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Polymerase chain reaction assay targeting cytochrome b gene for the detection of dog meat adulteration in meatball formulation

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ABSTRACT

A polymerase chain reaction (PCR) assay for the assessment of dog meat adulteration in meatballs was developed. The assay selectively amplified a 100-bp region of canine mitochondrial cytochrome b gene from pure, raw, processed and mixed backgrounds. The specificity of the assay was tested against 11 animals and 3 plants species, commonly available for meatball formulation. The stability of the assay was proven under extensively autoclaving conditions that breakdown target DNA. A blind test from ready to eat chicken and beef meatballs showed that the assay can repeatedly detect 0.2% canine meat tissues under complex matrices using 0.04 ng of dog DNA extracted from differentially treated meatballs. The simplicity, stability and sensitivity of the assay suggested that it could be used in halal food industry for the authentication of canine derivatives in processed foods.

1. Introduction

The prospects for Halal meats and meats products are rapidly expanding (Ali, Hashim, Dhahi, Mustafa, Man, et al., 2012). Currently, halal food consuming population has reached to 1.8 billion and the turnover of halal market has exceeded USD 661 billion (Ali, Kashif, et al., 2012). Several factors including increasing workloads are pushing potential halal consumers to spend more time in workplaces, leaving no time for self-cooking. Thus a growing number of people are increasingly being forced to eat readymade foods such as burger, pizza, hot dogs, meatball, soups and so on (Ali, Kashif, et al., 2012). Due to the specialized preparation of halal meats, the prices for halal brands are higher, especially in those countries where Muslims are the minority. Thus the fraudulent labelling of "halal" brands is prevalent (Ali, Hashim, Mustafa & Che Man, 2012). To cope up with the situation demands and business requirements, many countries including Malaysia, Indonesia, Thailand, Singapore, China, Brazil, Australia and Newzealand are having regulatory bodies to protect the sanctity of Halal food markets (Ali, Kashif, et al., 2012). Thus the food manufacturers, marketers and regulators need innovative, easily performable and improved authentication techniques for the verification of halal brands.

Meatballs made up with comminute meats are very popular throughout the world including Malaysia, Indonesia, China, Vietnam, India, the USA and the Europe (Ali, Hashim, Mustafa, Che Man, Dhahi, et al., 2012; Rohman, Sismindari, Erwanto, & Che Man, 2011). Dog meat is a potential adulterant in halal foods since stray dogs are available in many countries without any offered prices. Reports have been made for the consumption of dog meats in certain countries such as Vietnam, South Korea and China (Bartlett & Clifton, 2003; Podberscek, 2009). Foreign workers, especially from Myanmar and Vietnam origins, are reported to consume stray dog meat in Malaysia. However, no survey is made to verify the mixing of dog meats in commercial meat products across Malaysia or elsewhere in the world. The mixing of dog origin materials in food is a serious issue in many religions including Islam and Buddhism (Khattak et al., 2011; Mahanarongchai & Marranca, 2007).

Several methods such as SYBR green real-time PCR (Farrokhi & Jafari Joozani, 2011), molecular beacon real-time PCR (Yusop, Mustafa, Che Man, Omar, & Mokhtar, 2012), TaqMan probe real-time PCR (Ali, Hashim, Dhahi, et al., 2012), electronic nose coupled with gas chromatography–mass spectrometry (Nurjuliana, Che Man, Mat Hashim, & Mohamed, 2011), Fourier transform infrared spectroscopy (Rohman et al., 2011), enzyme-linked immunosorbant assay (Asensio, González, García, & Martin, 2008), PCR-RFLP (Ali, Hashim, Mustafa, & Che Man, 2012) and nanoparticle sensors coupled with optical or fluorescence spectroscopy (Ali et al., 2011) have been proposed for the authentication of meat species. Although cumbersome to some extent, the conventional species specific PCR assay is an easily affordable and reliable method for the routine analysis of animal meat products in food industry (Arslan, Ilhak, & Calicioglu, 2006; Matsunaga et al., 1999). For dog

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meat detection, a total of five PCR based assays have been documented (Abdel-Rahman, El-Saadani, Ashry, & Haggag, 2009; Abdulmawjood, Schönenbrücher, & Bülte, 2003; Gao, Xu, Liang, Zhang, & Zhu, 2004; İlhak & Arslan, 2007; Martín et al., 2007). However, most of them were of longer amplicon length (>150 bp) and none of them was tested under commercial matrices.

In this paper, we have described a short amplicon length conventional PCR assay targeting 100 bp region of cytb gene for the detection of canine tissues in ready to eat chicken and beef meatballs. We tested the assay under various backgrounds and processing conditions and high stability and sensitivity were obtained.

2. Materials and methods

2.1. Sample collections

Meat samples of 9 commonly available animals (chicken, duck, turkey, quail, goat, sheep, beef, buffalo and pig) and 3 plants species (onion, garlic and tomato) were purchased in triplicates from various supermarkets located at Serdang, Petaling Jaya and Kuala Lumpur in Malaysia. The typical fresh dog and cat meats were collected from euthanized stray dogs and cats from Jabatan Kesihatan Dewan Bandaraya Kuala Lumpur (DBKL), Malaysia. The post-mortem dog meat samples were also collected from the Faculty of Veterinary Science in University Putra, Malaysia. Commercial meatballs of five different halal brands were purchased from Serdang, Petaling Jaya and Kuala Lumpur in Malaysia in triplicates on three different days. All the collected samples were transported under ice chilled condition (4 °C) and were stored at −20 °C for further processing and DNA extraction.

