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SENSITIVE AND RAPID MODELS OF PCR-BASED METHODOLOGIES FOR ADMIXTURE DETECTION AND QUANTIFICATION OF DOMESTIC MEATS

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By

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SENSITIVE AND RAPID MODELS OF PCR-BASED METHODOLOGIES FOR ADMIXTURE DETECTION AND QUANTIFICATION OF DOMESTIC MEATS

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February 2016

Chair : Mohd Nasir Mohd Desa, PhD
Faculty : Halal Products Research Institute

This research project was undertaken to develop cost-effective, fast and sensitive PCR-based methods for fraud identification of meat-based products. Meats and meat-based product samples included in the analyses were pig, ruminant, avian and rabbit. Two methods were established for qualitative and quantitative evaluation of animal group contamination/ admixture in meat-based products. In this study, *NADH-dehyrogenase subunit 4* (*Nad 4*) gene of mitochondrial was used. *Nad 4* gene holds great potential for identifying meat type due to high interspecies variability of the gene sequence. To date, very little attention was given in meat identification using *Nad 4* gene. To further recognize the potentially important of *Nad 4* gene, the sequence variability has been explored in identifying differences in the designated meat types.

For qualitative analysis, a common primer multiplex PCR (CP-M-PCR) was developed by using a common forward primer, species-specific adapter reverse primers and a common adapter reverse primer.The designed primers were analyzed *in silico* and tested against pure meat DNAs. The primers successfully generated specific fragments of 267, 370, 504, and 548 bp lengths for pig, ruminant, avian and rabbit meats, respectively. A serial dilution of each reverse primer was used to determine and compare the sensitivity of CP-M-PCR to conventional multiplex PCR system. The detection limit of CP-M-PCR was evaluated with 10-fold serial dilutions of DNA concentration. The use of adapter sequence at the 5'-end of the species-specific reverse primers was shown to increase the efficiency of the PCR amplification and the application of a single forward primer reduced the complexity in multiplex PCR system. Bands of specific amplification can be detected from the PCR assays containing as low as 10^{-6} µM of adapter reverse primer. The CP-M-PCR limit of detection was as low as 0.01 ng of DNA for the four groups of meat which was deemed to be sufficient to qualitatively detecting accidental or intended contamination in meat products. The developed system was applied to 42 commercial meat-products and showed the presence of avian meat in analyzed ruminant (3/14), rabbit (2/2) and pig (1/11) samples.

In the subsequent work, a quantitative competitive PCR (QC-PCR) was developed to determine the percentage of contamination. Based on the CP-M-PCR results, six avian contaminated commercial meat-based products were quantitatively analysed using the calibrated QC-PCR. Prior to quantitative analysis, a competitor DNA of each animal meat group was constructed *via* site directed-mutagenesis processes. Site directed-mutagenesis was used to introduce a 40 bp fragment comprising a 30 bp insert and 10 bp repeated sequence. The constructed DNA competitors were coamplified with the target DNA at different ratios of concentration and results were analyzed using UVIgeltec imaging software version 12.1. The results of QC-PCR showed that the percentage of contamination was in the range of 0.27 to 5.08% in the collected samples. The developed methods in this study are considered practical and valid alternatives in the context of a routine diagnostic laboratory where they are cost-effective, efficient, and sensitive enough for qualitative and quantitative investigation of contamination or adulteration in meat products. The developed approaches are cost-effective because theydo not require expensive equipment, consumable and reagent, and high level of technical expertise to perform the analyses. Thus, the models may serve as templates for any screening system such typing of transgenic organisms, analyzing of forensic materials, detection of pathogens to cancer research, studying metagenomics, analyzing gene expression and monitoring environment.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

MODEL-MODEL YANG SENSITIF DAN PANTAS BERSANDARKAN KAEDAH PCR UNTUK PENGESANAN DAN PENGKUANTITIAN CAMPURAN PALSU DI DALAM DAGING TEMPATAN

