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Rapid, Sensitive and Label-Free Multiplex Meat Speciation Kit

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ABSTRACT

A label-free, one-step multiplex high-resolution melting (HRM) assay has been developed for the identification of five different meat species namely beef, pork, sheep, chicken, and duck. HRM involves PCR amplification of the region of interest in the presence of double-stranded DNA binding dye followed by melting at high resolution. The HRM assay is rapid, sensitive, cost-effective and allows simultaneous detection of multiple species in meat and meat products.

Keywords: Meat speciation; Halal; Beef; Pork; Chicken; Lamb; Duck; Multiplex PCR

1 Introduction

Porcine derivatives used in the meat processing industry include pork fat (lard), mechanically recovered meats (MRM), porcine gelatine and porcine blood plasma. Consumption of porcine derivatives is prohibited according to the Islamic law. The identity and authentication of ingredients in processed or composite mixtures have emanated into the appointment or formation of credible halal certification bodies [1]. Currently, many efforts have been made to develop more effective halal-authentication detection systems. However, there is no discernible trend in the methodologies employed for the verification of halal food from 1980 to 2021. Protein-based techniques have some limitations. They are limited when assaying heat-treated products due to the denaturation of proteins during thermal processing. Additionally, analyses of immunoassays, which rely on the use of antibodies raised against a specific protein, are often hindered by cross-reactions occurring among closely related species [2]. For these reasons, protein-based methods have been replaced by DNA-based ones. The PCR is an exceptionally sensitive method, but it requires post-PCR processing i.e., gel electrophoresis etc. Real-Time PCR methods require Taqman probes which are expensive. Moreover, these methods are limited to the detection of one pathogen at a time and require repeated and/or multiple tests for the detection of different pathogens.

Therefore, the present study aims to develop a simple and label-free detection method for the simultaneous detection of five different meats species namely pork, beef, chicken, duck, and lamb using multiplex PCR followed by high resolution melting (HRM) profile for detection.

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2 Methods

2.1 Primer Design and PCR

The target region of the amplicon and primers for the detection of chicken, duck, pork, sheep, and beef were essentially similar to the one described in the published reports [3-5] with modifications required to meet desired melting point (Tm). The modified primers were subjected to thorough scrutiny for specificity using BLAST analysis. The thermal cycling conditions are as follow: 3 mins of initial denaturation at 95˚C, followed by 30 cycles of 20sec of denaturation at 95˚C, 20sec of annealing at 55 ˚C, 30sec of extension at 72˚C and lastly 3 mins of final extension at 72˚C. Amplification was carried out in Applied Biosystems GeneAmp ® PCR System 9700.

2.2 Cloning of pGEM plasmid constructs

The target region of the DNA to be used for chicken, duck, pork, sheep, and beef was cloned into pGEM-T Easy Vector (Promega) following the manufacturer's protocol. The clones were confirmed by restriction digestion with Eco RI and by sequencing and used in further experiments.

2.3 Multiplex PCR and HRM Assay

The multiplex (5-plex) PCR reaction mixture contained plasmid templates of all 5 pathogens at a concentration of 30 to 3000 copies per reaction. The Rotor-Gene Q (Qiagen) was programmed to continue the melting of PCR products at a resolution of 0.02˚C between 70 and 90˚C after the completion of amplification cycles. Data generated after HRM was analysed using Rotor Gene-Q series software 2.0.2. The plasmids were replaced with genomic DNA after the optimization of the assay. For all the reactions, non-template control (NTC) was also used.

3 Results

3.1 Optimization of PCR conditions

The thermal cycling conditions were optimized to amplify all five different target DNA evenly. The PCR products for chicken, duck, pork, sheep, and beef were amplified efficiently as shown in Figure S1.

3.2 Standardization of HRM profile of individual pathogens

HRM assay of individual meat species was performed followed by PCR. Data generated after HRM was analysed using Rotor Gene-Q series software 2.0.2. The Tm of beef, pork, sheep, chicken, and duck showed Tm as 69.8, 73.7, 76.4, 80.0 and 83.0°C respectively (Figure 1).

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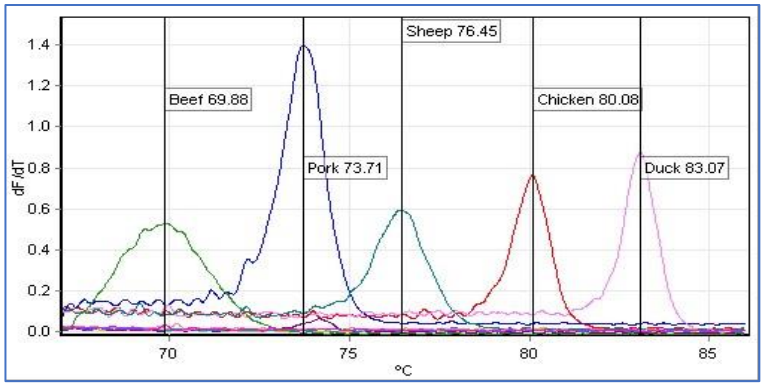

Figure 1: *Composite of melt curve of individual PCR amplicons of beef, pork, sheep, chicken, and duck merged. It can be observed that there is a 3.0°C difference between each profile/curve.*

3.3 Multiplex PCR and HRM

As described above, a 5-plex assay for beef, pork, sheep, chicken, and duck was developed using the plasmid templates and then evaluated with genomic DNA. To retain the Tm difference of 3°C between PCR products of the multiple species, the primers and amplification region underwent several changes and only the final set of data are presented here. Figure 2 presents the 5-plex HRM profile of the five meat species. There is a difference of about 0.3°C in Tm of these PCR products compared to the individual PCR/HRM reactions.

