DEVELOPMENT AND EVALUATION OF MULTIPLEX REAL-TIME PCR FOR PORCINE DNA DETECTION IN PROCESSED FOOD

by

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LIST OF ABBREVIATIONS AND SYMBOLS

Bp	Base pair(s)
BLAST	Basic Local Alignment Search Tool
°C	Degree Celsius
ddH_2O	Double-distilled water
DNA	Deoxyribonucleotide triphosphate
dsDNA	Double-stranded DNA
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
EDTA	Ethylenediaminetetraacetic Acid
et al.	and others
EtBr	3,8-diamino-5-Ethyl-6-phenylphenanthridinium Bromide
FAM	6-carboxyfluorescein
g	Gram
HEX	Hexachloro-fluorescein
L	Litre
LOD	Limit of detection
M	Molar
MCA	Melting curve analysis
$MgCl_2$	Magnesium chloride
µL	Microlitre
µM	Micromolar
min	Minute(s)
mL	Millilitre
mM	Millimolar
mtDNA	Mitochondrial DNA

NaCl	Sodium chloride
ng	Nanogram
NTC	No template control
OD	Optical Density
PCR	Polymerase chain reaction
pg	Picogram
qPCR	Real-time PCR
rpm	Rotations per minute
rRNA	Ribosomal RNA
RT	Room temperature
s	Seconds
SDS	Sodium dodecyl sulfate
SG	SYBR Green I
ssDNA	Single-stranded DNA
TAE	Tris-acetate-EDTA
Tris-Cl	Tris(hydroxymethyl)aminomethane chloride
UV	Ultraviolet
V	Volt(s)
VIC	2'-chloro-7'phenyl-1,4-dichloro-6-carboxy-fluorescein
w/w	Weight per weight
%	Percentage

PEMBANGUNAN DAN PENILAIAN SISTEM MULTIPLEKS 'REAL-TIME' PCR BAGI PENGESANAN DNA KHINZIR DALAM PRODUK MAKANAN TERPROSES

ABSTRAK

Pemprosesan daging merupakan satu kaedah yang digunakan untuk mengawet atau menambah perisa kepada daging namun kini ia disalahgunakan oleh pengeluar makanan cenderung untuk menggantikan daging bernilai tinggi dengan daging yang lebih murah untuk menambah keuntungan ekonomi mereka. Tindak balas rantai polimerase "real-time" berasaskan SYBR (qPCR) berserta dengan proses pengekstrakan DNA yang pantas telah dibangunkan dalam kajian ini untuk mengesan DNA *Sus scrofa* dalam makanan terproses. Sasaran DNA yang terlibat dalam qPCR merangkumi LINE-1, iaitu salah satu unsur berulang dalam genom khinzir, bersama dengan gen RNA ribosom 16S sebagai kawalan dalaman PCR. Kajian telah dijalankan untuk menentukan 1) spesifisiti, 2) sensitiviti dan 3) kesesuaian dalam mengkaji produk daging terproses. Ujian qPCR didapati dapat mengesan kewujudan spesies khinzir dalam produk makanan dengan spesifik apabila dinilai terhadap panel spesies yang biasa digunakan. Ujian ini mampu membezakan perbezaan antara daging lembu masak yang tulen dan daging lembu masak yang dicemarkan dengan 0.001% (w/w) daging babi dalam masa satu setengah jam. Seratus dua puluh satu sampel produk daging buatan tempatan telah disampel dari pasar raya di sekitar Pulau Pinang. Keputusan menunjukkan kesemua sampel produk daging berhalal yang dikaji adalah tulen sepenuhnya . Ujian ini amat berguna untuk ujian kawalan daging khinzir dalam industri makanan.

DEVELOPMENT AND EVALUATION OF MULTIPLEX REAL-TIME PCR FOR PORCINE DNA DETECTION IN PROCESSED FOOD

ABSTRACT

Meat processing is a method of enhancing the flavour or preserving the meat but now it was misused by food manufacturers, whereby the expensive meats in the processed food are substituted with cheaper or inferior meats for their economic gain. Complex composition of processed food today has made the species determination a more difficult task. A simple and practical procedure for identification of meat species origin in processed meat products was developed, whereby a SYBR Green-based duplex real-time polymerase chain reactions (qPCR) approach coupled with a rapid DNA extraction method was developed to detect *Sus scrofa* DNA in processed food. The qPCR targeted LINE-1, a repetitive element sequence exclusively found in pig genome, together with an internal control based on 16S ribosomal RNA gene. The assay was validated for 1) specificity, 2) sensitivity and 3) robustness on processed meat products. Results showed that assay was highly specific when evaluated against a panel of commonly consumed species. The developed assay is capable of capturing the DNA of 0.001% (w/w) adulterated pork meat in just one and a half hour. A total of 121 commercial meat products were tested. No pork adulteration was detected in all the halal-labelled samples. Similar results were obtained using both developed method and kit. The assay would be particularly useful as an alternative for pork control test in food industry.

CHAPTER 1

INTRODUCTION

1.1 Background

Since civilization began, consumers rely on package labelling in selection of proper food. Proper labelling of meat product is imperative to ensure fair-trade, and enable consumers to make informed choices. In fact, food labelling regulation requires the species of meat in food product have to be accurately declared to the consumer or the misleading food label is considered a scandal. Adulteration is associated with action that is taken to add or manipulate a food item or composite the food product with extraneous, substandard or inferior ingredients intentionally for economic profit. The adulterant has been hidden by evolving food processing technique to avoid detection by regulatory bodies or consumers. As a concern with regards to food safety as well as being of a food standard issue, adulteration and mislabelling should be monitored with reliable analytical methods.