Oleh

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Pengerusi : Mohd Nasir Mohd Desa, PhD
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Projek penyelidikan ini dijalankan untuk membina kaedah-kaedah yang menjimatkan, cepat dan sensitive untuk mengenal pasti kandungan daging tidak asli di dalam produk berasaskan daging. Sampel daging dan produk berasaskan daging yang digunakan di dalam analisis ini adalah terdiri daripada babi, ruminant, avian dan arnab. Dua kaedah telah dibangunkan untuk evaluasi kontaminasi/ percampuran secara kualitatif dan kuantitatif oleh kumpulan haiwan tersebut di dalam produk berasaskan daging. Di dalam kajian ini, gen mitokondria *NADH dehidrogenase subunit 4* (*Nad 4*) telah digunakan. Gen *Nad 4* mempunyai potensi yang besar untuk pengecaman jenis daging berikutan variasi interspesis yang terdapat di dalam jujukan gennya. Sehingga kini, tidak banyak perhatian yang diberikan terhadap gen *Nad 4*. Untuk lebih memperlihatkankepentingan potensi gen *Nad 4*, variasi jujukannya diterokai untuk mengenal pasti perbezaan-perbezaan yang terdapat di kalangan jenis daging.

Untuk analisis kualitatif, 'common primer multiplex PCR' (CP-M-PCR) telah dibangunkan dengan menggunakan primer forward sepunya, primer-primer reverse adapter spesis-spesifik dan primer reverse adapter. Primer-primer yang dicipta telah dianalisa secara *in siliko* dan diuji ke atas DNA daripada daging-daging yang asli. Primer-primer tersebut telah menghasilkan fragmen bersaiz 267, 370, 594 dan 548 bp untuk setiap kumpulan daging babi, ruminant, avian dan arnab. Pencairan bersiri primer telah digunakan untuk mengetahui dan membandingkan sensitiviti CP-M-PCR dengan sistem konvensional multiplek PCR. Had kemampuan pengesanan bagi CP-M-PCR telah dievaluasi melalui pencairan campuran DNA secara bersiri dalam gandaan 10. Aplikasi jujukan adapter pada hujung 5' primer-primer reverse adapter spesis-spesifik menunjukkan peningkatan efisiensi amplifikasi PCR dan penggunaan primer forward sepunya telah mengurangkan kompleksiti di dalam sistem PCR multiplek. Jalur-jalur terhasil daripada amplifikasi yang spesifik boleh dikesan daripada esei PCR yang mengandungi serendah 0.01 ng DNA untuk keempat-empat kumpulan daging tersebut di mana ianya mencukupi untuk mengesan secara kualitatif kontaminasi di dalam produk daging. Sistem yang dibangunkan ini telah diaplikasikan ke atas 42 produk

komersil daging dan menunjukkan kehadiran daging avian di dalam sampel ruminan (3/14), arnab (2/3) dan babi (1/11).

Kajian seterusnya, 'quantitatif competitive PCR' (QC-PCR) telah dibangunkan untuk mengesan peratus kontaminasi tersebut. Berdasarkan keputusan CP-M-PCR, enam produk daging yang dicemari oleh daging avian telah di analisa secara kuantitatif menggunakan QC-PCR yang telah dikalibrasi. Sebelum menjalankan analisis kuantitatif tersebut, DNA pesaing bagi setiap kumpulan daging haiwan telah dikonstruk melalui proses 'site-directed mutagenesis'. Proses ini menghasilkan fragmen bersaiz 40 bp yang terdiri daripad 30 bp jujukan tambahan dan 10 bp jujukan berulang. DNA pesaing yang telah dikonstruk tersebut telah di amplifikasi bersama-sama DNA target pada nisbah kepekatan berbeza dan hasil keputusan telah dianalisa menggunakan software pengimejan UVIgeltec versi 12.1. Hasil menunjukkan peratus kontaminasi adalah di dalam lingkungan 0.27-5.08% di dalam sampel-sampel yang diuji. Kaedah-kaedah yang dibangunkan di dalam kajian ini dianggap sebagai alternatif yang praktikal dan boleh diguna pakai di dalam konteks makmal diagnosis rutin di mana kedua-duanya adalah cukup murah, efisien dan sensitive untuk penyiasatan kontaminasi atau percampuran secara kualitatif dan kuantitatif di dalam produk daging. Pendekatan-pendekatan yang digunakan adalah murah kerana tidak memerlukan kepada peralatan, bahan dan reagen yang mahal, sertakemahiran teknikal yang tinggi untuk menjalankan analisa. Oleh demikian, model-model ini boleh menjadi templat untuk lain-lain sistem pengecaman seperti di dalam pengkelasan organisma transgenic, analisa bahan forensic, pengesanan patogen untuk kajian kanser, kajian metagenomik, analisa ekspresi gen dan pengawalan alam sekitar.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

