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SIMULTANEOUS DETECTION OF THREE FORBIDDEN ANIMALS (PORCINE, CANINE, AND RAT) IN HALAL FOOD BY USING HIGH RESOLUTION MELTING ANALYSIS

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Abstract

Halal food approved for Muslim consumers is strictly noticed that avoiding from contaminations that do contradict from Islamic regulations, such as the contamination of animal forbidden by Halal standards. Importantly, High resolution melting analysis (HRMA) is a potential molecular technique, which is used for identifying the species of organisms. Therefore, the research objective is to use the HRMA technique for simultaneous detection of the three forbidden animals (pigs, dogs, and rats) that have high opportunity to be adulterated in Halal food. The HRMA, targeting a fragment of NADH dehydrogenase (ND5), ATPase subunit 6 (ATP6), and Cytochrome B (Cytb) genes, were developed in order to authenticate the forbidden animal and their mixtures. Species-specific primers were designed and combined in a multiplex HRMA resulting in different sequences and therefore different melting behaviours for each species. The multiplex HRMA was then evaluated the PCR specificity against the targeted DNAs of targeted and non-targeted. It's demonstrated that the method had no cross-reaction with DNA from the experimental animal species (pigs, dogs, rats, cats, monkeys, chickens, horses, sheep, goats, and donkeys). The HRMA profile of amplified amplicons from the targeted animals produced uniquely melting peaks that were easily distinguished for each species in this study. Taken together, all data indicates that this multiplex HRMA is a simple, fast, specific, and cost-effective detection method for pig, dog, and rat in halal food. In order to carry out the analysis of commercial food products, 150 commercial food products was used to screen by species-specific primers for pigs, dogs, and rat were combined in multiplex HRMA. This method revealed that one sample was contaminated with pigs' DNA in the examined products. Therefore, the HRMA could be used as a halal verification technique for detecting aforementioned forbidden animals contaminated in halal food products.

Key words*: Halal food, Forbidden animal, Multiplex HRMA, species identification, Authentication.*

INTRODUCTION

"Halal" in an Arabic word means "permit" or "lawful" according to Islamic regulations, not only for food but non-foods and services as well.

Therefore, Muslims or the believers in Islam shall consume only Halal products and services and avoid "Haram" or "Forbidden" products.

Consuming Haram products would affect the faith and spirit of Muslims.

According to annually announced in coming 2050, the world population will continuously increase up to 9,000 million, which means resulting in increasing Muslim populations as well (Nakyinsige et al., 2012).

Besides, the world halal food industry exhibits high value of economics since Halal marketing is merely value of goods and services which have rapidly grown on demand and high consumer based that expanded gradually (Jafari and Scott, 2014).

Despite Thailand amongst the major food producers exporting varieties of food products to the world as well as to Muslim countries, almost all food manufacturing proprietors and producers are non-Muslims.

No matter how strict they comply with international food safety standards, the spiritual safety to protect faith and belief of Muslims has yet to be widely and clearly understood and often overlooked, which might result in

contaminating of forbidden composition regarding Islamic regulations from misunderstood or intended to produce halal products especially in meat processing industries (Dahlan, 2013).

From Islamic regulations, it indicates that various kinds of forbidden animals would not appear in Halal food such as pigs, dogs, rats, cats, reptiles, and others. The recently survey found that these animals have possibly mix and adulterated with qualified halal food to decrease the processing cost, which has occurred found in Thailand and other countries such as Vietnam, Indonesia and China (Dahlan, 2013; Ali et al., 2015).