Due to its nature, processed meat product has been a susceptible target in adulteration. The species origin of the mixed minced meat is extremely difficult to be identified with physical attributes. Due to the changes in biomarker morphology during food processing and complex composition of processed meat, authentication of species origin in processed meat product become even more challenging. The demand for reliable but less expensive species authentication test continues to grow

by leaps and bounds in conjunction with the overwhelming choices of processed food in the today market.

1.2 Problem statement

1.2.1 Pork adulteration

Pork is usually the source of adulteration of meats of higher value such as beef and veal (CHEN et al., 1998, Saeed et al., 1986, Nakyinsige et al., 2012, Premanandh et al., 2013, Ha et al., 2017). Pork-based meat derivatives such as the mechanically recovered meats, lard, blood plasma, gelatin and transglutaminase have been identified as the potential adulterants in meat products due to cheap in cost and readily available (Nakyinsige et al., 2012, Aravindran et al., 2016). First of all, pork adulteration in meat product leads to the unfair market competition among food producers (Man et al., 2007). In addition, consumption of food products with pork adulteration can be a source of allergen, besides increased risks associated with *Trichinella spiralis*, *Toxoplasma gondii* and *Yersinia enterocolitica* infections (Masiri et al., 2016). Apart from that, pork adulteration also raised religions concern, particularly among the Muslims and Jews as pork and its derivatives are strictly forbidden in their religions (Nakyinsige et al., 2012). As such, it is imperative to have proper regulation and detection system to address the pork adulteration issue which causing economic, health and religious concerns.

Pork adulteration has been of international concern. To cope with the problem, Halal certification bodies were established in many countries, including the United Kingdom, the United States and developing countries in Southeast Asia to monitor the import and export of Halal food products (Nakyinsige et al., 2012). In fact, the

Halal certification has been made mandatory for all meat-based food imports in Middle East and many other Islamic countries (Nakyinsige et al., 2012). In Malaysia, Malaysian Standard on Preparation and Production of Halal Food (MS 1500:2009) was introduced by Department of Islamic Development Malaysia (JAKIM) to ensure that the Halal concept is applied from farm to table by constant monitoring of Halal food production process to be compliant with the Syariah laws.

Despite clear regulation, incidences of pork adulteration are still reported globally. Pork DNA has been detected in a number of meat products supplied to supermarkets despite being certified with Halal logo (Calvo et al., 2002, Karabasanavar et al., 2014, Yusop et al., 2012, Ha et al., 2017). A recent study in Portugal indicated that up to 40% of Halal products did not comply with their Halal labeling as traces of pork were detected in the samples (Amaral et al., 2017). There have been a number of Halal fraud incidences in the news lately. Over 20 tons of fake beef made up of chemically treated pork were seized in China in September 2013 (Tan, 2013). Over 120 tons containers containing approximately RM2 million of pork adulterated meat was seized in a cargo shipment area at Port of Tanjung Pelepas, Johor in recent raid (Salim, 2017). These incidences are certainly a tip of the iceberg and revealed the gaps in low enforcement towards quality assurance of processed meat.

1.2.2 Limitations of current method

qPCR assays have recently revolutionized the scene of analytical food biotechnology with the capability specifically detecting a few copies of DNA in complex processed food (Salihah et al., 2016). The commercial qPCR kits available for porcine DNA

detection are foodproof SL Porcine Species Detection Kit, Mericon Pig Kit, Agilent Porcine Detection Kit, Techne Sus scrofa Speciation Kit, Kogene Pig Kit and Progenus TagPro Pig Quantification Kit. There are only a few of the qPCR kit come together with DNA extraction kit, and often expensive due to incorporation of advanced spin column-based technology to deliver quality DNA for subsequent PCR analysis. These rapid and simple detection methods with advanced technology although expensive, have circumvented the limitation of conventional method which are laborious and time consuming.

In a recent study, Ahmed et al., (2016) patented a porcine DNA detection method whereby an in-house DNA extraction method is implemented in its simplest form with no additional reagent or specialized instrument required, prior to PCR analysis (Patent no WO/2016/028138). The idea enables obtaining ample and appropriate DNA in a cheaper and less laborious way. Absence of co-extraction PCR inhibitors from samples is important to ensure a better capacity for PCR amplification process. In the in-house method, crude DNA is used without compromising the capacity of PCR amplification. Furthermore, no hazardous chemicals were used in the protocol thus there is no safety and disposal issue. However, the protocol requires an overnight incubation to extract DNA out of the meat, which results in prolonged screening process.

Thus in this study, a combination of DNA extraction mentioned above and SG qPCR assay was introduced for development of a porcine DNA detection method. Attempt was made to optimize the time taken for the in-house DNA extraction while developing a more rapid and cost-effective qPCR assay, to facilitate the pork adulteration inspection program in our food industry. The developed assay was

evaluated for its 1) specificity, 2) sensitivity and 3) robustness to ensure its reliability in processed meat products analysis.