A	Adenosine
AFLP	Amplified fragment length polymorphism
AP-PCR	Arbitrarily primed PCR
APPI	Atmospheric pressure photoionization ionization
APPI-MS	Atmospheric pressure photoionization ionization mass spectrometry
ATPase	Adenosine triphosphate synthase
BGH	*Bovine growth hormone* gene
BOLD	Barcode of Life Database
BSE	Bovine spongiform encephalopathy
C	Cytosine
CE	Capillary electrophoresis
CE-SDS	Sodium dodecyl sulphate polymer-filled capillary gel electrophoresis
COX	Cytochrome c oxidase
CP	Common primer
CP-IRMS	Continuous flow isotope ratio mass spectrometer
CP-M-PCR	Common forward multiplex polymerase chain reaction
CSP-M-PCR	Common single primer multiplex PCR
CTAB	Cetyl trimethyl ammonium bromide
GMP	Guanosine monophosphate
cytb	*Cytochrome b* gene
DART	Direct analysis in real time
DART-TOF-MS	Direct analysis in real time time-of-flight mass spectrometry
ddPCR	Droplet digital PCR

DI-MS	Direct-infusion mass spectrometry
D-loop	Displacement loop
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleick acid
DSC	Differential scanning calorimetry
DVS	Department of Veterinary Services
2D-DIGE	Two-dimentional difference gel electrophoresis
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetate
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
EMA	Economically motivated adulteration
E-Nose	Electronic nose
ESI	electrospray ionization
ESI-MS	Electrospray ionization mass spectrometry
EU	European Union
FFN	Food Fraud Network
FISH	Fluorescent in situ hybridization
FT-ICR	Fourier transform ion cyclotron resonance
FTIR	Fourier transform infrared spectroscopy
G	Guanosine
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography mass spectrometry
GDF-8	Growth differentiation factor 8
GFSI	Global Food Safety Initiative

GH	Growth hormone
GHP	Good Hygiene Practice
GMO	Genetically modified organism
GMP	Good Manufacturing Practice
HCCP	Hazard Analysis and critical Control Point
HCl	Hydrochloric acid
HDC	Halal Industry Development Corporation
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled mass spectrometry
IEF	Isoelectric focusing
ILE-2	Interleukin-2 precursor
IMP3	The Third Industrial Master Plan
IPTG	Isopropyl-β-D thiogalactopyranoside
IR	Infrared
IRMS	Isotope-ratio mass spectrometry
IT	Ion trap
JAIN	Jabatan Agama Islam Negeri (State Islamic Religious Department)
JAKIM	Jabatan Kemajuan Islam Malaysia (The Malaysia Islamic Development Department)
kb	Kilobase
kbp	Kilobase pair
KCl	Kalium chloride (potassium chloride)
KPDNHEP	Kementerian Perdagangan Dalam Negeri dan Hal Ehwal Pengguna (Ministry of Domestic Trade and Consumer Affairs)
K^+	Kalium ion (potassium ion)
LB	Luria-Bertani (culture medium)
LB	Lithium borate (electrophoresis buffer)

LC	Liquid chromatographic
LC-MS	Liquid chromatography mass spectrometry
LINE	Long interspersed nuclear elements
LOD	Limit of detection
M	Molar
MAIN	Majlis Agama Islam Negeri (The State Islamic Religious Councils)
MALDI	Matrix-assisted laser desorption/ionization
MALDI-ToF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer
PAGE	Polyacrylamide gel electrophoresis
PAGIF	Polyacrylamide gel isoelectric focusing
PCR	Polymerase chain reaction
PCR-DGGE	Polymerase chain reaction denaturing gradient gel electrophoresis
PCR-RFLP	Polymerase chain reaction-restriction length fragment polymorphism
PCR-SSCP	Single strand conformation polymorphism polymerase chain reaction
pI	Isoelectric point
PNA-FISH	Peptide nucleic acid fluorescent in situ hybridization
ppt	Part per trillion
PTR-MS	Proton transfer reaction mass spectrometry
PTR-TOF-MS	Proton transfer reaction mass spectroscopy time-of-flight mass analyzer
Q	Single quadrupole
QC-PCR	Quantitative competitive polymerase chain reaction
qPCR	Quantitative polymerase chain reaction/ real-time polymerase chain reaction
QUID	Quantitative Ingredient Declaration
RAPD	Randomly amplified polymorphic DNA
RAPD-PCR	Random amplified polymorphic DNApolymerase chain reaction