Therefore, rapid and reliable methods is needed for these forbidden species identification. DNA-based PCR methods have become widely used to identify meat species authentication. As a result of high stability under high temperatures, pressures, and chemical treatments of DNA. It can be investigated on raw meat or cooked meat (You et al, 2013). High resolution melting analysis (HRMA) is one of DNA-based PCR method that allows genotyping and fingerprinting by discrimination separation DNA sequence variant such as single nucleotide polymorphisms (SNPs) and small insertion and deletions (indels) based on the shape of melting transitions (Tm) of real-time PCR. This method can also be applied for screening for the existence of unknown sequence variations without a sequencing process. However, simplex HRMA is laborious, expensive, and complex. To reduce cost and increase the speed of HRMA, a Multiplex HRMA was developed (Druml and Cichna, 2014; Iacumin et al, 2015). In this study we aim to apply the Multiplex HRMA for simultaneous detection of the three forbidden animals composed with pigs, dogs, and rats that have high opportunity to be adulterated in Halal food.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

Among the three target forbidden animals pigs (n=4), dogs (n=4), and rats (n=4). Porks were collected from Samyan market in Phatumwan district, Bangkok, Thailand. Dog meats were collected from the Faculty of Veterinary Science, Chulalongkorn University, while rat meats were collected from Taladthai market Phatumthani province, Thailand. Meats and whole bloods from other species (cats, monkeys, donkeys, horses, chickens, sheep, and goats) were collected from various markets, zoological park organization of Thailand and the Faculty of Veterinary Science, Chulalongkorn University. Then, all samples were DNA extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Finally, the extracted DNA concentrations were examined by a Nanodrop 2000 (ThermoScientific, USA). The quantified DNAs were stored at -20 °C until use.

Multiplex HRMA

All three of species-specific primer pairs used in this study are listed in (Table 1). Verification of the specificity of each primer pair was performed using NCBI BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

To develop technique, both simplex and multiplex HRMA were carried out by using a LightCycler®480 instrument (Roche, Germany).

In a total volume of 20 µL containing 20 ng of genomic DNA, 10 µL 2XQIAGEN Multiplex PCR (Qiagen, Hilden, Germany) 0.2 µM of forward and reverse primer, and 1x LightCycler ResoLight dye. The amplification condition was 1 cycle of 5 min at 94°C; 29 cycles of 30 sec at 94°C; 40 sec at 56°C; 1 min at 72°C followed by HRMA ramping from 60°C to 99°C with 50 acquisitions. PCR products were analyzed by QIAxcel Capillary Electrophoresis system (Qiagen, USA).

The HRMA developed in this study was validated for its specificity and reliability. In terms of the specificity test, the assay was cross tested with all three target species and seven other animals (cats, monkeys, donkeys, horses, chickens, sheep, and goats).

Finally, for reliability (real-world performance testing), the developed assay was used to test 150 Halal food products, including 50 meat processing products, 30 dairy products, 20 seasonings, 20 snacks, and 30 bakery products.

Table 1. Details of primer sequence, PCR product size, and Tm (C^o)

Species	Primer	Sequence (5' to 3')	Product size	Tm (C^o)	References
Porcine	ND5-For ND5-Rev	5'-GGCGCCATCCCAATTATAATATCCAACTC-3' 5'-TGATTATTTCTTGGCCTGTGTGT-3'	144bp	81.50-81.57	Ali et al., 2015
Dog	ATP6-For ATP6-Rev	5'GAGGTGCGGAAGCGGAGGGGCGGGGGCTCT AGCCGTTCGAT -3' 5'-GTGATAAAAGCTGTGGTCG -3'	138bp	85.71-85.83	In this study
Rat	Cytb-For Ctyb-Rev	5'-TAACCACTCCTTCATCGACCTT -3' 5'-CCCCGTTGGCGTGTAAATA -3'	218bp	83.49-83.50	Yanita.I.W et al., 2015

Table 2. Cross reaction of the HRMA (*n=4), (+) amplification, (-) no amplification

Species*	Scientific name	Species specific primer		
		Pig-ND5	Dog-ATP6	Rat-Cytb
pig	*Sus scrofa*	+	-	-
Dog	*Canis lupus familiaris*	-	+	-
Rat	*Rattus argentiventer*	-	-	+
Cat	*Felis catus*	-	-	-
Monkey	*Macaca fascicularis*	-	-	-
Donkey	*Equus asinus*	-	-	-
Horse	*Equus caballus*	-	-	-
Chicken	*Gallus gallus*	-	-	-
Sheep	*Ovis aries*	-	-	-
Goat	*Capra aegagrus*	-	-	-