2.2. Ternary admixture preparation

To evaluate the performance of the PCR assay for canine meat detection in processed food, ternary admixtures composed of different percentages of dog meats were prepared according to Ali, Hashim, Mustafa, and Che Man (2012). Briefly, to obtain 1%, 0.5%, 0.2%, 0.1%, and 0.01% of dog meat admixtures, dog meat was mixed with chicken meat and wheat flour in the ratio of 2:98:100, 1:99:100, 0.4:99.6:100, 0.2:99.8:100 and 0.02:99.98:100. Finally, hundred millilitres (100 ml) of deionized water was added to the admixtures and vigorously grinded with a blender to obtain a homogenous semi solid-slurry. All admixtures were prepared on three different days by three independent analysts and autoclaved at 120 °C under 45-psi pressure for 2.5 h. Thus prepared samples were kept at −20 °C for further DNA extraction.

2.3. Meatball preparation

Pure meatballs were prepared according to Rohman et al. (2011) with balanced amount of grinded chicken, beef and dog meat with cooking salt, garlic and other ingredients as shown in Table 1. To obtain dog meat contaminated meatballs 1%, 0.5%, 0.2%, 0.1%, and 0.01% of dog meat were added with 100 g of chicken and beef meat in the formulation. The meats with all other ingredients were mixed well by vigorous blending and the emulsified homogenous meat mixtures were mechanically given into ball shape. To simulate cooking and extensive autoclaving effect thus prepared raw meatballs were subjected to cooking at 100 °C for 90 min and autoclaved at 120 °C under 45-psi pressure for 2.5 h. All samples were prepared on three different days by three independent analysts and were stored at −20 °C for DNA extraction.

Table 1
Ingredients used in meatball preparation.

Ingredient	Chicken meatball	Beef meatball	Dog meatball
Minced meat	100 g[a]	100 g[a]	100 g
Breadcrumbs	7.5 g	7.5 g	7.5 g
Chopped onion	5 g	5 g	5 g
Ginger freshly chopped		1.5 g	1.5 g
Cumin powder		1.25 g	1.25 g
Garlic powder	1.25 g	1.25 g	1.25 g
Black pepper	0.14 g		0.14 g
Milk	0.01 l		0.01 l
Butter	3.28 g		3.28 g
Tomato paste		2.5 g	2.5 g
Salt	0.05 g	0.05 g	0.05 g

[a] 1%, 0.5%, 0.2%, 0.1% and 0.01% of dog meat were mixed with a balanced amount of chicken and beef meat to make 100 g specimen of each meatball meat.

2.4. DNA extraction

DNA was extracted from 25 mg of raw and treated meat samples using NucleoSpin® Tissue DNA extraction kit (Macherey-Nagel, Germany) following manufacturer's instructions. DNA was extracted from plants, admixed and commercial samples from 100 mg specimen using the CTAB method and subsequent purification was performed using Promega Wizard™ DNA isolation kit (Promega Corporation, Madison, USA). Extracted DNA was analyzed by gel image capturing after running the total DNA in 1% agarose gel containing 1 μg/ml ethidium bromide in 0.5% Tris Borate buffer (TBE) for 45 min at 100 volt. The concentration and purity of DNA were determined using a spectrophotometer (Biochrom Libra S80—Cambridge, England).

2.5. Canine specific primer design

The hyper variable region of the dog cytb gene (Dog: JF489119.1) was identified through alignment analysis with the cytb genes of 8 common halal meat species (Chicken: EU839454.1, Turkey: HQ122602.1, Duck: HQ122601.1, Quail: EU839461.1 Beef: EU807948.1, Buffalo: D32193, Sheep: EU365990.1, Goat: EU130780.1), 2 non halal meat species (Pig: GU135837.1, Cat: AB194817.1) and apocytochrome b (cob) gene of 3 plant species (Tomato: XM004251454.1, Garlic: AF356823.1, Onion: GU253304.1) using mega 5 software (Tamura et al., 2011) and clustalW alignment tool (Thompson, Higgins, & Gibson, 1994). Thus found hyper variable regions were used to design a pair of canine specific primers (Forward 5′ CCTTACTAGGAGTATGCTTG 3′ and Reverse: 5′ TGGGTGACT GATGAAAAAG 3′) using primer3plus software (www.bioinformatics.nl/cgi-bin/primer3 plus/primer3plus.cgi). The canine specificity of the designed primers was theoretically confirmed through "BLAST" analysis in NCBI data base (http://www.ncbi.nlm.nih.gov/blast). Primers were purchased from the 1st BASE Laboratories Pte Ltd (Selangor, Malaysia).

2.6. PCR assay optimization

PCR assay was run in a gradient thermocycler (Eppendorf, Germany), using 20 μl of reaction mixture composed of 1x PCR master mix (Promega, Promega Corporation, Madison, USA) containing 50 units/ml of Taq DNA polymerase (supplied in a proprietary reaction buffer pH 8, 400 μM each dATP, dGTP, dCTP, dTTP and 3 mM MgCl2), 100 nM of each primer and 20 ng of total DNA. PCR cycling was done using an initial denaturation at 94 °C for 3 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 5 min. The separation of PCR products was performed in 1% agarose gel (Promega, Madison, USA) in 1x LB buffer of pH 8.0 at a constant voltage of 170 V for 15–20 min, pre-stained with 6x loading dye and using a 100 bp DNA ladder (Fermentas, USA) as reference standard. PCR product was visualized on ethidium bromide stained agarose gel using a gel image documentation system (AlphaImager HP; California, USA).

2.7. Pair wise distance and phylogenetic tree

For pair wise distance and phylogenetic analysis, sequencing results obtained from 100 bp PCR product were aligned with the retrieved cytb/