RASFF	Rapid Alert System for Food and Feed
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RP-HPLC	Reverse phase-high performance liquid chromatography
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase PCR (RT-PCR)
URF	Unassigned reading frames
USP	US Pharmacopeia
V	Volt
vCJD	Creutzfeld Jacob Disease
VTNR	Variable number tandem repeat
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside
WCO	World Customs Organization
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Meat authenticity had long been a global issue. Food fraud is committed either by cross-contamination or intentional adulteration including economically motivated adulteration (EMA). Adulteration usually refers to non-compliance with health, safety standards and religious issues. In 2014, meat products were the most investigated foodstuffs amongst other cases handled by EU Food Fraud Network (Food Fraud Network Activity Report, European Commission, 2014). Consumers are concerned about the honesty of ingredient declaration because the misdescription of food contents on the product labels may affect their lifestyles, health or religious concerns. In 2002, European legislation (EC Regulation 178/2002) was adopted to provide the general principles and requirements of food law and thus ensure the foodstuffs are safe and authentic for human consumption and animal feed. Another example is halal certification has become important in many countries supported by the increasing number of world's Muslim population.Halal status for all meat and meat-based products has been made mandatory in almost Islamic countries.

Meat has become the most targeted for species substitution in food products due to the high market price. For example, Cawthorn *et al.* (2013) reported that processed meats substituted with pork, chicken and also unconventional species such as donkey, goat and water buffalo were discovered and has become commonplace in South Africa; and the substitution of beef with rat meat in Indonesian market (Rohman *et al.*, 2011). Nowadays, more concern about meat authenticity has increased from the public and the governments in many countries especially after the incident of horse meat scandal in February 2013 (ZhongMeng *et al.*, 2015).

Malaysia has set to become a global halal hub in 2020. In relation to this, numerous methods were developed to identify species origin in meat mixtures and meat products including the employment of FTIR spectroscopy (Rahmania et.al, 2015; Rohman *et al.*, 2011), electronic nose and gas chromatography mass spectrometer (Nurjuliana *et al.*, 2011; Kurniawati et, al, 2014); polymerase chain reaction assay (Rahman*et al.*, 2014a, Rahman*et al.*, 2014b; Man *et al.*, 2007); PCR-RFLP (Murugiah*et al.*, 2009); and real-time polymerase chain reaction (Ali *et al.*, 2014). At the same time, rapid on-site detection kits (PORKline MEAT, Olipro® Meat ID gene chip, USM Porcine DNA Detection Kit) and portable detection machine (Hafys™) with high sensitivity and rapidity for sample screening had been developed for meat authenticity identification. Presentlya, an effort for the establishment of DNA barcoding for identification of meat species through microbial content (Faculty of Biotechnology and Cell Molecular Biology, Universiti Putra Malaysia) and the development of One-Minute Halal Detection kits (IAB Halal Quality Assurance Research Laboratory, Universiti Selangor) are underway to assist halal food monitoring.However, some of the testing methods are costly to be used commercially and do not suit for processed meat analysis. Adoption of cost-effective and suitable analyses for processed meat products as well as rapid, specific and robust, have important purpose towards health, religious and economic implications

for consumers, the food industries, quality control laboratories and regulatory agencies. As PCR-based technique provides a rapid and high throughput procedure even at a very small amount of sample, it was selected for the development of sensitive, simple and much cost effective (no need for sophisticated equipment or expensive reagents) methods for qualitative and quantitative analyses. The analytical framework designed in this study focused on the methods of identification and quantifying food adulteration or admixed with specific emphasis on controlling the cost of analyses.

Since multiplex PCR has been introduced in 1988 by Chamberlain, the identification of meat species in food and animal feed has become very popular until today (*e.g.* Ali *et al.*, 2015; Iwobi *et al.*, 2015, Safdar *et al.*, 2015; Kitpipit *et al.*, 2014). However, the application of common primer multiplex PCR (CP-M-PCR) system in food especially in meat identification is still very little. It may be that this technique requires detail knowledge of primer selection criteriacoupled with a working knowledge of primer reaction chemistry in the PCR assay.