RESULTS AND DISCUSSIONS

Halal food safety has now become an important issue not only for food producers but also for consumers who desire and concern to quality food that obedience to health, religion, and fair prices (Rahman et al., 2014). Many researches have been reported that the halal food products collected from market surveillance were adulterated with forbidden meats such as pork, dogs, rats, monkeys, and others (Cai et al.,2012 ; Dahlan, 2014; Kitpipit et al., 2014 ; Ali et al., 2015 ; Yanita.I.W et al., 2015). HRMA is a molecular method that allows detecting and differentiating DNA amplicon by discriminating DNA sequence variants such as single nucleotide polymorphisms (SNPs) and small insertion and deletions (indels) based on the specific melting behaviour of the DNA amplicon (Sakaridis et al., 2013). This method can be applied to detect adulteration of forbidden animals DNA contaminated in food products by evaluating different melting behaviours of each forbidden animal species. To save cost and reduce the time, in this study, The multiplex HRMA was conducted by employing 40 DNAs from 10 animals (pigs, dogs, rats, cats, monkeys, donkeys, horses, chickens, sheep, and goats) (Table 2). Moreover, three pair of species-specific primers targeting the intra-species conserved and inter-species hyper variable regions of mitochondrial ND5, ATP6 and Cytb gene were used to set up multiplex HRMA. Importantly, each set of primers was firstly evaluated the PCR specificity against the targeted DNAs of all animals. Each pair of primers was tested against its targeted forbidden animals with the other species. The results showed that only specific target was testily amplified (Table 2). The HRMA profiles of amplified amplicons from targeted animals were also assessed in this study, the raw melting curve data to form a normalized melting curve and melting peaks. In this study, the method highlighted uniquely melting curves and melting peaks that were easily distinguished for each species. To be illustrated, all species were distinguished by their species-specific melting curves and melting peaks. Generally, it can be seen that different genotypes have their own unique transitions that are merely shown by their HRMA profile (Figure 1). The accuracy of method was investigated in the testable conductivity in all assays were completely done in forty tested DNAs. The results signify-cantly indicated that no cross amplification even on repetition in blind experiment and showed 100% accuracy (Figure 2).

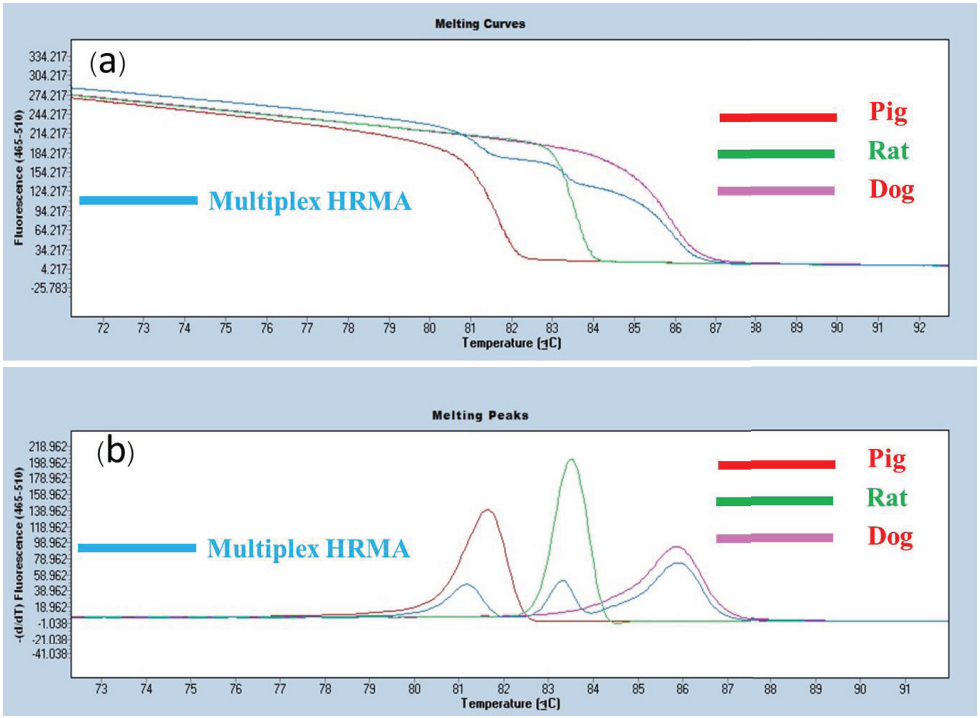

Figure 1. The HRMA profiles of amplified amplicons from species targeted animals:
a normalized melting curve (a) and a melting peak (b)