1.3 Research objectives

The main goal of research is to develop and validate a real-time PCR assay for porcine DNA detection that is applicable to both raw and processed meat-based food products. Specific objectives include:

1) To design primers for both porcine-specific and internal control target in real-time PCR assay.
2) To develop and optimize the real-time PCR assay.
3) To determine the analytical sensitivity and specificity of assay.
4) To evaluate and validate the optimized real-time PCR assay with food samples as compared to a commercial kit.

CHAPTER 2

LITERATURE REVIEW

2.1 Food labelling

Food labelling refers to any written, printed or graphic information that is present on a food product, including those for product promotion and disposal information (Commission, 1985a). A generic food labelling provides information on name of food, a description of the food, list of ingredients, net contents and drained weight, name and address of manufacturer, date marking, explanation of function and storage condition. The food labelling is deemed to be an effective way for food producers to disclose the product information to the society which effectively bridge the informational gap between producers and consumers (Messer et al., 2017). Thus it should neither be false, deceptive or misleading.

Codex Alimentarius Commision is an ad hoc intergovernmental body founded in 1963, by World Health Organization (WHO) and Food and Agriculture Organization (FAO), which responsible to create a unified international food standard. The joint FAO/WHO Food Standards programme is implemented with the major purpose of to protect health of consumers and to ensure fair trade practices (Commission et al., 2007). In Malaysia, food standard or food labelling is primarily regulated by the Food Safety & Quality Division of the Ministry of Health Malaysia through authority vested in the Food Act of 1983 and Malaysian Food Regulation of 1985 (as of February 2018). According to the Malaysian law, any person who

prepares, packages, labels or sells any food in a manner that is false, deceptive or misleading is regarded as commits an offence. No person shall prepare or sell fraudulent labelled food (adulterated food) or offenders are liable to a fine not exceeding RM 5000 or to prison sentence of up to 2 years as prescribed in Section 272 of the Penal Code (2002).

2.2 Food adulteration

The United States Food and Drug Administration (Safdar and Abasıyanık) defined economically motivated adulteration as "fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production" (Johnson, 2014). The malpractice is not new as the first case of food adulteration in meat was recorded in thirteenth century A.D at Florence in Italy (Thornton, 1968). Today, it was estimated that the global food fraud totalled up to $40 billion in value annually across the globe, according to Michigan State University's Food Fraud Initiative (Schlesinger, 2016). Increased exposure of food adulteration cases has raised significant public awareness on the food composition consumers' purchase. Based on the "NFU Mutual Food Fraud Report 2017", one third of the correspondents are less trusting of products and retailers compared to 5 years ago (Devlin, 2017). Recent example of food adulteration in Malaysia, "the instant durian coffee mixture" incidence that took the country by storm has caused at least five people to be hospitalized on December 2017 (Dermawan, 2018). The cases of food adulterations not only slowly eroded the customer trust on food labels and the composition but also put our public health at risk. As such, identification of species origin in food is essential to provide

consumers accurate information about the product consumed to avoid fraudulent labeling as well as promote fair trade.

2.3 Meat adulteration

Animal meat has been routinely consumed in human diet as premium source of proteins and fat (Valsta et al., 2005). In general, the species origin of unprocessed animal meat can be easily distinguished based on the morphological traits. Conversely, it is no longer a straightforward task to identify the species origin in processed meat. Processed meat products including sausages, salami, luncheon, meatball and canned meat are generally made of comminuted meat or leftover meat, which often enriched with synthetic ingredients such as additives, flavourings and colours, in order to enhance the general quality of food and to preserve the food. For many years, processed food is in high demand among the urban population as it tastes good, is convenient and available at lower cost. The natural shape, texture and colour of meat were altered during processing and packaging step. The comminuted meat used in modern food is therefore susceptible to adulteration by the dishonest food manufacturers who eager to gain more profit by intentionally substituting premium meat with cheaper meat or other materials (Singh and Neelam, 2011).

2.4 Methods used to determine species origin in meat

2.4.1 Conventional method

In order to protect the consumers from fraudulent meat, appropriate methods to verify the meat source has been developed. Early food verification detection attempts

were based solely on physical inspection (Winterhalter, 2007). In the late nineteenth century, the fraud detection methods were improved by using analytical balances and microscopic methods (Hahn, 1999, Van Raamsdonk et al., 2007). While meat constituents of different animal origin can be detected, the microscopic methods could not assign the species origin in mixture samples. A number of molecular analytical methods were developed based on protein or DNA analysis over the pass decades (Ali et al., 2014).

2.4.2 Protein-based method

Protein-based methods are specific and sensitive in raw meat analysis but they have limitations in processed meat as; (1) extensive processing (heat, pressure, chemical) of the meat in industrial practices might denature the proteins (Yada, 2017, Davis and Williams, 1998); (2) different tissues or organs may have a different protein profile (Ibarguren and Villamarín, 2017). Additionally, cross reaction of the antibodies used against proteins from closely related species may occur in immunoassays. Hence, protein-based approach may be less appropriate in processed food analysis (Van Raamsdonk et al., 2007).