There is also a requirement to indicate on the food labelthe quantity (in percentage) of each ingredient contained in the food, known as the Qualitative Ingredient Declaration (QUID) labelling.QUID is important because the nutrition label alone cannot provide the significant information to the health benefit.As a result, in this study a quantitative competitive PCR (QC-PCR) method was developed to determine the percentage of contamination in meat products.

Nowadays, real-time quantitative PCR (qPCR) and free-flow isoelectric focusing become common practices in DNA quantification. But financial cost is a major contributing factor to these types of analysis because the equipment, reagents and consumables are expensive in which many laboratories are still unaffordable. Therefore, this method relying only mainly on normal thermocycler, is deliberated as an economic alternative to quantification method. As meat-based companies are growing, this method can be applied for meat-based food screening in any laboratory including in the rural area with minimal equipment.

Main objective:

> To protect consumers from meat fraud and misdeclaration by developing sensitive, cost-effective and rapid qualitative and quantitative methods for monitoringmeat contents of complex food products.

Specific objectives:

1) To develop a highly sensitive common-multiplex-PCR method for the rapid identification of meat in meat-based products by the application of common primers for routine analysis.
2) To validate the common-multiplex-PCR system for sensitive and accurate identification of meat species.
3) To develop a simple quantitative method for quantitative monitoring of meat-based product mixtures with a synthetic gene as a control template.
4) To determine the percentage of foreign DNA content in meat products as a direct reflection of meat contamination using competitive DNA.

Hypotheses of the study:

1) The common primerand adapter primer increase the amplification efficiently in CP-M-PCR assay.
2) The QC-PCR method enables detection of other fraudulent constitutions at minimal amount and reducing the existing inter-laboratory differences by using DNA competitor as an internal control.

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BIODATA OF STUDENT

Ummi Kalthum binti Hanapi was born on 20th March 1975 in Johor Bahru, Johor, Malaysia. She entered a primary school at Sekolah Kebangsaan Pulai Sebatang, Pontian, Johor and then continued her education at Sekolah Menengah LKTP Kahang Timur, Kluang, Johor and Sekolah Menengah Perempuan Sri Aman, Petaling Jaya. Upon finishing her matriculation at Universiti Malaya, she started her undergraduate education at the same university and graduated in 1999 with Bachelor of Science (Hons.), majoring in Biochemistry. In 2008, she received her Master of Science degree in Plant Genetic Engineering and Molecular Biology from Universiti Putra Malaysia. She has been working as a research assistant at Malaysian Agricultural Research and Development Institute (1999-2001), Universiti Putra Malaysia (2001-2005), UKM Medical Molecular Biology Institute (2006-2006), as a biotechnologist at Felda Agricultural Services Sdn. Bhd. (2006-2009) and as a lecturer at Universiti Selangor (2009-2011). Currently, she is pursuing her Ph.D. programme in Halal Product Science at Halal Product Research Institute UPM. Her study is supported by research grants received from the Ministry of Science, Technology and Innovation (ScienceFund) and Universiti Putra Malaysia (Research University Grant Scheme) and scholarship from the Ministry of Education (MyPhD).

LIST OF PUBLICATIONS

This thesis dissertation resulted in the following publications:

Publications

Hanapi, U. K., Desa M. N. M.*, Ismail, A., & Mustafa, S (2014). A Higher Sensitivity and Efficiency of Common Primer Multiplex PCR Assay in Identification of Meat Origin Using NADH Dehydrogenase Subunit 4 Gene. *Journal of Food Science and Technology*, 1-10. (JIF 2014 = 2.203).