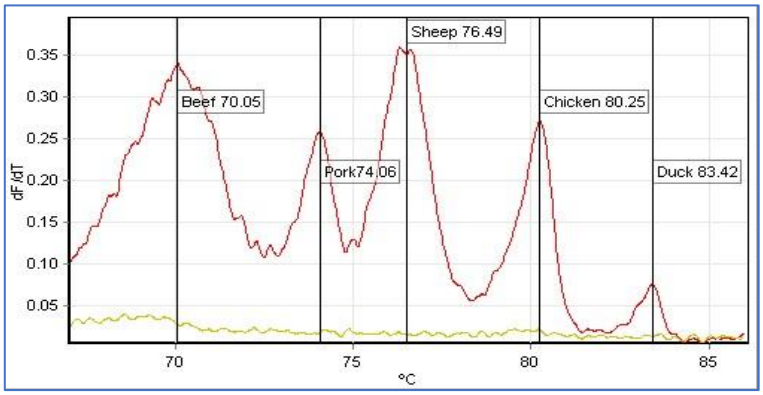

Figure 2: *Melt curve of 5-plex PCR amplicons showing beef (70.0°C), pork (74°C), Sheep (76.4°C) chicken (80.2°C), and duck (83.4°C).*

3.4 Specificity multiplex HRM assay

To examine the specificity and cross-reactivity of the primers, PCR/HRM assays were performed within the presence of all five pairs of primers and with only one of the 5 templates. The results indicated no cross-reactivity between the five different target DNA sequences as the melt curves are specific as designed.

3.5 Sensitivity of multiplex PCR and HRM assay

Our initial real-time qPCR results revealed 3 copies as the limit of detection of individual DNA target. pathogens. To determine the minimum copy number of template DNA required for detection in multiplex conditions, Multiplex PCR followed by HRM experiments were performed by mixing equal copies of all 5 different plasmid templates. The results indicated

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that a minimum of 2000 copies are required for even amplification of all 5 templates. Whereas, in 2-plex and 3-plex, distinguishable melt curves can be seen with low copy numbers (data not shown).

4 Discussion

HRM analysis is a post-PCR analysis method used to identify variations in nucleic acid sequences. The 5-plex HRM assay was initially optimised with plasmid templates followed using genomic DNA templates from the five meat species. The HRM profile/melt curve carried out with plasmid templates showed only minor differences in Tm e.g., amplicons from genomic DNA templates. The slight increase in Tm is likely since the genomic DNA of the meat species used are very long compared to the plasmid templates. It has been demonstrated that melting temperature depends on a variety of factors, such as the length of DNA the nucleotide sequence composition and salt concentration [6].

Patent

The authors have filed a Singapore provisional patent (SG10202011271W).

Supplementary Materials

The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Agarose gel electrophoresis of PCR products resolved in 2% gel; Table S1: title. Sequences of primers used in PCR and HRM assay.

Author Contributions

Conceptualization, A.A, K.P and S.M.; methodology, M.M and T.N; formal analysis, A.A, M.M and T.N.; writing—review and editing, A.A and K.P.; supervision, A.A and K.P.; project administration, A.A. and MM.; funding acquisition, A.A and K.P.

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References

1. Nakyinsige K., Man Y.B., Sazili A.Q. Halal authenticity issues in meat and meat products. *Meat Sci.* **2012**,91(3), pp 207-14.
2. Fajardo V., González G., Rojas M., García T. and Martín R. A review of current PCR-based methodologies for the authentication of meats from game animal species. *Trends in Food Science & Technology* **2010**, 21 (8), pp 408-421, https://doi.org/10.1016/j.tifs.2010.06.002.
3. Tanabe S., Hase M., Yano T., Sato M., Fujimura T. and Akiyama H. A real-time quantitative PCR detection method for pork, chicken, beef, mutton, and horseflesh in foods. *Bioscience, Biotechnology, and Biochemistry*, **2007**, 71 (12), pp 3131 – 3135.
4. Singh V.P., Pathak V., Nayak N.K., Verma A.K. and Umaraw P. Recent developments in meat species speciation-a review, *Livestock Science*, **2014**, 5 pp. 49-64.

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5. Amaral J.S., Santos C.G., Melo V.S., Costa J., Oliveira M.B.P. and Mafra I. Identification of duck, partridge, pheasant, quail, chicken and turkey meats by species-specific PCR assays to assess the authenticity of traditional game meat Alheira sausages, *Food Control,* **2015**, 47, pp. 190-195.
6. Khandelwal G., Bhyravabhotla J. A phenomenological model for predicting melting temperatures of DNA sequences. *PLoS One*. **2010** ,26;5(8), e12433.

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