2.4.3 DNA-based method

On the other hand, DNA-based methods has been proven to be more effective, sensitive and reliable in processed food analyses (Ballin et al., 2009). DNA-based methods are often preferred for processed meat as; (1) DNA sequence is identical in all cells type of a given organism as all cells are originate from a single cell (Alberts

et al., 2002, Lockley and Bardsley, 2000); (2) DNA is more stable bio-molecule and it is thus more likely to be covered from processed meat that has been exposed to environmental and technology factors, compared to proteins (Danezis et al., 2016). Furthermore, most DNA-based methods rely on the polymerase chain reaction (PCR) technique for its specificity, sensitivity and simplicity. In conventional PCR techniques, target DNA is copied and amplified to thousand and million-fold using species specific oligonucleotide so that the PCR product can be detected via gel-electrophoresis and image analysis (Fajardo et al., 2010) (Figure 2.1). An overview of conventional PCR technique is provided in figure below:

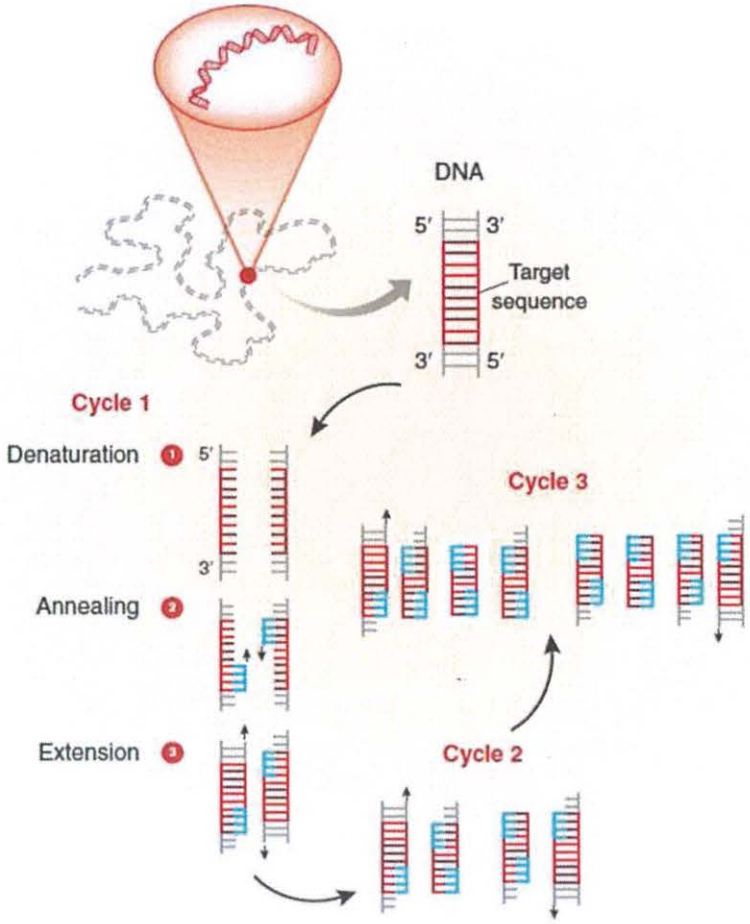

Figure 2.1: A representation of PCR principle (Garibyan and Avashia, 2013). DNA was synthesized with the aid of oligonucleotide primers and DNA polymerase.

An advanced version of PCR technique, real-time PCR has emerged as the leading tool for detection of DNA origin in meat samples over the past decade, providing advantages that include: (1) increased dynamic range of detection for a better sensitivity; (2) closed-tube system to reduce the cross-contamination risk; (3) an automated system to eliminate the post-PCR analysis such as the gel electrophoresis (Navarro et al., 2015). It has been regarded as a "gold standard" for detecting minute quantities of DNA from highly damaged DNA sources present in complex processed food due to its perceived simplicity, sensitivity, specificity and speed (Pegels et al., 2012).

2.5 Real-time polymerase chain reaction

Real-time polymerase chain reaction (qPCR) was first introduced by Higuchi in 1993. qPCR is an advanced molecular technique used to monitor the progress of PCR amplification based on fluorescent signal (Higuchi et al., 1993). An overview of qPCR principle is provided in Figure 2.2.