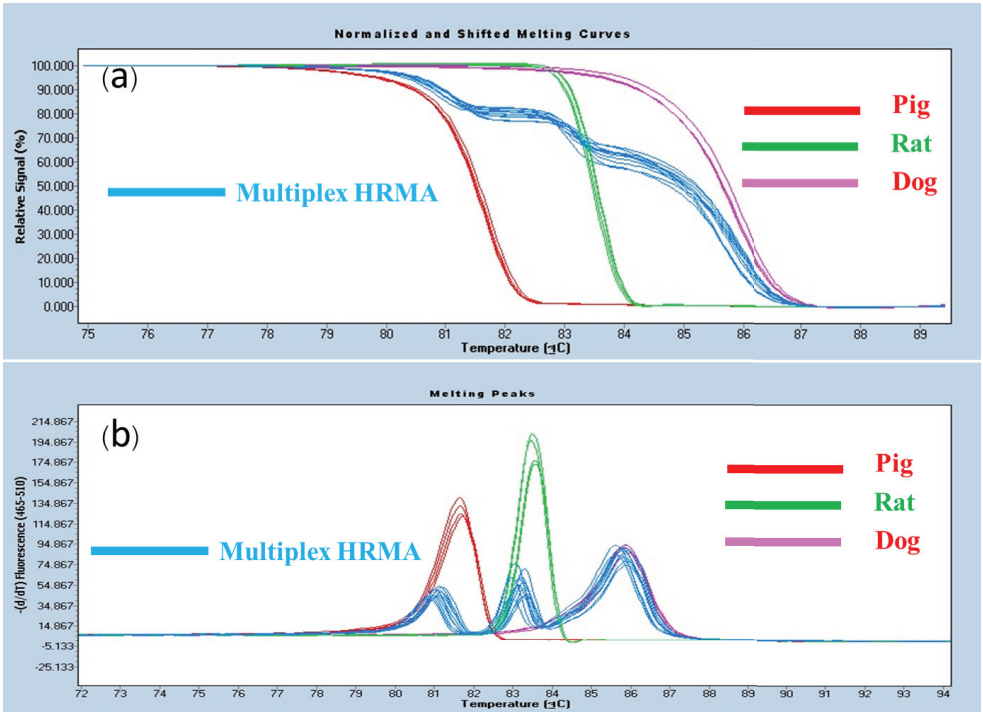

Figure 2. The HRMA profiles of forty blind experimental animals;
a normalized melting curve (a) and a melting peak (b). .

This revealed that there was high specificity of the established primers. In order to carry out the analysis of commercial food products, 150 commercial food products was used to screen by the developed multiplex HRMA.

The results indicated that one sample was contaminated with pigs' DNA, suggesting its usefulness for detection of pigs, dogs and rats. Therefore, the HRMA method could be used as a halal verifycation technique for detecting aforementioned forbidden animals contaminated in halal food products. Furthermore, the HRMA assay ultimately showed that it is simple, cheap, and rapid method, the cost per sample is comparatively lower than CE analysis (Ulca, 2015; Ali et al., 2015).

CONCLUSIONS

The HRMA method was scientifically developed in this study and it had been specifically proven in specificity and reliability for simultaneous detection of pig, dog, and rat DNA standards. The assay was also successfully validated to detect these three forbidden animal species in halal food products. Therefore, it could be potentially applied as a simple and rapid tool for halal verification technique to detect the forbidden contamination in halal food manufacturing.

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