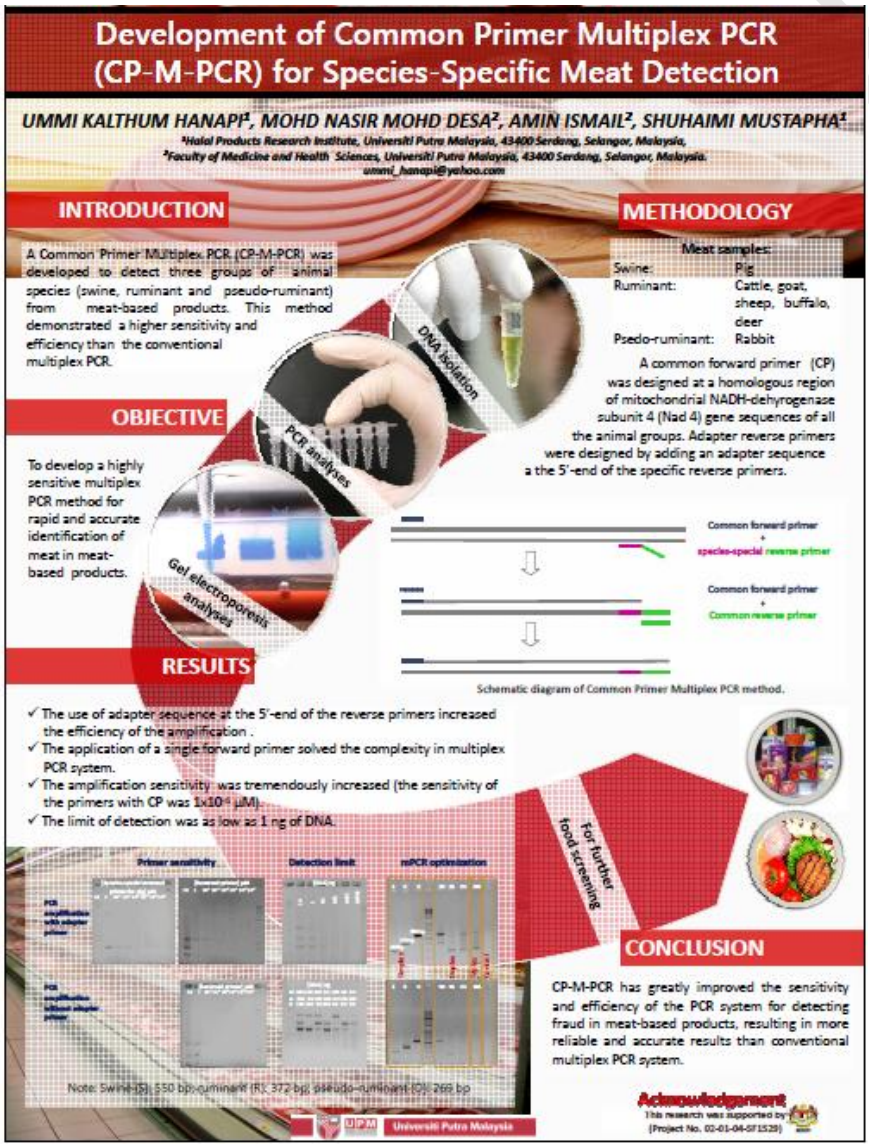
Hanapi, U. K., Esa, M. N.,Desa, M. N. M*., Ismail, A., & Mustafa. PCR-based dual detection for differentiating chicken from avian meat in processed meat products *Food Analytical Research,* submitted (JIF 2014/2015 = 1.956).

Hanapi, U. K., Esa, M. N., Desa, M. N. M*., Ismail, A., & Mustafa. Quantitative Determination of Avian DNA in Meat Products Using a Quantitative Competitive PCR. *International Food Research Journal,* submitted.

Proceedings/ Conferences

Hanapi U. K., Desa, M. N. M*, Ismail A., Mustafa S. Rapid and reliable identification of meat origin in meat products using CP-M-PCR [No. ID025m, ORAL]. In: *Malaysian International Halal Research & Education*. Putrajaya: Halal Products Research Institute, Universiti Putra Malaysia, December 2-4, 2014.

Ummi, K. H., Desa, M. N. M*, Ismail A. & Mustafa S. Development of common primer multiplex PCR (CP-M-PCR) for species-specific meat detection. [Abstract no. PP39, p.95; POSTER]. In: *1st International Conference on Molecular Diagnosis and Biomarker Discovery*. Penang: Institute of Research in Molecular Medicine, Universiti Sains Malaysia, October 23-25, 2013. [Abstrat Published in; Ummi, K. H., Desa, M. N. M.*, Ismail, A., & Mustafa (2014), Development of Common Primer Multiplex PCR (CP-M-PCR) for species-specific meat detection. *Asian Pacific Journal of Tropical Disease*, 4(3), 250.]



Award

Gold Medal

Research Title: "A High Sensitivity and Efficiency of Common Primer Multiplex PCR Assay in Identification of Meat Origin Using NADH Dehydrogenase Subunit 4 Gene".

Organizer: RMC, Universiti Putra Malaysia.

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