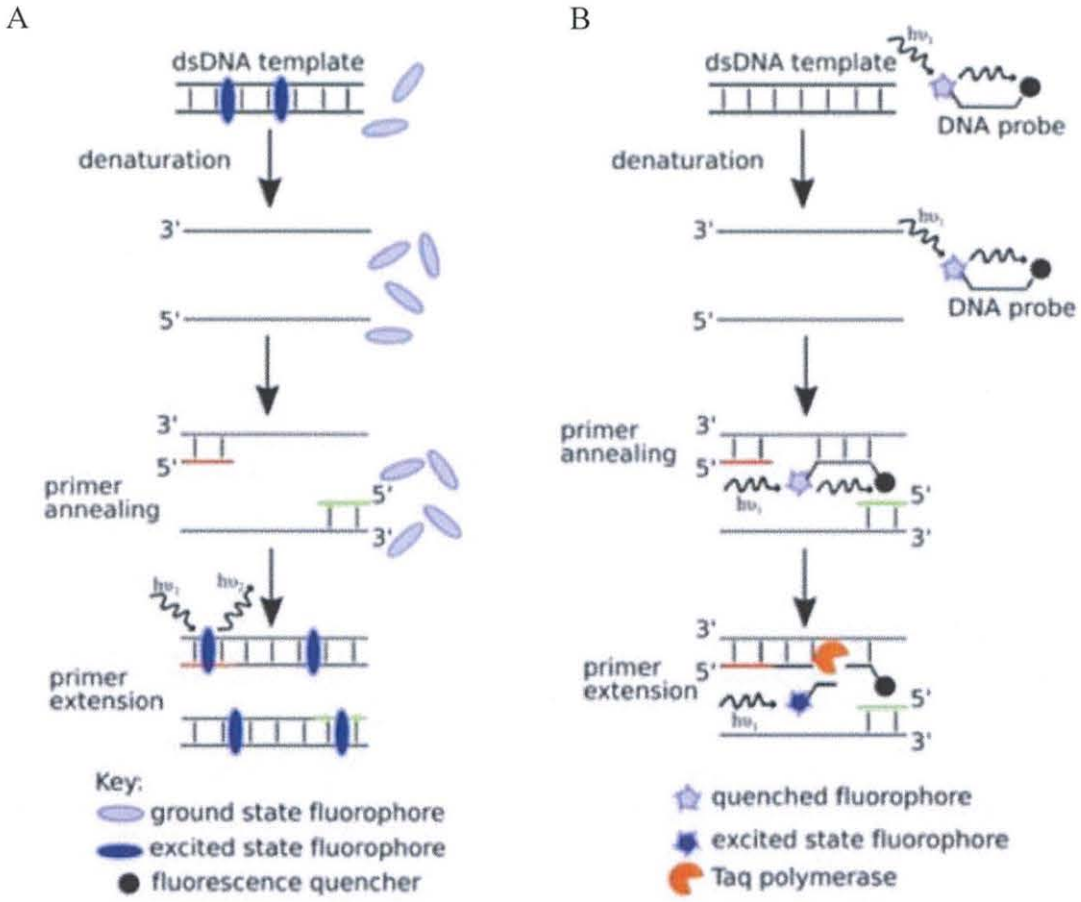

Figure 2.2: A representation of qPCR working principles of (A) intercalating dye-based real-time PCR and (B) oligonucleotide probe-based real-time PCR. Figure adapted from https://bitesizebio.com/29508/real-time-pcr-digest/. (Accessed on 12th March 2018, 5.30 pm).

As a well-established and comprehensive technology, it has been widely used in gene expression analysis, diagnosis of disease purpose, forensic, but also for species differentiation and detection in food samples (Nour et al., 2014). Like standard PCR, qPCR involves an *in vitro* enzymatic method to achieve billion-folds amplification of a specific DNA sequence. Each amplification reaction consists of DNA template, oligonucleotide primers, deoxynucleotide triphosphate (dNTP), thermostable DNA polymerase enzyme, divalent cation (e.g. Mg^{2+}) and PCR reaction buffer. The generic qPCR analysis involves a pre-incubation at 95 °C for 2-5 min to ensure the secondary structure of dsDNA molecules are separated into ssDNA, followed by up to 40 cycles of DNA template denaturation, primers annealing and growing strand extension to exponential amplify targeted DNA sequences, also known as the PCR product. In conventional method, the PCR product is visualized at the end of the 40 repeated amplification cycles by gel-based post-PCR analysis. On the contrary, the accumulated PCR product in qPCR is measured at the end of each of the 40 amplification cycles. In qPCR technique, PCR amplification progress can be monitored throughout the course of amplification by addition of fluorophores that bind to the accumulating PCR amplicon. With each amplification cycle, the intensity of fluorescence signal will increase in proportion to the increased concentration of PCR amplicon.

2.5.1 Real-time PCR amplification plot

Consequently, an amplification curve can be plotted from the fluorescent signals of each amplification cycle. An ideal amplification curve is in sigmoid shape and it can be divided into three phases (Figure 2.3). During the initial cycles (lag phase), the

increase in fluorescence signal cannot be detected as the amount of DNA is too little, compared to the baseline signal. The exponential phase (log phase) occurs when the amount of amplified DNA is sufficiently high that the fluorescence signal starts to rise above the background level. This exponential amplification is detected at relatively short period of time and it is usually used to speculate the amplification efficiency. Log phase is followed by a plateau phase as reaction component become limited and PCR inhibitors accumulated in late amplification cycles (Freeman et al., 1999). In analysis, a fluorescence threshold level is set above the baseline signal. The cycle number at which amplification curve intercept the threshold level is defined as threshold cycle (C_T) value. This C_T value can be directly correlated to the initial concentration of the target DNA sequence in the sample. Relatively higher the amount of target DNA in sample will have relatively lower C_T value (The term of C_T is used interchangeably with C_P, crossing point).

