Brien, Ramos, & Antunes, 2008; Kitpipit et al., 2014). On the other hand, moderate evolutionary rate and clear evolutionary patterns have made cytochrome b (cytb) gene a suitable candidate to study phylogenetic evolution at the intra- and inter-species levels and target for specific primers and probes (Brown, George, & Wilson, 1979; Xin et al., 2006). These features grew our interest to design species-specific primers targeting *ND5* gene for pig and monkey, *ATPase 6* for dog and rat, and *cytochrome b* for cat species. The whole genome sequences of pig (AF034253.1), monkey (FJ906803.1), dog (NC_002008.4), rat (NC_012374.1) and cat (NC_001700.1), were retrieved form NCBI database (http://www.ncbi.nlm.nih.gov/) and were aligned using ClustalW sequence alignment tool (Thompson, Higgins, & Gibson, 1994) to select the inter-species hyper-variable and intra-species conserved regions. Thus found regions of the noted gene sites were used to design species-specific primer pairs for pig [Forward: (5′-CCATCCCAATTA TAATATCCAACTC-3′) and reverse (5′-TGATTATTTCTTGGCCTGTGT GT-3′)]; monkey [Forward (5′-TG AGACCTCCAACAAATACTAGC-3′)