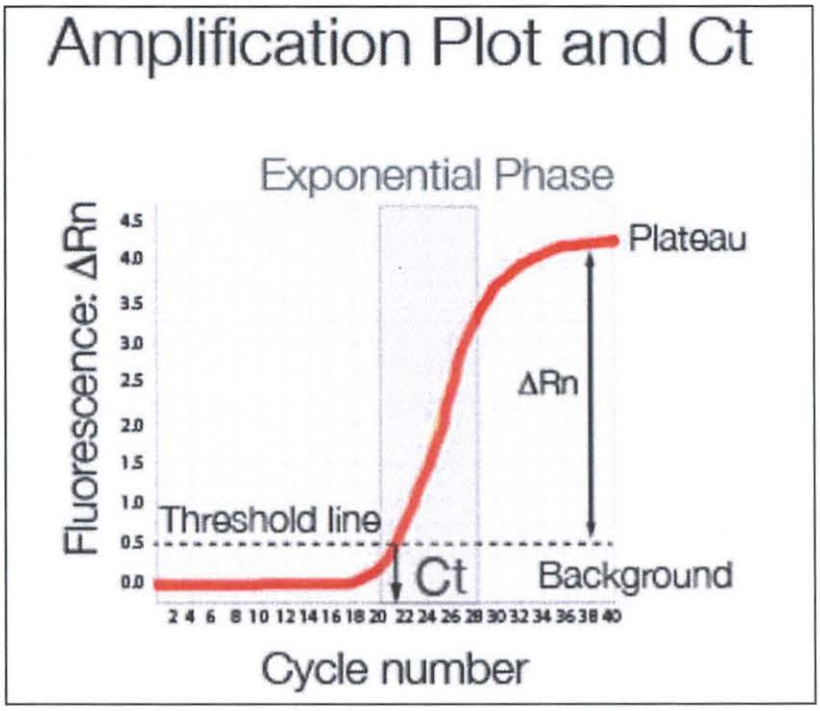

Figure 2.3: Graphical representation of an ideal amplification plot to be obtained over 40 cycles of qPCR. Figure adapted from https://www.cogentech.it/realtime-pcr-eng-technical-details.php. (Accessed on 12th March 2018, 5.30 pm).

2.5.2 Real-time PCR chemistries

Fluorophores are chemical compounds that can emit fluorescence light upon excitation. Different fluorophores have been employed to detect PCR product in q PCR system. Fluorophores can be excited upon absorption of energy from the light of specific wavelength and emits a transient fluorescent light of longer wavelength when the active molecules returned to the ground state. The two major classes of fluorophores used in the qPCR are either: (1) DNA-intercalating dyes such as the SYBR Green I and EvaGreen; and (2) oligonucleotide probes such as the TaqMan probes and Beacon. Both assays are rapid and sensitive, although the cost-per-assay are different due to their principle of detection (Arya et al., 2005, Ponchel et al., 2003).

2.5.2(a) Oligonucleotide probes

The trend is to monitor the amplification reaction with inclusion of fluorescent-labelled oligonucleotide in the qPCR reaction. In addition to the PCR primers, the PCR system includes a third oligonucleotide, conjugated with a reporter dye at 5' end and quencher moieties at 3' end, and known as a probe. This oligo probe therefore it acts as an additional specificity for the probe detection system. Fluorescence resonance energy transfer (FRET) mechanism was incorporated in the probe where both reporter and quencher are placed in close proximity so that no fluorescence is detected by nature (Figure 2.4). The probe is designed to anneal in between of both forward and reverse primers. As polymerase extends on growing strand, it will encounter the 5' end of the probe. The 5' exonuclease activity of Taq DNA polymerase then separates the 5' reporter from 3' quencher. The free reporter dye

now can be detected, which produces a fluorescent signal that is proportional to the amplicon yield. This probe-based was found to be 4 times less cost effective compare than the use of classical methods due to the application of additional probe oligonucleotides involving FRET mechanism (Marín et al., 2010).

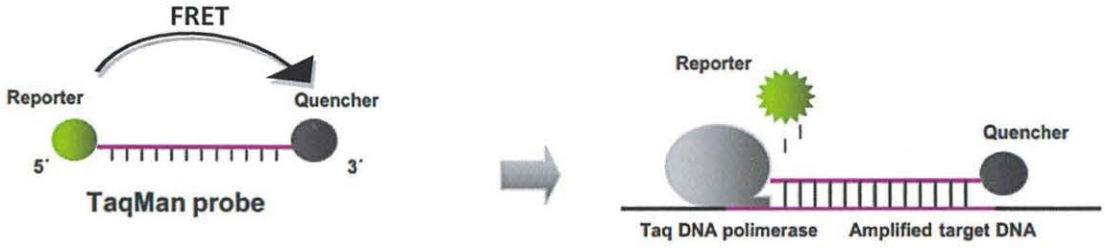

Figure 2.4: Schematic mechanism of fluorescence resonance energy transfer (FRET) held in TaqMan probe (A) before and (B) after DNA amplification (Navarro et al., 2015).

2.5.2(b) DNA-intercalating dyes

Fluorescent DNA-intercalating dye, such as the SYBR Green I (SG) has a high affinity to double-stranded DNA (dsDNA) in a sequence-independent way. In the buffer solution, non-binding fluorescent dyes exhibit low fluorescence because the excitation energy can be dissipated through vibration energy. As increasing target DNA target is amplified during the PCR reaction, the fluorescent dyes undergo a conformational change when intercalating with the minor groove of duplex DNA. The conformational change dampens the intra-molecular mobility, hence excitation energy is emitted as fluorescence, resulting in the over thousand-fold increase in signal intensity (Dragan et al., 2012).

One shortcoming of qPCR assay based on DNA-intercalating dyes is the non specific binding to all dsDNA, including primer dimers and non-specific DNA amplification that may exhibit false positive fluorescence. Therefore an additional step is often carried out to verify the amplicon species. Non-specific amplification can be detected by performing a melting curve analysis on the PCR product (Ririe et al., 1997).

2.5.3 Melting curve analysis in SG qPCR

Melting curve analysis (MCA) is a heat dissociation analysis that is performed after the completion of real-time PCR amplification reaction. MCA allows the differentiation of PCR amplified DNA utilizing the intrinsic melting temperature (T_m) property of different DNA sequences. The analysis is carried out with a stepwise increase in temperature from around 50 °C to 95°C. As the increasing temperature unravels DNA duplexes, less intercalating dye can bind to the DNA and the fluorescence intensity decreases. The change in the fluorescence intensity over increasing temperature is used to determine the T_m value. The T_m is identified as a peak value in the first negative derivative of MCA plot representing the changes of fluorescent signal with respect to time (-d(RFU)/dT) against the temperature (Figure 2.5), which corresponds to the temperature where 50% of the base pairs of PCR amplicon duplex is uncoupled.

The T_m value of a PCR amplicon generally depends on its concentration, length and nucleotide composition and it should be unique and consistent. It is a critical point particularly in the presence of more than one population of amplicon, where multiple peaks will be reflected on the derivative melting curve to represent

each amplicon species. By the same token, the non-specific amplicon will generate a different T_m peak on the derivative melting curve, and can thus be discriminated from specific amplicon. This reveals the specific identification of melting peak permits more than one target sequence to be co-amplified in the same reaction and still can be distinguished (Hoorfar et al., 2004). Consequently, multiplexing is made possible in SG qPCR system, utilizing two or more sets of primers that amplifies DNA product with different T_m.

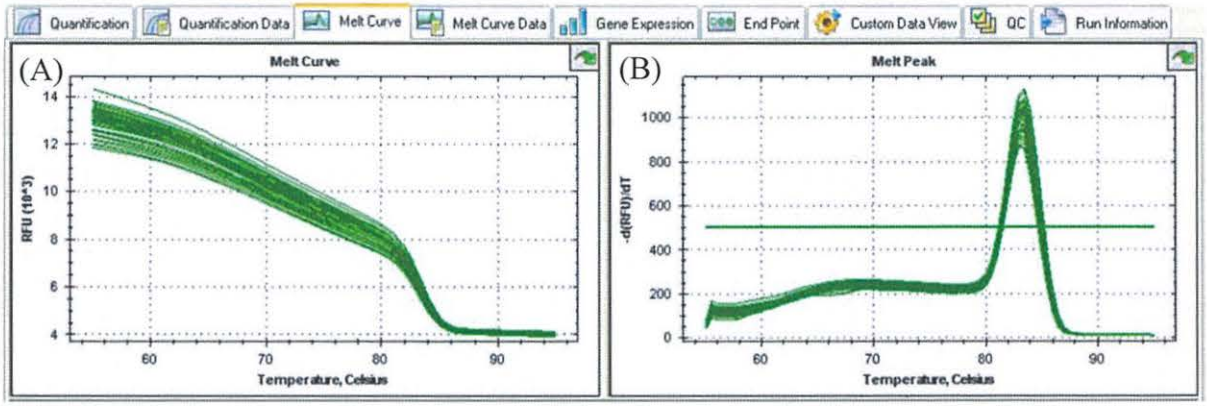

Figure 2.5: The typical MCA results can be presented in either melting curve (A) or melting peak (B) (derivative melting curve) format. Figure adapted from http://bascompalmer.org/documents/CFX-Connect-Instruction-Manual-Part3.pdf. (Accessed on 1st April 2018, 8.48pm).

2.6 Multiplex PCR

The amplification of more than one target DNA sequence within a single PCR reaction is called multiplexing. A singleplex assay is designed to amplify a single gene of interest in a single reaction. A duplex assay involved combination of two primer sets to simultaneously amplify two genes of interest in a single reaction. The advantages of multiplexing include increased throughput, reduced reagent usage, reduced sample usage while allowing more data to be generated from the same starting material. In addition, multiplexing allows the inclusion of an internal control reference DNA sequence to devoid of false negative results, thereby significantly

improve data validity. Melting curve analysis (MCA) can be carried out at the end of amplification cycle in SG qPCR to identify the amplification of target DNA that has been amplified.

2.7 Internal amplification control

In PCR assay, internal amplification control is a positive control used as a strategy to rule out the false negativity. Internal control can be from an exogenous source (recombinant plasmid) or endogenous source (genomic DNA). Both are used to check if the PCR inhibitor is present in the reaction. This is imperative as multiple ingredients in clinical, environmental samples and food products have been proven to be the PCR inhibitors and compromise the assay (Schrader et al., 2012). However, the endogenous internal control has additional benefits to assess: (1) if the template extracted at good quality, (2) whether the DNA is successfully extracted from a complex sample.

No template control (NTC) is the negative control where the DNA template is replaced by double distilled water to determine the false positive result from DNA contamination. The possible sources of contamination are: (1) cross-contamination between samples; (2) carry-over contamination of PCR product from previous PCR runs. In qPCR assay, no fluorescence signal should be observed in the NTC sample ideally. At the very least, the C_T value should be 5 cycles away from positive samples ideally (Hu et al., 2013). However, if the C_T value of NTC is less than 5 cycles delayed compared to samples containing template, the assay is considered not valid as reagent might be contaminated.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

The chemicals used throughout the study were listed in Table 3.1.

Table 3.1: List of chemicals and sources

Chemical	Manufacturer
Sodium acetate, anhydrate	Bio Basic Inc (Canada)
30% Acrylamide/bis solution, 19:1 **TEMED**	Bio-Rad (Hercules, USA)
10X Reaction buffer $MgCl_2$ FREE **Magnesium chloride solution (50mM)** **dNTP mix (10mM)** **DNA polymerase (1U/µL)**	Biotools (Australia)
Ammonium persulfate	Calbiochem (USA)
Tris Base	Fisher Scientific (USA)
Ethanol **Hydrochloric acid, c(HCl)= 1 mol/L** **Sodium chloride** **Proteinase K**	Merck Millipore (Darmstadt, Germany)
Sodium Dodecyl Sulfate (SDS)	Promega (Madison, USA)
Sodium hydroxide pellet	R&M Chemicals (Essex, UK)
Boric acid, ≥99.5% **EDTA** **Ethidium bromide** **Phenol:chloroform:isoamyl alcohol 25:24:1,pH 8.0**	Sigma-Aldrich (USA)

3.1.2 Buffers/solutions

The buffers used were listed in Table 3.2.

Table 3.2: Composition of the buffer used in the study

Buffers	Components
Digestion buffer	100mM NaCl, 10mM Tris-Cl (pH 8.0), 25 mM EDTA and 0.5% SDS.
LightCycler® 480 SYBR Green I Master	FastStart Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), SYBR Green I dye, and $MgCl_2$.

3.1.3 Animals Meat

Raw meat from pig (*Sus scrofa*), cow (*Bos taurus*), goat (*Capra hircus*), chicken (*Gallus gallus*), sambar deer (*Rusa unicolor*), pomfret fish (*Bramidae*) and whiteleg shrimp (*Litopaneous vannamei*) were purchased from local markets. All animal meat were morphologically identified and processed immediately or stored in -20°C till further use.

3.1.4 Commercial meat-based products

A total of 121 commercially processed meat-based products of different brands were randomly purchased from local supermarket in Northern Region of Peninsular Malaysia from March 2015 to August 2017 (list in Appendices, from PT001 to PT121). Each sample was cut into cubes with 1-2 mm edge length by using a disposable scalpel on petri dish. Samples were collected separately in 50 mL centrifuge tubes and stored in -20°C until further use.

3.1.5 Commercial kits

The commercial kits used throughout the study were listed in Table 3.3.

Table 3.3: List of commercial kits and its description

Material/ Manufacturer	Description
LightCycler® 480 SYBR Green I Master, Roche Applied Science (Mannheim, Germany), (Lot#: 04707516001)	Real-time PCR kit based on SYBR® Green I dye, used for sensitive detection of defined DNA sequences.
PorcineTrace Real-time PCR Kit with PorcineTrace Food DNA Extraction Kit, 7FoodPillars Sdn Bhd (Selangor, Malaysia), (Lot#:CQ00303G)	Real-time PCR kit based on TaqMan™ probes, used for specific detection of domesticated pig and wild boar in food samples.

3.2 Methods

3.2.1 Genomic DNA preparation by Ahmed's method

Total genomic DNA from pig and cow species was extracted using a lysis buffer-based method as described by Ahmed with some modification (Ahmed et al., 2016). First, 200 mg of raw beef and pork were placed in fresh 1.5 mL microcentrifuge tubes. Meat samples were ground into paste form with tissue grinder pestle (Axygen) and suspended with 1 mL of digestion buffer. Samples were well-mixed by vortex followed by incubation with 200 rpm at 55°C overnight (for 16 hours) on Thermo-Shaker TS-100C. Crude lysates in viscous liquid form were obtained in the next day. Supernatant were obtained from centrifugation at 14,000 g at 25°C for 2 minutes. One volume (0.7 mL) of phenol:chloroform:isoamyl alcohol, pH 8.0 (25:24:1 (v/v); Sigma Aldrich) was carefully mixed with the supernatant. The mixture was centrifuged at 12,000 g at 25°C for 10 minutes. The top aqueous layer was

transferred to a new tube and mixed with 1/10 volume of 3M sodium acetate, pH 5.2 and 2 volume of absolute ethanol. The mixture was centrifuged at 12,000 g at 25°C for 12 minutes. The supernatant was discarded while pellet was rinsed with 1 mL of 70% ethanol. Supernatant was removed by centrifugation at 13,000 g at 25°C for 2 minutes. The remaining pellet was air dried for 5 minutes and further resuspended in 50 µL of ddH_2O. The purity and concentration of extracted DNA were determined by measuring the absorbance at 230 nm, 260 nm and 280 nm with NanoPhotometer (IMPLEN). The extracted DNA was stored at -20°C until further use.

3.2.2 Optimization of in-house DNA extraction method

Two different temperatures and a series of incubation time were assessed in optimization of DNA extraction method proposed by Ahmed et al., (2016). The crude lysates was then evaluated in the gel-based PCR assay to determine the minimum time required for DNA extraction for species detection in PCR analysis. One percent (w/w) adulterated pork in beef mixture was ground into paste form with tissue grinder pestle (Axygen) and suspended with 1 mL of digestion buffer. Samples were well-mixed by vortex followed by incubation with 200 rpm at 55°C and 65°C, for 10 min, 20 min, 30 min, 60 min and 180 min respectively to obtain the crude lysates. After centrifugation at 14,000 g at 25°C for 2 minute, 2 µL of supernatant was diluted in 200 µL ddH_2O before subjected to PCR analysis.